Stem cell niche ontogeny during lateral root development in *Arabidopsis thaliana*

Dissertation

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Combined Faculty of Natural Sciences and Mathematics

of the Ruperto Carola University Heidelberg, Germany

for the degree of

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Béatrice Berthet

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List of abbreviations

A	ACR4	ARABIDOPSIS CRINKLY4
	AGL42	AGAMOUS-LIKE 42
B	BCECF-AM	2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
	BRAVO	BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER
C	CAM	Cambium
	CC	Central cells
	CEI	Cortex- endodermis initials
	CFP	Cyan fluorescent protein
	CK	cytokinins
	CLE	CLAVATA3-ESR
	CLV	CLAVATA
	Col-0	Columbia
	CRN	CORYNE
	CSC	Columella stem cells
	CYCD	D type Cycline
	Cytrap	Cell Cycle Tracking in Plant cells
D	DCR	DEFECTIVE IN CUTICULAR RIDGES
	Dex	Dexamethasone
	DMSO	Dimethylsulfoxyde
	DNA	Deoxyribonucleic acid
E	EdU	5-ethynyl-2´-deoxyuridine
	EDZ	Elongation and differentiation zone
	Epi/LRC	Epidermis/Lateral root cap initials
	ERF115	ETHYLENE RESPONSE FACTOR115
G	GFP	Green fluorescent protein
	Gif	GRF-INTERACTING FACTORs
	GRF	GROWTH REGULATING FACTOR
	GUS	β-glucuronidase
H	H2B	Histone H2B
	HAM	HAIRY MERISTEM
I	IAA	Indole acetic acid
L	LB	Lysogeny broth

	LR	Lateral root
	LRCi	Lateral root cap initials
	LRFC	Lateral root founder cells
	LRP	Lateral root primordium
	LRR	Leucin-rich repeat
M	MES	2-(N-morpholino)ethanesulfonic acid
	MOL	MORE LATERAL GROWTH
	MP	MONOPTEROS
	mPS-PI	Modified pseudo-Schiff propidium iodide
	MS	Murashigo and Skoog
O	OC	Organizing center
	OE	Over-expressor
P	PBS	Phosphate buffer saline
	PFA	Paraformaldehide
	PI	Propidium iodide
	PIN	PIN-FORMED
	PIP	Plasma membrane intrinsic protein
	PLT	PLETHORA
	PM	Proximal meristem
	PR	Primary root
	Pre-Em	Pre- emergence
	PXY	PHLOEM INTERCALATED WITH XYLEM
Q	QC	Quiescent center
R	RAM	Root apical meristem
	RBR	RETINOBKASTOMA-RELATED
	RFP	Red fluorescent protein
	RLK	receptor like kinase
S	SAM	Shoot apical meristem
	SC	Stem cells
	SCN	Stem cell niche
	SCR	SCARECROW
	SHR	SHORTROOT
	SMB	SOMBRERO
	STM	SHOOTMERISTEMLESS
T	T-DNA	transfer DNA
	TA	Transit amplifying cells

	TZ	Transition zone
W	WOX	WUSCHEL-RELATED HOMEOBOX
	WT	Wild type
	WUS	WUSCHEL
X	X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
	XPP	Xylem pole pericycle
Y	YFP	Yellow fluorescent protein

Summary

Stem cell niches are critical elements of plant organs, supporting their design, growth and maintenance. Typically, a stem cell niche (SCN) is composed of stem cells, able to asymmetrically divide to self-renew and generate a daughter which further proliferates before differentiating into a given tissue. Stem cells are maintained undifferentiated by an organizing center cells (OC). In the model plant *Arabidopsis thaliana*, three stem cell niches are driving the main axis of growth. The shoot apical meristem, at the apex of the shoot, generate aerial tissues. The root apical meristem, at the tip of roots, drive soil exploration for nutrients, water and anchorage, while the cambium is in charge of the radial expansion of plant organs. The root system of *Arabidopsis thaliana* is critical for the plant adaptability to its environment. It is composed of a primary root formed during embryogenesis, and lateral roots originating from cells deep inside the primary root tissues. Lateral root are formed post-embryonically and robustly reproduce the primary root organization, therefore being a convenient model to investigate de novo stem cell niche establishment. Early lateral root (LR) development has been well characterized. However, a comprehensive view on the ontogeny of lateral root stem cell niche establishment from the earliest stages of LR development up to post emergence is still to be proposed.

The aim of this thesis is thus to characterize the process of stem cell niche establishment in *Arabidopsis thaliana* lateral roots from the beginning of lateral root development until it reaches a primary root-like organization.

First, I defined a developmental frame of reference to follow LR development from stage I up to 2 mm post-emergence using cell wall staining and confocal microscopy of LR transverse section. The analysis of topological features in relation to the LR length indicate a progressive onset of a primary root like stem cell niche topology, post emergence. Transcriptional reporters of genes involved in the primary root stem cell niche patterning overlap to delineate the stem cell niche and its organizing center in short emerged LR, ranging from 80 to 250µm.

Second, I investigated the behavior of the SCN and its OC. To this end I assessed cell proliferation in LR of different length. While the stem cell niche organizer is proliferative prior emergence, quiescence is acquired post-emergence in short lateral roots. Genotoxic stress sensitivity assay and lineage tracing suggest a progressive onset of the organizing center functions within the stem cell niche.

Finally, I challenged the proliferative behavior of the stem cell niche organizer using mutants and ectopic expression of OC quiescence regulators. However those approaches did not strongly affected the stem cell niche organization.

The originality of this work is the investigation of the functional aspect of SCN establishment with a focus at the cellular resolution spanning lateral root development from initiation up to 2mm post-emergence.

The observations collected in this study may contribute to broaden the current view on stem cell niche establishment during lateral root development. Possibly by expanding the focus of further investigations up to post-emergence.

I hope that the findings presented here will be helpful for deepening our understanding of plant organogenesis.

Zusammenfassung

Stamzellnichen sind essentielle Elemente pflanzlicher Organe, relevant für deren Gestaltung, Wachstum und Erhaltung. Typische Stammzellnichen (SCN) bestehen aus Stammzellen mit der Fähigkeit zur asymmetrischen Teilung. Eine Tochterzelle dient der Stammzellerhaltung, während die andere sich weiter teilt bevor sie zum entsprechenden Gebwebstyp ausdifferenziert. Die Undifferenziertheit der Stammzellen wird dabei durch 'organizing center cells' (OC) erhalten. 3 Stammzellnichen sind dabei hauptverantwortlich für die primäre Wachstums-achse der Modelpflanze *Arabidopsis thaliana*. Das Spross-apikalmeristem in der Sprossspitze produziert die oberirdischen Gewebe. Das Wurzel-apikalmeristem in der Wurzelspitze hingegen bildet Wurzelgewebe zur Bodenverankerung, Mineralstoff- und Wasserversorgung. Das meristematische Gewebe des Kambiums sorgt dabei für die notwendige radiale Ausdehnung der pflanzlichen Organe. Das Wurzelsystem spielt eine essentielle Rolle in der pflanzlichen Anpassung an die Umwelt. Bestehend aus einer während der Embryogenese angelegten Primärwurzel, wird es ergänzt durch sekundäre Seitenwurzeln die aus Zellen tief im Primärwurzelgewebe entspringen. Seitenwurzeln entstehen postembryonal und reproduzieren dabei in robuster Weise die Organisation der Primärwurzel, was sie zu einem idealen Untersuchungsmodel für die *de novo* Etablierung von Stammzellnichen macht. Die frühe Seitenwurzelentwicklung wurde bereits gut untersucht. Ein umfassender Überblick der vollständigen Ontogenese von der Entstehung der Stammzellniche von Seitenwurzeln fehlt jedoch bisher.

Ziel dieser Doktorarbeit ist die Charakterisierung dieser Prozesse, die von der Etablierung einer Seitenwurzel-stammzellniche bis zum Erreichen einer Primärwurzel-gleichen Organisation.

Startpunkt ist dabei die Definition eines Entwicklungsrahmens mit Referenzstadien der Seitenwurzelentwicklung von Stadium I bis 2mm Länge nach Erscheinen der Seitenwurzel. Die Analyse topologischer Merkmale in Bezug zur Seitenwurzellänge deuten auf einen progressiven Anfang einer primärwurzel-gleichen Topologie nach dem Erscheinen der Seitenwurzel außerhalb des Primärwurzelgewebes. Transkriptionsreporter für Gene die typischer Weise während der Stammzell-nichen Bildung der Primärwurzel aktiv sind, überlappen nun. Sie grenzen dabei in der 80 bis 250µm kurzen, gerade aus der Primärwurzel hervorgetretenen Seitenwurzel die Stammzellniche von ihrem Organizing Center ab. Im zweiten Schwerpunkt meiner Arbeit, untersuchte ich das Verhalten von Stammzellniche und ihres Organizing Centers in entstehenden Seitenwurzeln. Zu diesem Zweck beobachtete ich die Zellteilung in Seitenwurzeln verschiedener Längen. Vor dem Erscheinen der Seitenwurzel ist Teilungsaktivität im Organizing Center der Stammzellniche detektierbar, während der Ruhezustand nach Erscheinen in den kurzen der Seitenwurzel Seitenwurzeln erreicht wird. Experimente mit genotoxischer Stress-sensitivität sowie Zelllinien-verfolgung, deuten auf eine progressive Aktivierung des Organizing Centers innerhalb der Stammzelniche hin. Zuletzt untersuchte ich das Zellteilungsverhalten des Stammzellnichen Organizers mittels Pflanzen linien für Mutanten- und über-expressoren von Regulator-elementen des Ruhezustandes des Organizing Centers. Diese Ansätze belegten jedoch keine starke regulatorische Rolle für die untersuchten Elemente.

Die Originalität dieser Arbeit basiert auf den Untersuchungen der funktionalen Aspekte der Etablierung der Stammzellniche auf zellulärer Ebene von der Initiation bis hin zu 2mm Länge nach Erscheinen der Seitenwurzel.

Die hier dokumentierten Beobachtungen können zu einer Vervollständigung momentaner Betrachtungen der Etablierung von Stammzellnichen während der Seitenwurzelbildung beitragen und mögliche Ausweitungen der Studien auf Seitenwurzeln nach ihrem Erscheinen initiieren.

Introduction

1. Plant organogenesis, coping with and growing in an ever changing environment.

1.1 De novo organogenesis and post-embryonic development

Plants can sustain living through changing and challenging environment thanks to their capacity to produce tissues and organs all along their life (Birnbaum & Alvarado, 2008; Dinneny & Benfey, 2008). This aptitude is linked to the presence of meristems, a structure containing the stem cell niches able to generate and regenerate tissues, organ and whole organisms (Gaillochet & Lohmann, 2015). De novo organogenesis, or the formation of new organs after embryonic development has completed, is particularly interesting in plants considering their plasticity and robustness. Indeed, many cells can revert to a pluripotent state and develop into a specific organ, in absence of embryonic blueprint. Understanding that process is about determining the sequence of events leading to a new functional organ. How do cells divide, coordinate and get functionalized to create the 3D structure able to contribute to the organism survival and growth?

The thorough study of the orientation and order of cell division within a growing organ informs about the topological patterning leading to the physical organization of that organ. Directional cell growth is also involved in this process and all together, it traces back the developmental history (ontogeny) of an organ. Morphogens gradient formed by the diffusion or the active transport of molecules, are important regulators of growth, and morphogenesis (organ shape formation). Morphogens can be hormones, proteins or peptides, small RNAs or reactive oxygen species (Jourquin et al., 2020; F. Ercoli et al., 2016; Furuta & Lichtenberger, 2012; van Norman et al., 2011).

The most common plant hormones are auxin, cytokinins (CK), gibberellic acid, brassinosteroids (BR), abscisic acid, ethylene and jasmonate. Among those, auxin and cytokinin are involved in a wide range of developmental processes such as root and shoot development, or gravitropism (Ditengou et al., 2008; Woodward & Bartel, 2005). The antagonistic signalling of auxin and cytokinin is critical for meristem establishment and regulation (Greb & Lohmann, 2016). Most plant hormones follow the same sequence of steps to integrate environmental and developmental inputs : local biosynthesis, directional transport / diffusion, perception, signal

transduction, transcriptional response yielding cellular changes, thus generating patterning at the whole tissue and organ level (Guseman et al., 2015).

1.2 Challenges of plant development

The survival of an organism is determined, by its ability to gather nutrients and water. In plants, this is guaranteed by the root system. It is composed of roots formed either during embryogenesis or afterward. The later ones provide an interesting example of how de novo formation of an organ allows adaptation to changing environmental conditions (Greb & Lohmann, 2016). Lateral root patterning along the primary root, defines areas, pre-branching sites, with the potential to grow lateral roots. However environmental input can affect this patterning as well as influence and/or stop LR development at different stages. Plants face the additional challenge that their cells too cannot move, as they are trapped into a cell wall matrix. As a consequence the formation of new organ is based on the activation of meristematic cells, cell reprogramming, oriented cell division and elongation, and the coordination of neighbouring cells to integrate both developmental and environmental signals.

1.3 The *Arabidopsis* root system as a model to study de novo organ formation

Arabidopsis thaliana has a tap root system composed of 3 types of roots : the primary root (PR), lateral roots (LR) and adventitious roots (Atkinson et al., 2014). The primary root meristem is formed during embryogenesis. Its main function is to support the growth of the primary root and therefore drive soil exploration for resources, and determine the position of the overall root system. LR are generated post-embryonically from pericycle cells within the PR (Figure Intro-6 B). Adventitious roots are also post-embryonic root, but produced from non-root tissues. An interesting and useful feature of the *Arabidopsis thaliana* root system is that all the roots formed post-embryonically are similar to the primary root in their organization despite the different ontogeny (Tian, De Smet, et al., 2014; Waidmann et al., 2020). Since the PR meristem has been well studied it can be used as a basis for comparison when studying the de novo onset of a similar structure.

The primary root of *Arabidopsis thaliana* is organized in concentric layers of tissues. Each layer is one-cell thick and constitute a cylindrical tissues, composed of cell files generated in the meristem at the tip of the root (L. Dolan et al., 1993). Because of this organization, the

developmental timeline of a given cell file can be followed along the longitudinal axis of the root. Furthermore, the radial symmetry of the root allows simplifying developmental questions. Indeed, the observation of a single transverse section of the root can be used to infer processed occurring within the whole volume of the root.

2. Stem cells niches

2.1 Cellular organisation and common regulators

2.1.1 Cellular organisation

A stem cells niche (SCN) is a group of stem cells (SCs) associated to an organizing centre (OC) (Figure1).

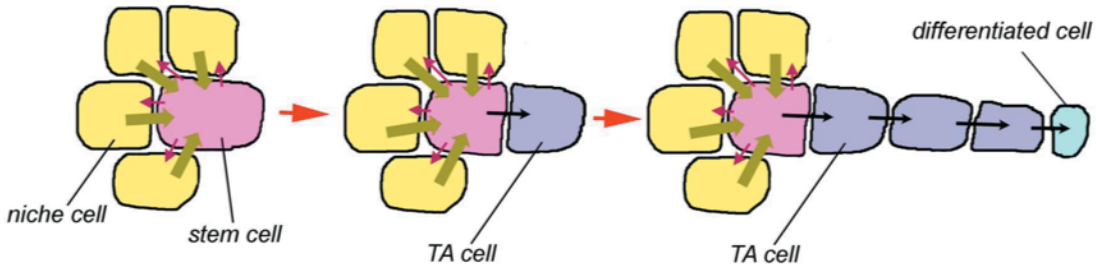


Figure Intro-1 : Schematic depiction of stem cell niches components.
Stem cells (pink), are in direct interactions (gold and magenta arrows) with cells from the niche (yellow), including the organizing center. The stem cell divide asymmetrically to self-renew and generate a transit-amplifying daughter cell (purple). The transit amplifying cell further divide before differentiating (blue) .TA : transit amplifying cells. Adapted from Stahl et al. 2005.

SCN generate organs and maintain their organisation. A key function of stem cell niche is the continuous production of cells at the appropriate location to ensure proper tissue organisation and growth or damage recovery. SCs divide asymmetrically to regenerate themselves and produce a daughter cell, which further proliferate before differentiating into a given tissue. The OC acts in a non-cell autonomous manner to maintain stem cells in an undifferentiated state (Figure Intro-1, (Pi et al., 2015; Scheres et al., 2002)). Additionally the OC is believe to play the role of "back-up cell population" able to replenish damaged stem cells. The stem cells and their organizer, are characterized by different proliferative capacity. Indeed the stem cell niche organizing centre is often quiescent, therefore decreasing the risk of DNA damages.

Primary stem cell niches are formed during embryogenesis, while secondary stem cell niches are formed post-embryonically. The shoot and root apical meristems are formed during embryogenesis and support longitudinal growth of the plant above and under-ground respectively (ten Hove et al., 2015). Those two types of stem cell niches are re-iterated post-embryonically to form secondary meristems such as lateral root meristems and axillary meristems (Q. Wang et al., 2016; Y. Wang & Li, 2008; Yang & Jiao, 2016). A third type of meristem, the cambium, is responsible for the radial growth of organs (J. Zhang et al., 2014).

2.1.2 Common molecular regulators

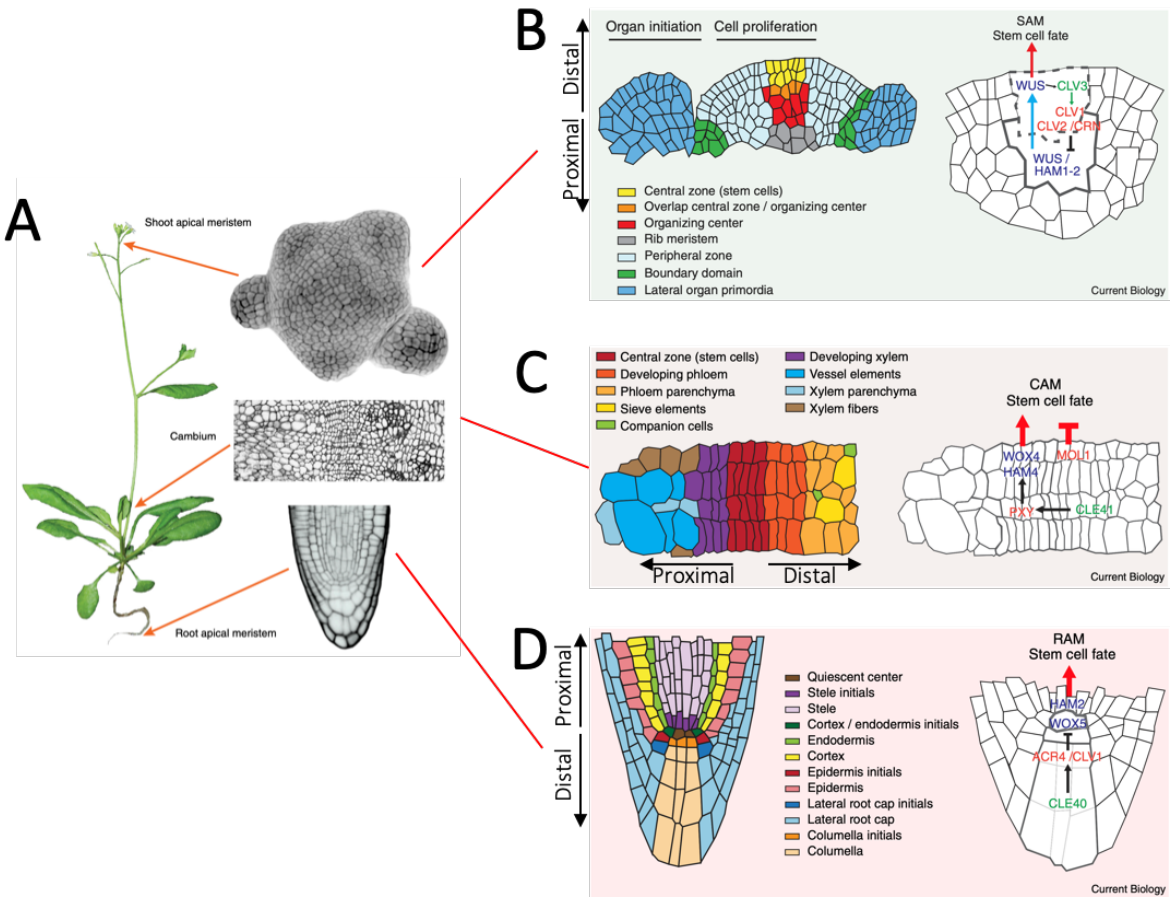


Figure Intro-2 : Arabidopsis thaliana stem cell niches.
(A) Images of an Arabidopsis thaliana plants, and the 3 types of stem cell niches present in the plant. (B,C,D) Schematic representation of stem cell niche organizations and tissue distribution (left), and distribution of WOX-related transcription factor and mobile peptide network (right) regulating the stem cell niche maintenance. (B) Longitudinal section through the middle of the shoot apical meristem (SAM). (C) Cross section through the cambium (CAM), (D) Transverse section of the root apical meristem (RAM). ACR4 : ARABIDOPSIS CRINKLY4, CLE : CLAVATA3-ESR, CLV : CLAVATA , HAM : HAIRY MERISTEM, MOL : MORE LATERAL GROWTH, PXY : PHLOEM INTERCALATED WITH XYLEM, WUS : WUSCHEL, WOX : WUSCHEL-RELATED HOMEOBOX. Adapted from Greb and Lohmann, 2016.

The three types of primary stem cell niches present in Arabidopsis, are using similar regulators. At a local scale, genes from the WOX (WUSHEL RELATED HOMEOBOX) family are expressed in the organizing centre of the SCN. The WOX transcription factor moves to the neighbouring cells where they prevent stem cell differentiation. The WOX transcription factors is regulated by a negative feedback loop. This feedback loop is based on the expression, secretion and migration of peptides belonging to the CLAVATA3/ESR-RELATED (CLE) family, and the interaction of these peptides with a leucin-rich repeat (LRR) receptor like kinase (RLK) receptor (Figure Intro-2, (Gaillochet & Lohmann, 2015; Greb & Lohmann, 2016)). Furthermore, the patterning and the maintenance of the SCN organisation are regulated at medium to long range via hormone gradients (such as auxin and cytokinins), diffusion of peptides and proteins (such as the PLETHORAs and GROWTH REGULATING FACTORs or GEF-INTERACTING FACTORs, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptides), and diffusion of micro RNA (such as miR396, or miR160) (F. Ercoli et al., 2016; M. F. Ercoli et al., 2018; Galinha et al., 2007; Hong et al., 2015; Shi et al., 2018). Auxin and cytokinin gradients contribute to the patterning of the stem cell niche by defining areas with high and low proliferation (Greb & Lohmann, 2016). Additionally, integration of BR signalling occurs in both RAM and cambium, to maintain stem cells undifferentiated and regulate the proliferation of the OC (Vilarrasa-Blasi et al., 2014a).

2.2 Specificities of the shoot apical meristem

The shoot apical meristem (SAM) is located at the apex of the above ground tissues of the plant. Its function is to generate all the green parts (Figure Intro-2. A-B). In Arabidopsis the SAM is a group of small proliferative cells, forming a dome expressing the transcription factor

SHOOTMERISTEMLESS (STM)(Long et al., 1996). In the SAM the stem cells are slowly dividing and located in the 3 outermost layers, while the organizing centre is directly under the stem cells deeper into the SAM. The OC expresses the WUSCHEL transcription factor, which then moves towards the stem cells where it triggers the expression of a short mobile peptide CLAVATA3 (CLV3). CLV3 is then secreted and diffuses between cells and binds to LRR-receptors in this OC and causes reduction of WUS expression (Figure Intro-2.B, (Schoof et al., 2000)). Furthermore, cytokinins are positive regulators of WUS expression and cell proliferation, while auxin induces cell differentiation (Besnard et al., 2014).

2.3 Specificities of the cambium

The cambium is located within the vasculature, between the xylem, a vascular tissue in charge of water transport, and the phloem, vascular tissue enabling transport of signalling molecules and metabolic products over long distances (Figure Intro-2. A-C). It supports radial growth, or thickening, of organs. In this context, WOX4 is the key WUS related gene involved in SCN regulation (Zhou et al., 2015). It is expressed in the central area of the cambium, where the stem cells are located. CLE41/42/44 peptides are expressed in the distal area of the cambium, then move to the undifferentiated part of the cambium and bind to the PHLOEM INTERCALATED WITH XYLEM (PXY) receptor to trigger WOX4 expression (Figure Intro-2.C, (Etchells & Turner, 2010; Fukuda & Hardtke, 2020; Hirakawa et al., 2008, 2010)). Notably, in the cambium, the CLE-Receptor modules positively regulate stem cell fate, instead of inhibiting it as in the SAM and RAM.

A cambium characteristic is to be organized and to function as a bifacial stem cell niche, able to produce tissue both in distal and proximal direction. However, unlike the RAM, the same stem cells are producing tissues in opposite directions.

Regarding hormone patterning, auxin is promoting cell proliferation (Suer et al., 2011). Strong auxin signalling within and around the cambium stem cells defines the position of the stem cells ´ organizer (Smetana et al., 2019; Suer et al., 2011). Similar to WOX5 in the RAM, WOX4 is downstream auxin signalling.

2.4 Specificities of the root apical meristem

The root apical meristem is located at the tip of roots (Figure Intro-2. A-D). It is more structured than the SAM, and leads to the growth and the patterning of root tissues. Contrary to the SAM, but reminiscent of the cambium, the RAM contains a bi-directional stem cell niche. Different populations of initials are producing the distal and proximal tissues, while the OC can replenish any of the initials (L. Dolan et al., 1993). In the RAM, the SCN organizer is quiescent, hence it is named Quiescence Centre (QC).

In the root, auxin promotes cell proliferation and position the SCN within the root meristem (Mähönen et al., 2014) while cytokinins induce cell differentiation. Downstream auxin, key transcription factors, the PLETHORA family, create a gradient of protein, diffusing through the meristem to position and pattern the stem cell niche (Aida et al., 2004; Galinha et al., 2007; Mähönen et al., 2014; Salvi et al., 2020). In the RAM the stem cell niche organizer is specified by the WUSCHEL-RELATED HOMEOBOX 5 gene (Sarkar et al., 2007). Similar to the SAM, WOX5 moves from the OC to the neighbouring columella stem cells to maintain their stem cell state (Forzani et al., 2014b; Pi et al., 2015). Additionally WOX5 integrates auxin signalling (Tian, Wabnik, et al., 2014). Similar to the shoot, WOX5 is under the control of the CLAVATA3-ESR related 40 peptide, and its receptors CLV1 and ARABIDOPSIS CRINKLY4 (Stahl et al., 2013); Meyer et al. 2015) (Figure Intro-2. D). Interestingly, here the peptides and receptors are not expressed in stem cells, but in the differentiated columella cells (Stahl et al., 2013; Wink et al., 2009)

3. Ontogeny of the primary root apical meristem and its stem cell niche.

3.1 Embryonic development

During the development of the root apical meristem, the stem cell niche needs to be formed. Cells with the appropriate tissue identity and arrangement relative to one-another need to be specified and maintained.

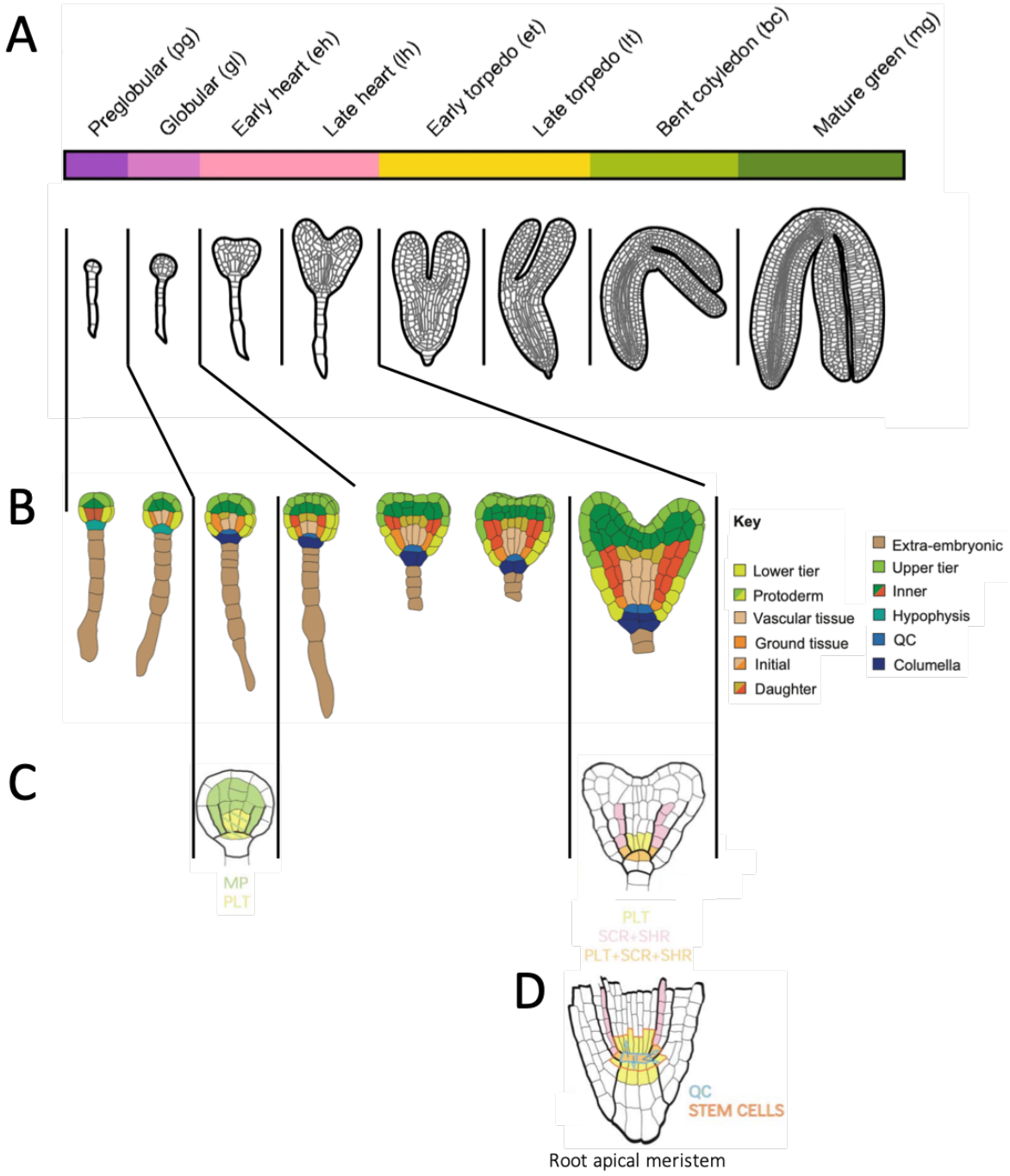


Figure Intro-3 : Lateral root development relies on patterning mechanisms similar to the one of the primary root during embryogenesis.

(A)Schematics of embryo development stages. (B) Schematics of embryo development highlighting tissue identity distribution. (C) Root stem cell niche patterning by PLETHORA and SCR-SHR pathways at globular and late heart stage during embryo development, and post-embryogenesis (D). QC : quiescent center, MP : MONOPTEROS gene, SHR : SHORTROOT gene, SCR : SCARECROW gene, PLT : PLETHORA genes.

(A) Adapted from Hofmann et al. 2019. (B) Adapted from ten Hove et al. 2015.(C and D) Adapted from Aida et al. 2004.

The primary root is first initiated at the 8-cell stage of embryogenesis as the hypophysis is specified (Figure Intro-3. B, (Schlereth et al., 2010)). This cell later on gives rise to both the columella and the organizing centre of the root stem cell niche. As the embryo reaches the late globular stage, epidermis, ground tissues and vascular tissues are defined. The stem cell niche generating the RAM is established at the heart stage of embryogenesis (Figure Intro-3. A-C, (L. Dolan et al., 1993; Scheres et al., 1994)). The *SCARECROW* (SCR) and *SHORTROOT* (SHR) belong to the GRAS family of transcription factors, and define the radial patterning of the stem cell niche. Together those genes specify the QC, the endodermis identity, as well as establishing the stem cells generating both the cortex and endodermis, the cortex- endodermis initials (CEI)(Figure Intro-3. C). SCR is expressed in the QC, the CEI and the endodermis while SHR is expressed in the QC, the CEI and the vasculature. However the SHR protein moves to the endodermis to trigger SCR expression. While SCR is first expressed in the hypophysis of the early globular stage, and SHR in the provascular cells of the late globular stage, it is only at the transition stage of embryo development that the patterns of expression of SCR and SHR are similar to the one observed in the post-embryonic primary root (Liam Dolan, 1997; Helariutta et al., 2000; Laurenzio et al., 1996; Nakajima et al., 2001). Additionally SCR and SHR regulate the interplay between auxin and cytokinin to enable stem cell niche establishment in the embryonic PR (Salvi et al.2018).

WOX5 is expressed at late globular stage in the lens shaped cells which later will become the QC (Haecker et al., 2004). Additionally, PLT1 expression at late globular stage covers QC and vasculature, to contribute to QC fate specification (Aida et al., 2004). Furthermore auxin signalling and polar auxin transport also contribute to the specification of the QC and columella during embryogenesis. Cytokinin signalling is also involved by being first expressed in the hypophysis at the globular stage, then later in the lens shape cell, precursor of the QC, and finally in the QC as the embryo reaches the heart stage (Müller & Sheen, 2008).

As the embryo matures, the RAM forms the radicle, structurally similar to the primary root post-embryonic meristem. It contains a stem cell niche and a short transit amplifying compartment. All the tissues constituting the root are present. From the outside to the inside : the epidermis, the outermost tissue layer, the cortex, the endodermis, the pericycle, and the vasculature at the centre of the root (L. Dolan et al., 1993). At the tip of the root, the stem cell niche contains the QC surrounded by the initials. The quiescent centre , and is located in the Central Cells (CCs). The CCs are cells between the T-divisions but not directly contributing to them. The T-divisions

are a cell wall arrangement formed by the cortex-endodermis initial (CEI), the first cell of cortex, and the first cell of endodermis (Laurenzio et al., 1996)(Figure Intro-5).In the RAM, almost all stem cells are in direct contact with the organizing centre of the SCN. The vascular initials are proximal, shootward to the QC, while the columella stem cells (CSCs), are distal, rootward to the QC. Only the shared initial for the epidermis and lateral root cap (Epi/LRC), are not in direct contact with the QC cells. Covering the SCN, the root cap is composed of the columella, cells directly distal to the QC, and lateral root cap, flanking the columella (L. Dolan et al., 1993; Scheres et al., 1994). At germination the radicle protrudes out of the seed coat and root growth occurs (Finch-Savage et al., 2006). This leads to the formation of several developmental zones along the primary root. Those developmental zones are, from the tip to the shoot (Figure Intro-4):

-the root cap, the gravity sensing apparatus of the root.

-the stem cell niche, from which each tissue originate. The SCN is critical for continuous maintenance of tissue organisation within the root.

-the transit amplifying compartment, characterized by high cell proliferation which increases the amount of cells per tissues.

-the transition zone, where cells transition from cell proliferation to cell differentiation.

-the elongation zones, the initial steps of differentiation start as cells elongate

-the differentiation zone, the part of the root where tissues have reached their final differentiated state. (Liam Dolan et al., 1993; Scheres et al., 2002b)

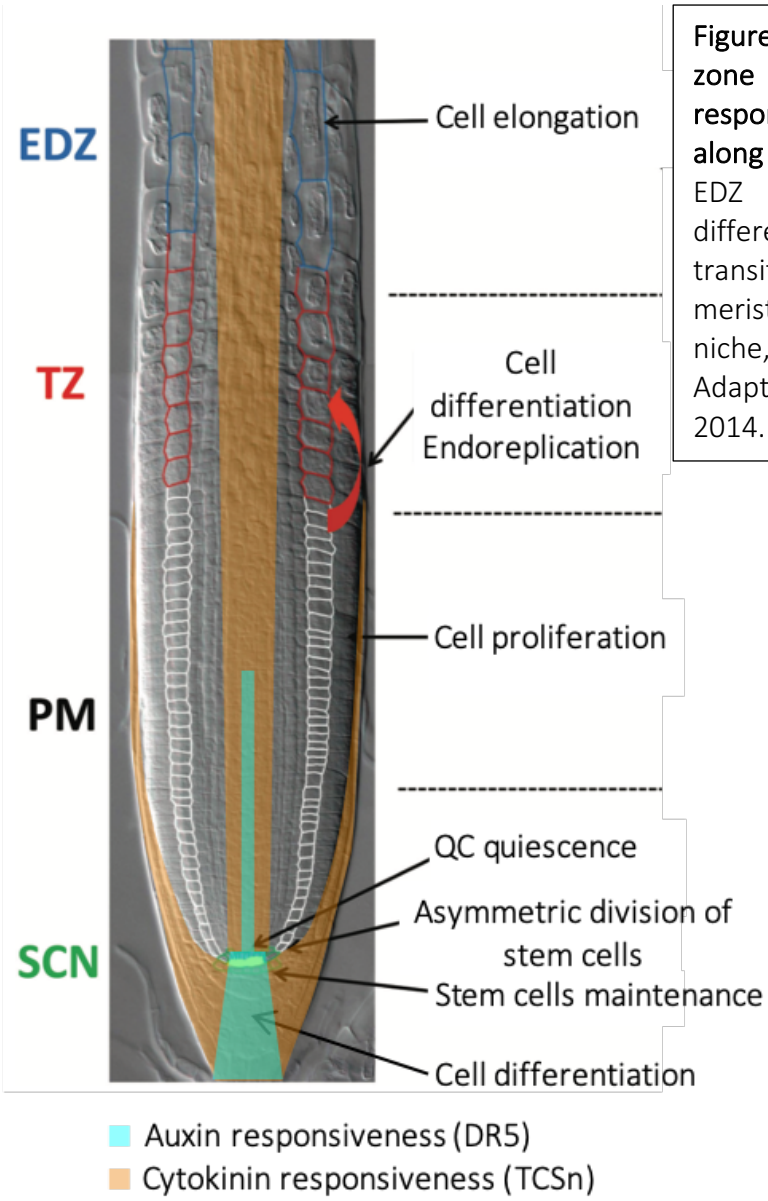


Figure Intro-4 : Developmental zone and auxin/cytokinin responsiveness distribution along the primary root.
EDZ : elongation and differentiation zone, TZ : transition zone, PM : proximal meristem, SCN : stem cell niche, QC : quiescent center. Adapted from Takatsuka et al. 2014.

The different developmental zones along the primary root axis, spans a gradient of proliferation and a gradient of differentiation (Cruz-Ramírez et al., 2013; Efroni et al., 2016; Rahni & Birnbaum, 2019). The QC is quiescent and undifferentiated with a division rates of about once every 4.5 days at minimum (Cruz-Ramírez et al., 2013; Rahni & Birnbaum, 2019). Several genes are involved in the regulation of QC quiescence. WOX5 regulates QC proliferative behaviour by down regulating the expression of the cyclin CYCLIN D1;1 (CYCD1;1), and cyclin CYCD3;3 in the QC cells (Forzani et al., 2014b). SCARECROW (SCR) and RETINOBLASTOMA-RELATED (RBR) protein interact with one another to maintain quiescence in the OC (Cruz-Ramírez et al., 2013). The transcription factor BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO) is expressed in both QC and vascular initials, and represses QC division (Vilarrasa-Blasi et al., 2014a). ETHYLENE RESPONSE FACTOR 115 (ERF115)

is a transcription factor which, like BRAVO, integrates brassinosteroids signalling but promotes QC division upon stress conditions or stem cells damage (Heyman et al., 2013; Vilarrasa-Blasi et al., 2014a).

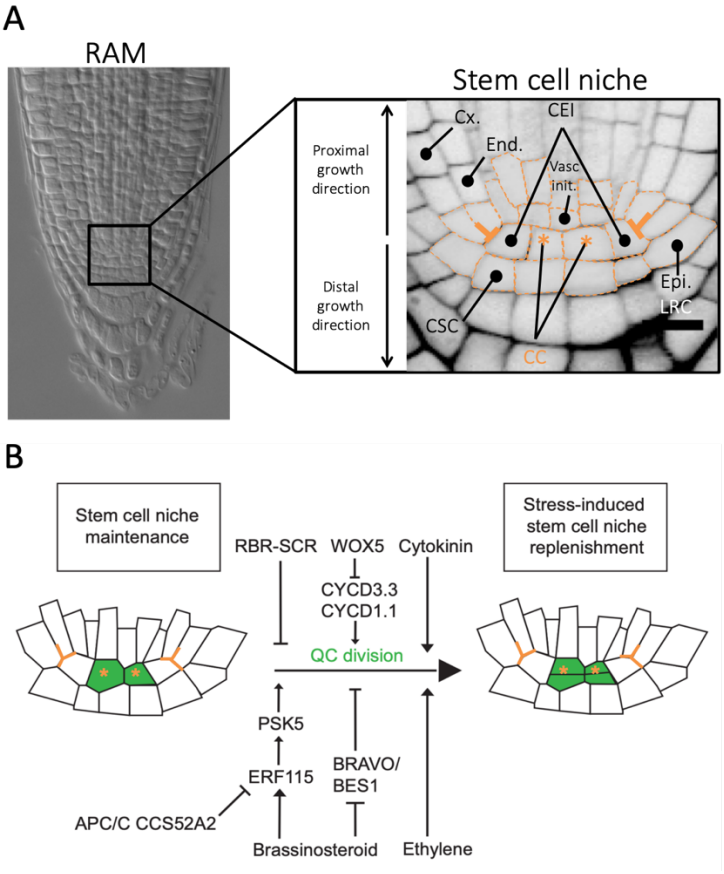


Figure Intro-5 : Topology of the primary root stem cell niche and regulators of QC division.
(A)The stem cell niche of the, primary root is at the tip of the root apical meristem (RAM). The Central Cells (CC) are surrounded by the columella stem cells (CSC) in the distal direction, the vascular initials (Vasc init.) in the proximal direction and the cortex (Cx.)/endodermis (End.) initials (CEI) on the side. Epidermis and lateral root cap initials : (Epi. LRC). The CCs, marked with an orange asterisc, are between but not directly contributing to the T-divisions (orange "T").
(B) In the root apical meristem, the CCs are quiescent, thus named quiescent center (QC). However under specific conditions, the QC can devide. The state of quiescence of the QC is regulated by : the interaction between RETINOBLASTOMA-RELATED (RBR) and SCR : SCARECROW; WUSCHEL-RELATED HOMEOBOX 5 (WOX5), CYCLIN D3;3 (CYCD3;3) and CYCD1;1; PHYTOSULFOKINE PRECURSOR 5 (PSK5); ETHYLENE RESPONSE FACTOR 115 (ERF115); BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO); BRI1-EMS-SUPPRESSOR 1(BES1); anaphase-promoting complex/cyclosome/CELL CYCLE SWITCH 52 A2 (APC/CS52A2(APC/C CCS52A2).
(B) Adapted from Gaillochet et al. 2015

The surrounding stem cells have a higher proliferative activity, dividing in average every 58 h (Rahni & Birnbaum, 2019)The daughters of stem cells create the transit amplifying compartment with the highest proliferative activity of the meristem ranging from about 16h to

24h between 2 divisions, depending on the tissue (Cruz-Ramírez et al., 2013; Efroni et al., 2016; Rahni & Birnbaum, 2019).

Interestingly, as the radicle grows out of the seed coat, there is a transition from meristem establishment (occurring during embryogenesis) to meristem maintenance.

3.2 Root meristem maintenance during post- embryonic growth

Post embryonically, the primary root adopts the typical RAM organization. Stem cell niche positioning is maintained through the auxin gradient. The auxin gradient directs a gradient of the PLETHORA gene products which regulates stem cell niche and meristem activities through long range signalling (Mähönen et al., 2014). PLTs were found to interact with WOX5 to regulate QC quiescence and distal stem cell fate and differentiation (Burkart, et al. 2019). Stem cell niche patterning and definition of the QC is maintained through two genetic pathways : the PLETHORA pathway and the SCR-SHR pathways (Scheres, 2007; Shimotohno et al., 2018). Auxin distribution within the meristem is regulated by cytokinin to control meristem size (Di Mambro et al., 2017, 2019). The mutually exclusive interaction between auxin and cytokinin signalling define the boundaries of the meristem, while integrating inputs form the PLT genes (Bishopp & Benkova, 2011; Salvi et al., 2020).

Radial patterning of the RAM is maintained through the action of the SCR-SHR pathway, where the expression of the CYCD6;1 (CYCLIN D6;1) in the CEI continually induce the formative division leading to the formation of cortex and endodermis layers (Cruz-Ramírez et al., 2012; Cui, 2007; Gallagher & Benfey, 2009; Nakajima et al., 2001; Sozzani et al., 2010). Additionally the SCR protein interacts with the RETINOBLASTOMA-RELATED (RBR) protein to maintain quiescence in the quiescent centre, therefore protecting the stem cell niche organizer from accumulating DNA damages (Cruz-Ramírez et al., 2013). The PLETHORA(PLT) gene family is another important player in stem cell niche patterning and maintenance in the primary root (Aida et al., 2004; Galinha et al., 2007).

4. Lateral root development

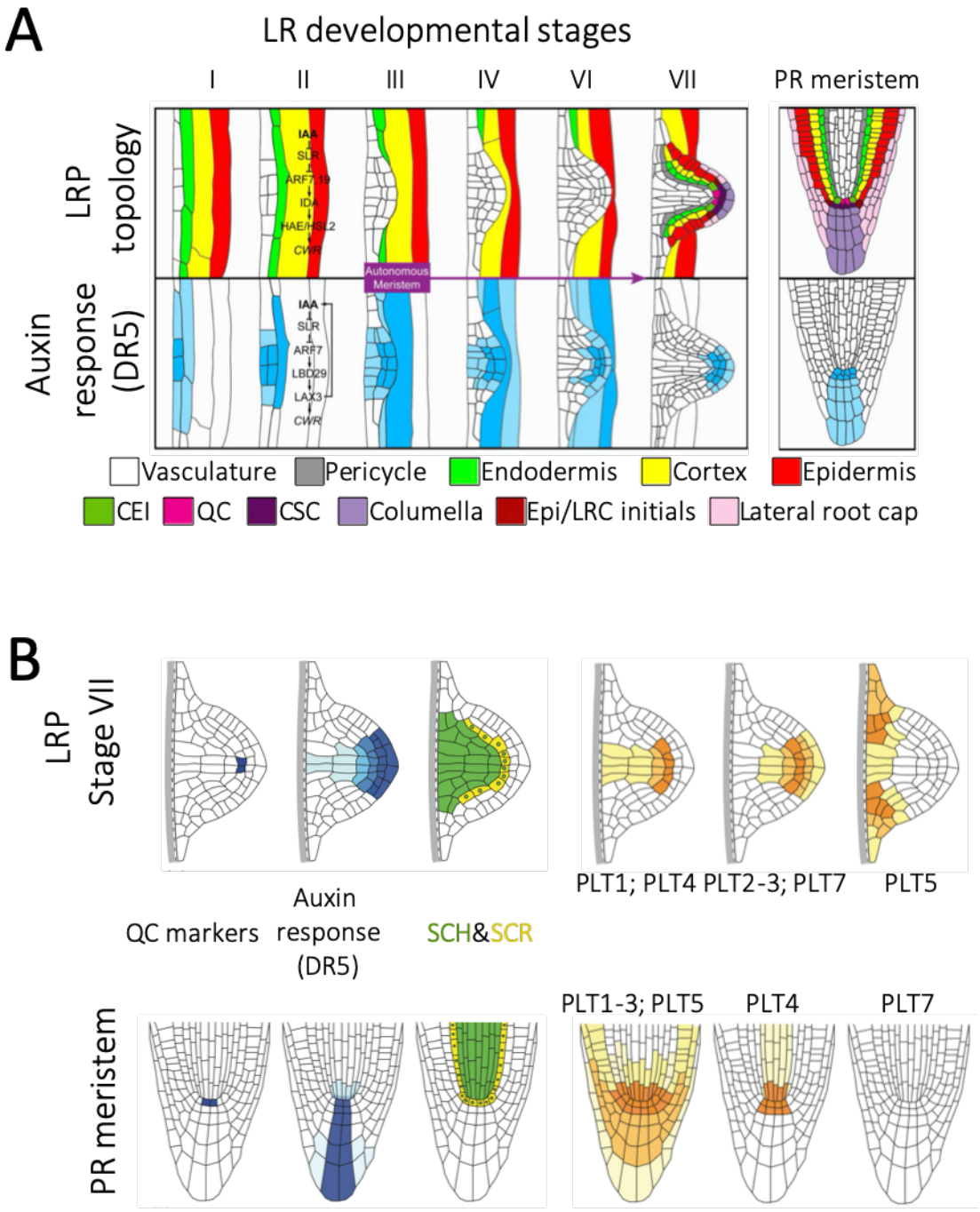
4.1 Cell and tissue organisation

The post-embryonic development of lateral roots starts by the specification of lateral root founder cells (LRFC) within the pericycle cell files abutting the xylem. The specification of LRFC occurs at the exit of the root apical meristem (De Smet et al., 2007). Oscillating auxin signalling combined with signals from apoptotic lateral root cap cells confers to LRFC the capability to yield lateral root development, LRFC become primed (Van den Berg et al., 2018; Laskowsky et al., 2017; Moreno-Ruisero et al., 2010). Higher up the primary root, in the differentiation zone, lateral root development begins with initiation (Dubrowsky et al., 2000). Two to eleven LRFCs start accumulating auxin from the vasculature and show high auxin signalling activity (Casimiro et al., 2001). Asymmetric cell swelling and nuclear migration toward the cell wall shared by neighbouring LRFCs lead to a formative, anticlinal, asymmetric cell division (Goh et al., 2012). Short central cells, flanked by longer cells are thus creating a stage I lateral root primordium (LRP) (de Smet, 2012; Malamy & Benfey, 1997; Vilches-barro & Maizel, 2015; Vilches Barro et al., 2019).

Lateral root development prior emergence is divided into 2 phases : the formation of a lateral root primordium, and the formation of a lateral root meristem, with the capacity to autonomously support LR growth. It was further described that the formation of PR-like meristem topology occurs shortly after the establishment of an autonomous meristem ((Goh, Toyokura, Wells, Swarup, Yamamoto, Mimura, Weijers, Fukaki, Laplaze, Bennett, & Guyomarc, 2016; Laskowski et al., 1995). Malamy and Benfey described lateral root development prior emergence as a

Figure Intro-6 : Lateral root development relies on patterning mechanisms similar to the one of the primary root.
(A)Schematic representation of the transverse section of lateral root primordia up to emergence . Lateral root development is divided in 7 stages, where the overlaying endodermis (green), cortex (yellow) and epidermis (red) are crossed, as lateral root emerge and reproduce an organization similar to the one of the primary root. The gradient of auxin responsiveness (light to dark shade of blue) is progressively established as LR emerge. (B) The key pathways involved in SCN patterning and maintenance in the primary root are also at play during lateral root development, to established the new stem cell niche. CEI : cortex endodermis initials, QC : quiescent center, CSC : columella stem cells, Epi/LRC initials : epidermis/ lateral root cap initials, PR : primary root, SCH : SHORTROOT gene, SCR : SCARECROW gene, PLT : PLETHORA genes.
(A) Adapted from Du et al. 2017. (B) Adapted from Trinh et al. 2018.

succession of seven stages (Figure Intro-6 A).



Characteristics of the LR such as the number of cell layers, the number of cells in a specific layer and the tissue crossed by the emerging LR were assessed on fixed tissues, at the transverse section of the primordium (Malamy & Benfey, 1997). At Stage I, the LRP is formed by pairs of xylem pole pericyle cells, where the anticlinal asymmetric cell division took place. Stage II to IV are characterized by an increase in the number of cell layers composing the primordium. The

endodermis of the primary root is crossed at stage IV. The crossing of the cortex occurs as the LRP grows through stages V and VI. Specific landmarks of stage VI are the periclinal division of the second outer most layer of the LRP to form what is becoming the primordium cortex and endodermis (Figure Intro-6 A.(Malamy & Benfey, 1997)). Additionally, cells at the centre of the LRP are elongated and resemble vasculature. Already at that stage, the general aspect of the LRP is similar to the one of the primary root meristem. And finally the LRP crosses the epidermis to extrude out of the primary root body through the stage VII (Malamy & Benfey, 1997). Post emergence, lateral root development is not described with a clear consensus. Kiss et al. proposed a qualitative classification of emerged lateral roots on the basis of the angle at which they grow in regard to the perpendicular to the axis of the primary root. Type 1 lateral roots are emerging through the epidermis of the PR and growing at 90° from the primary root. Type 2 LRs, are about 500 µm long in average and grow with 26° angle to the PR. Type 3 LRs grow with a 37° average angle and measure about 1 mm. Type 4 LRs grow with a 60° angle, and an average root length of 2.5 mm. Type 5 and 6 grow almost vertically, similar to the PR. (Kiss et al. 2002). This static description of lateral root development was further completed by 3D live-imaging-based analysis (Wangenheim et al., 2016). The pattern of cell division during LR development is not stereotypic, no specific sequence of cell divisions with a defined orientation occurs (Lucas et al., 2013; Wangenheim et al., 2016). The overall LRP shape is also not strongly affected by the pattern of division within the primordium, however, the occurrence of periclinal division leading to new cell layer is correlated with the number of cells within the LRPs (Lucas et al., 2013; Wangenheim et al., 2016). LR development is a self -organizing and non-deterministic process (Wangenheim et al., 2016). Furthermore, as the endodermis accommodates the growth of the primordium, LR radialization takes place (Lucas et al., 2013; Vermeer, 2014). The primordium shape transitions from bilateral to radial symmetry (Lucas et al., 2013). This developmental transition seems to be associated with a change in tissue organization within the LRP. Cell layers are stacked on top of one-another in the dome shaped primordium. As radialization occurs, the LRP adopts a new organization where the different tissues form one cell thick cylinders, arranged in concentric layers (Lucas et al., 2013). During emergence, the LRP crosses through the overlying tissue, generating strong mechanical constraints (Lucas et al., 2013). However, as the LR grows, the overlying tissues accommodate the passing root by relying on auxin signalling induced water transport and cell wall remodelling. (Reinhardt et al., 2016; Roycewicz et al., 2014; Benitez-Alfonso et al., 2013; Kumpf et al., 2013; Peret et al., 2012; Swarup et al., 2009). As lateral root emerge out of the primary root,

their structural composition is similar to the one of the PR (Figure Intro-6 (Bennett & Scheres, 2010; L. Dolan et al., 1993; Scheres, 2007)). However a detailed characterization of the organization and behaviour of LR, post emergence still remains to be done.

4.2 Patterning of hormone and tissue identity

4.2.1 Auxin and cytokinin role in lateral root development

Auxin is involved in LR patterning along the PR, by defining the pre-branching site during the priming of lateral root founder cells (Dubrovsky et al., 2008; Smet et al., 2007; Moreso-Risueno et al., 2010). Later on, auxin plays an important role in LR initiation (Celenza et al., 1995; Casimiro et al., 2001 Dubrovsky et al., 2008; Rybel et al., 2010; Vilches-barro & Maizel, 2015; Vilches Barro et al., 2019). Further in LR development, auxin plays a role in emergence out of the primary root, both by promoting growth within the LR primordium (Celenza et al., 1995; Dubrovsky et al., 2008) and by inducing remodelling of the tissues covering and surrounding the emerging lateral root (Porco et al., 2016; Vermeer, 2014; Benitez-Alfonso et al., 2013; Péret et al., 2012; Swarup et al., 2008). At stages I and II, auxin signalling is spread throughout the LRP, as indicated by DR5 synthetic reporter (Figure Intro-6 A(Benkova et al., 2003)). However, in later stages, until emergence, a gradient of auxin signalling is established. The tip of the LRP shows the highest DR5 signal while the flanks and basis show low to no expression, thus establishing an auxin maxima, positioning the new stem cell niche of the LR (Figure Intro-6 A(Benkova et al., 2003)). In the LRP, auxin gradient in controlled by polar auxin transport mediated by the PIN-FORMED (PIN) (Tang et al., 2017;; Guyomach et al., 2012; Petrasek et al., 2009, Benkova et al., 2003) proteins. Post emergence auxin is to also involved in meristem activation and maintenance. For instance the mutant *alf3-1* shows LRs arrested shortly after emergence, a phenotype which can be rescued by exogenous IAA application (Celenza et al., 1995).

While cytokinins are key players of meristem regulation in the primary root, much less is known about their involvement in lateral root development. However cytokinin signalling affect LR positioning along the PR, by inhibiting LR initiation in the neighbourhood of already existing LRs (Bielach et al., 2012; Jing & Strader, 2019). Furthermore, exogenous CK application prevents LR initiation, and affect the organization of LRP which managed to develop (Laplaze et al., 2007).

Additionally CK signalling affect auxin signalling by modifying auxin transport (Marhavý et al., 2011).

4.2.2 Identity patterning during LR development

Malamy and Benfey, first described tissue patterning during LR development using an enhancer trap approach (Malamy & Benfey, 1997). The authors describe that epidermis identity is visible at stage VI, while cortex identity is established just before emergence. Endodermis identity is first visible at stage V in the second outermost layer of the primordium while vascular identity is seen only post emergence in central part of the LR at its basis (Malamy & Benfey, 1997). However more recent descriptions indicate that endodermis identity, revealed by a transcriptional reporter of the SCR gene, is first visible in stage II LRPs (Du & Scheres, 2017; Goh, et al. 2016), while epidermis identity was found visible at stage VI (Du & Scheres, 2017). Furthermore, the organizing centre identity has been reported as early as stage V (Goh, et al. 2016). However, key regulators of SCN patterning such as SHR, SCR the PLT or WOX5 are already expressed in stage II LRP (Du & Scheres, 2017; Hofuis et al., 2013).

4.3 Open questions

Despite the extensive knowledge about lateral root development prior emergence, it still remains unclear when exactly a functional, primary-root-like stem cell niche is established. In other words, when is the formation of the new meristem completed, and lateral roots start foraging their environment to support growth of the whole plant.

AIM of the study

Lateral root founder cells are a subset of xylem pole pericycle cells, primed in the primary root (PR) meristem. In the differentiated part of the PR, those cells re-enter cell cycle and form a lateral root primordium which grows into replicating the tissue organisation of the primary root. During this process a stem cell niche is established newly.

Whereas the mechanisms responsible for the maintenance of SCNs in the PR are well described, there are in comparison little known about how a SCN is post-embryonically established de novo. Lateral roots are therefore a convenient model to study de novo stem cell niche formation. While lateral root development has been well characterized, a detailed and comprehensive analysis of the ontogeny of the SCN establishment is still to be proposed.

The main objective of this thesis is thus to characterize, in the model plant *Arabidopsis thaliana*, the process of stem cell niche establishment from the beginning of lateral root development until it reaches a primary root-like organization.

First, I established a developmental frame of reference to follow LR growth from stage I up to 2mm post emergence, addressing the question of how are the topology and patterning of the SCN established. This will be covered in Chapter 1.

Second, I did a functional characterisation of the behaviour of the SCN and its OC during LR development. To this end, I assessed cell proliferation in the SCN and the function of the OC using EdU incorporation, lineage tracing and sensitivity to genotoxic stress. This will be covered in Chapter 2.

Finally, to provide insights into the role and the relevance of the timing of OC proliferation during LR development I challenged the system by manipulating the proliferative behavior of the OC. For this, I used mutants and ectopic expression of key transcription factors to modify OC behavior This will be covered in Chapter 3.

Results

Chapter 1: Cell organisation and patterning during lateral root stem cell niche establishment

1.1 Progressive establishment of a primary root-like stem cell niche in the developing lateral root

Lateral roots grow into mimicking the tissue organisation of the primary root, including the presence of a stem cell niche with well identified central cells. In this part, I analysed the ontogeny of the stem cell niche in lateral roots, focusing on two questions :

1) At which stage are the central cells first visible ?

2) At which stage is the number of central cells stabilized at 2 in the root transverse section?

To compare lateral roots to the primary root, both are imaged at their transverse section by confocal microscopy after cell wall staining with Calco Fluor White (Ursache et al., 2018). I focused on the number of central cells that serve as organiser of the stem cell niche in the primary root and the number of columella cell layers. Central cells (marked with asterisks (*)) are located between the two T divisions (marked with a "T"), and their number is equal to the number of cells between these two divisions minus 2 to account for the stem cells (see Figure 1A).

Classification of LR development is typically done with qualitative terms (stages I to VII) before emergence (Malamy & Benfey, 1997) but no consensus toponymy exist post-emergence. To circumvent this, I standardize the stage of LR development by using their length. Lateral root length is measured from the basis, the junction with the vasculature of the PR, up to the tip (Figure 1. A). Roots from different genotypes, across experiments and at different developmental stages can thus be compared in an impartial manner.

To simplify the analysis, root lengths are binned in different categories : "Pre-emergence", spanning all lateral root primordium stages up to 80µm length; "Short" emerged lateral roots from 80 to 250µm. "Medium" lateral roots from 250µm to 1000µm. "Long" lateral roots from 1000 to 2000µm and finally the "Very long" lateral roots, longer than2000µm.

1.1.1 Variability in number of central cells diminishes during lateral roots development.

In the primary root 1 to 2 central cells are visible in the transverse section in ~90% of cases (Figure 1C and (L. Dolan et al., 1993)). In the lateral root, this number is more variable. Before emergence, it ranges from 0 to 6 in lateral root primordia, with two central cells only visible in ~40% of the cases. As LRs develop post-emergence, the variability progressively diminishes (Figure 1. C). This indicates that in lateral roots the emergence of a cell organisation as encountered in PR is a progressive process.

In the primary root the number of cells in the gravity sensing columella is maintained homeostasically. Indeed, the tight coordination of the columella stem cell activity with the sloughing off of the outer most layer of root cap leads to columella of 5 layers in PR of 5days post germination (dpg)(Dubreuil et al., 2018; Fendrych et al., 2014). In lateral roots, we assume that similar to the PR, a topological stability of the stem cell niche is associated to the homeostasis of the lateral root columella. To ascertain this, I documented the organisation of the columella during LR development.

Here (Figure 1), the first columella layer counted is the one directly abutting the CC in the distal direction. Cell layers are counted up to the furthest layer of cells still attached to the root cap. Before emergence, there are mostly 2 layers of columella in the LR. This number progressively increase as LR grow. At emergence, LR length ranges from 60 to 80µm, and the columella reaches 3 layers. Three full layers of columella can be observed in short LR as early as ~100µm. Shortly before LR reach medium size, the columella transitions toward a 4 layer. The columella size is maintained at 4 layers up to 2000µm length. The columella reaches 5 layers only in very long lateral roots (Figure 1. D).

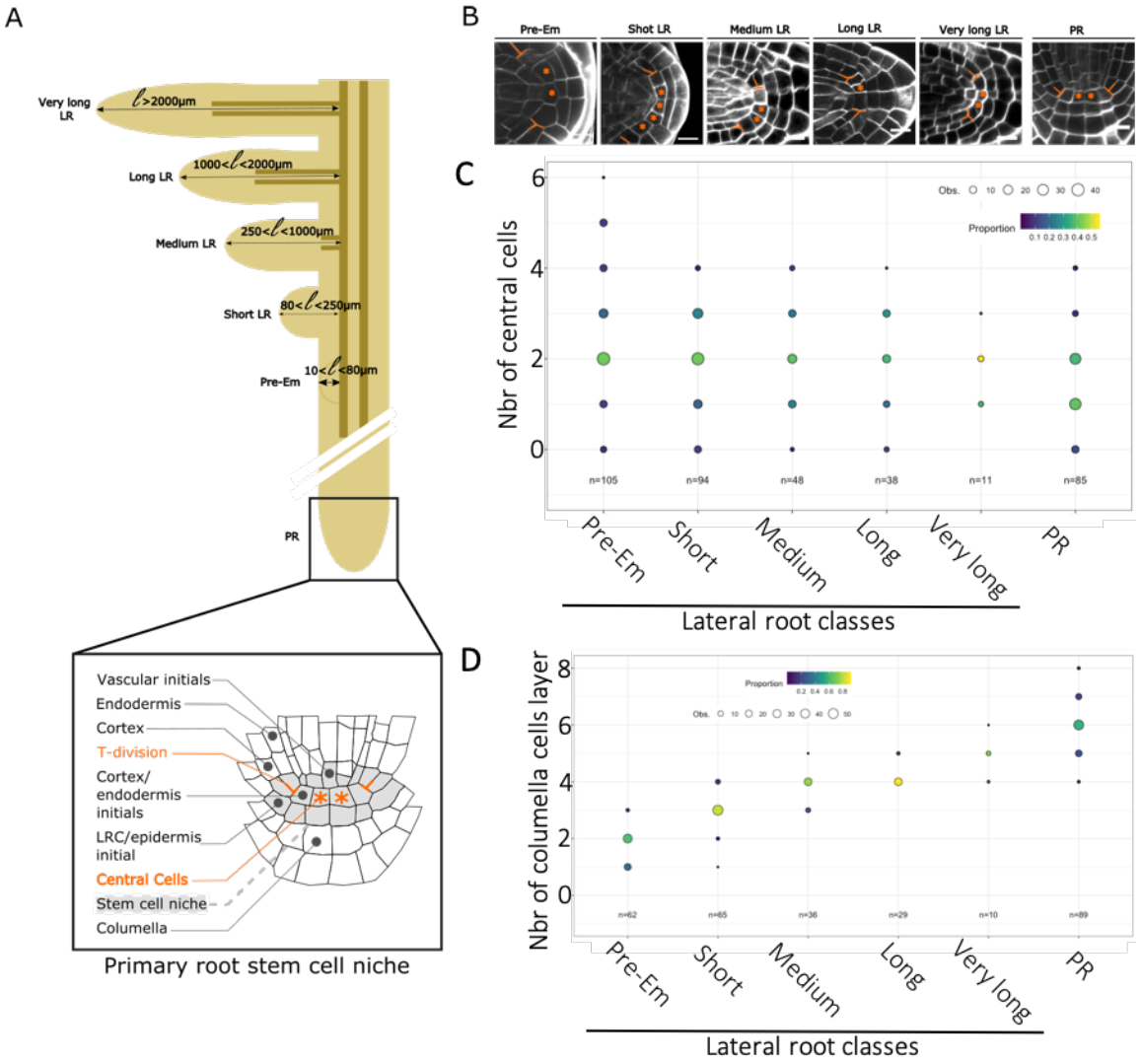


Figure 1 : Ontogeny of lateral root stem cell niche topology during lateral roots development.

(A) Lateral root length (l) is measured in the transverse section of cell wall stained lateral roots, from the connexion to the xylem of the primary root, to the tip of the lateral root. Measurements are aggregated into the indicated length classes. The primary root (PR) is used as a reference for the stem cell niche topological features such as the T-division (orange T) and the central cells (orange asterisks) assessed on transverse section of roots. (B) Confocal micrographs of the transverse section of LR SCN representative of each length class. Number of central cells (C) and of columella layers (D) for the different classes of roots. The size of the circle is proportional to the number of observations (Obs.), and the colour represents the relative proportion of observations within a given class. Scale bar : 10µm.

These observations suggest that homeostasis in the root cap is established very late in LR development, far after the SCN topology is established.

1.1.2 Topological changes during emergence of the embryonic primary root and lateral root topology follow different trends.

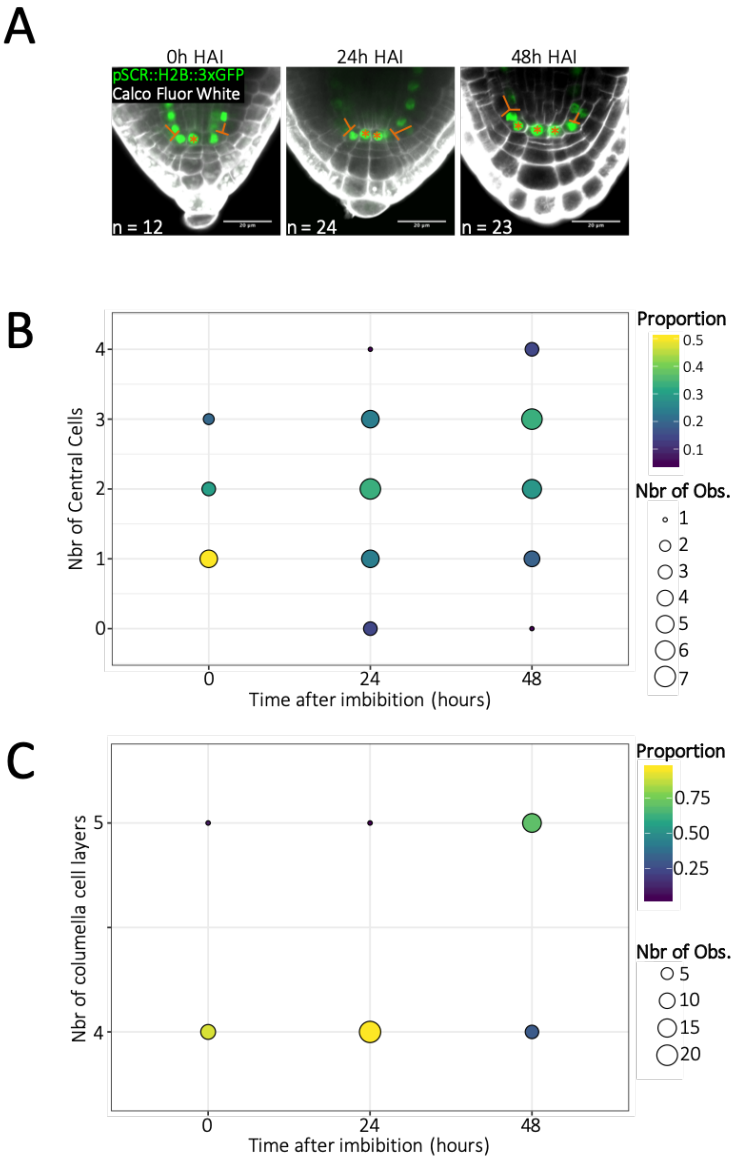


Figure 2: Ontogeny of lateral root stem cell niche topology during lateral roots development.
(A) Stem cell niche topological features (the T-division (orange T) and the central cells (orange asterisks)) are assessed on transverse section of cell wall stained radicles expressing the SCR transcriptional reporter. The topology is assessed at different time after imbibition, spanning the emergence of the radicle out of the seed coat.
(B) Quantification of the number of central cells (C) and of the number of columella cell layers. n : number of seedling observed The size of the circle is proportional to the number of observations (Nbr of Obs.), and the colour represents the relative proportion of observations within a given class. Scale bar : 20µm.

Based on the similarities between the primary root and lateral roots, we assumed that the topology of the embryonic primary root, the radicle, could be reminiscent of the one of emerging lateral roots. To test this, I assessed the number of central cells and the number of columella cell layers at the transverse section of cell wall stained radicles emerging out of the seed coat. Radicle emergence takes place at about 24h post imbibition (Mansfield & Briarty, 1991).

Before radicle emergence (0h after imbibition), the primary root meristem and its stem cell niche are already formed (Figure 2, (Scheres et al., 1994)). The SCN is most frequently observed with 1 CC and 4 columella layers (Figure 2 A and B). After emergence from the seed coat, the

number of CC is more variable and increases, while the number of columella cell layers remains stable until 24h post imbibition, then increases up to 5 layers.

The time frame of 24h to 48h after imbibition is comparable to the 24h following lateral root emergence out of the primary root tissues. This roughly corresponds to the class of short lateral roots. While both the PR and LR undergo emergence, their respective SCN topological features are different.

1.2 Patterning of the stem cell niche during lateral root development.

In plants, fate commitment and identity acquisition are predominantly determined by position rather than by lineage (Berg et al., 1995; Kidner et al., 2000; Pierre-Jerome et al., 2018). The objective of this section is to investigate how, within the evolving topology described above, the molecular markers associated with the SCN and the tissues it generates, are distributed.

1.2.1. Auxin and Cytokinin signalling locate the stem cell niche at the tip of the short lateral roots.

Hormone gradient provide morphogenetic cues involved in the patterning and delineation of developmental zones (Salvi et al., 2020). In the primary root, auxin and cytokinin signalling define the meristematic zone (Bishopp & Benkova, 2011; Salvi et al., 2020; Su et al., 2011), the position of the stem cell niche and its organizer. The transcriptional activity triggered by auxin and cytokinin signalling is visualized with the DR5v2 and the TCSn reporters respectively.

In the primary root, *DR5v2::Venus* (Heisler et al. 2010;(Liao et al., 2015)) is highly expressed in the OC and in the columella whereas the TCSn reporter, driving the expression of 2xVenus-NLS, highlights cytokinin responsiveness (Network et al., 2013). TCSn is expressed in the root cap and in the vasculature, just above the OC, as well as at the beginning of the differentiation zone (Figure 3. A, (Network et al., 2013)). Thus TCSn reporter marks the end of the meristematic zone, and surrounds the stem cell niche.

In lateral root, the pattern of auxin and cytokinin signalling has been mostly studied before emergence (Adamowski, 2015; Benkova et al., 2003). I could confirm the observations that similar to the primary root, they delineate the position of the SCN (Figure 3). DR5v2 is expressed throughout the transverse section of LRPs from stages I to IV (Figure 3.B, (Benkova et al., 2003)). As LRPs grow further, the DR5v2 expression is further restricted to the tip (Figure 3). However

in emerging LR, the highest DR5v2 expression is between the T-divisions and in the cells positioned in the columella area (Figure 3). The pattern remains similar in longer LR, therefore reproducing the auxin signalling visible in the primary root.

TCSn expression in not visible in LR primordium up to emergence (Figure 3A, Chang et al. 2015), as described in (Bielach et al., 2012). However it is detectable in the vasculature under the LRP, and in the tissues directly overlying the growing primordium (Bielach et al., 2012).. In short LR, TCSn signal is acquired sequentially, first in the vasculature, then in the columella. In longer LR, TCSn expression is present in the 2 to 3 outermost layers of columella (Figure 3A). This expression pattern in reminiscent from the one of the primary root.

Taken together these observations indicate that auxin signalling positions the SCN very early during LR development, while the complementary cytokinin signalling patterns is established only post emergence. In short LR, the SCN is therefore restricted to the few cells which strongly express DR5v2 between the vasculature and the root cap. Additionally the expression of TCSn in both the vasculature and the root cap suggest that in short LR, up from ≈200µm, two directions of differentiation are established (from the SCN toward the basis of the LR, and from the SCN toward the tip of the root). This could indicate the presence of a SCN that supports bidirectional growth, similarly to the primary root (Rahni et al., 2016).

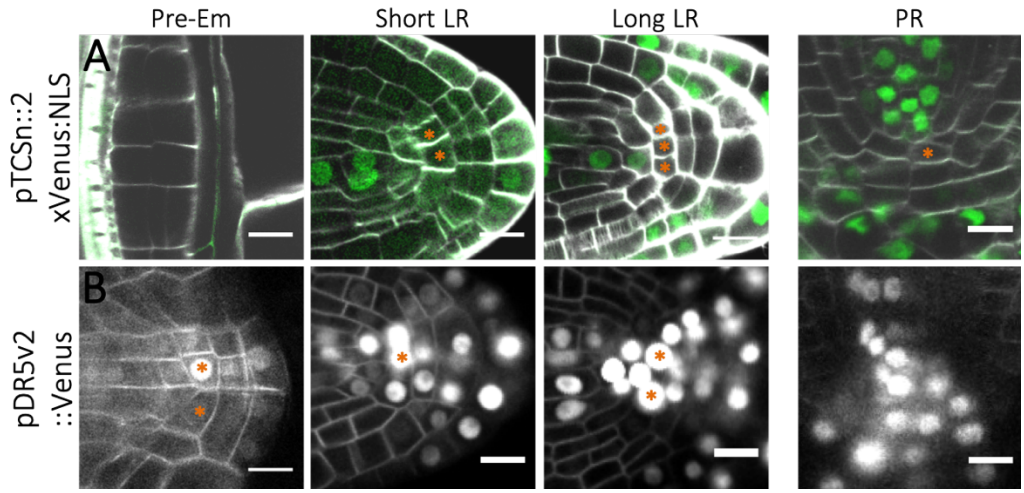


Figure 3: Auxin and Cytokinin transcriptional output during lateral root development.
Cytokinin (A) and Auxin (B) transcriptional output visualized on transverse sections of lateral root of different developmental stages. In (A) cell contour are highlighted by cell wall staining (white lines), whereas in (B) signal is coming from a GFP-tagged plasma membrane PIP2 protein. The central cells are marked with an orange asterix when possible. Scale bar 10µm.

1.2.2. Patterning of stem cell niche identities.

While the transcriptional read out of hormonal cues defines different zones within an organ, it does not describe strictly the identity

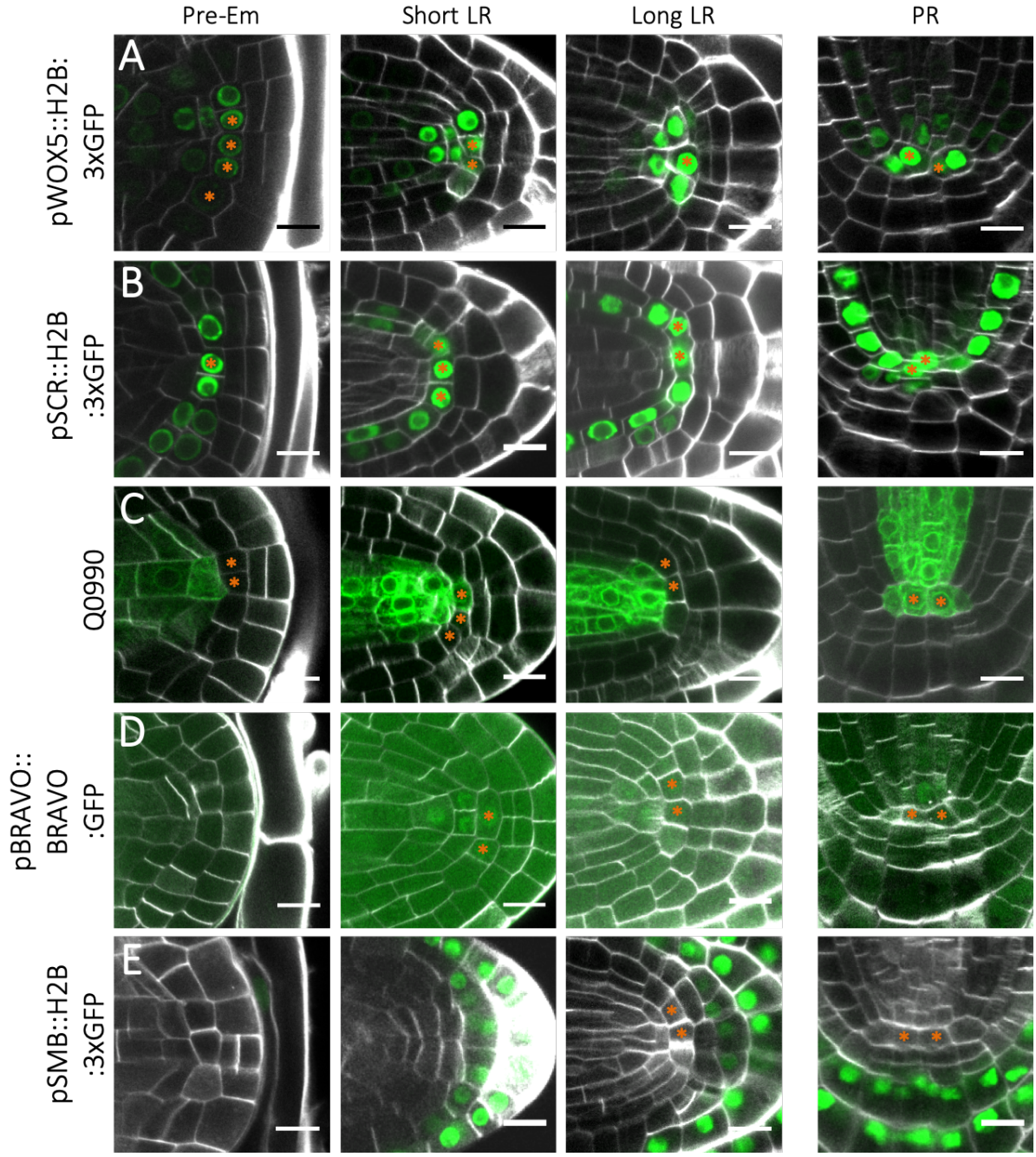


Figure 4 : Ontogeny of SCN markers expression during LR development.
Stem cell niche identities reporters (A-E) at the transverse section of cell wall stained lateral root of different developmental stages. Central cells are marked with orange asterisks. Scale bar 10µm.

patterning taking place during the development. Tissue identity can be extrapolated from the expression of enhancer trap lines or from transcriptional reporters of genes known to be specifically expressed in a given tissue. I used 2 categories of reporters/enhancer trap lines. The first category

marks several components of the stem cell niche, for instance by being expressed in both the OC and a sub-population of stem cells. The second category marks only a specific stem cell type or the OC.

The reporters which marks both the OC and other components of the SCN are WOX5, SCR and BRAVO reporters and the Q0990 enhancer trap line (Radoeva et al., 2016). WOX5 is a transcription factor involved in the organizing centre function of the CC in the primary root (Sarkar et al., 2007). It is highly expressed in the CC, and the CEI. To a lower intensity its expression can also be detected in the vascular stem cells, but not in the columella initials (Figure 4.A and (Pi et al., 2015)). The transcriptional fusion *pWOX5::H2B:GFPx3* is assumed to label OC and more the whole SCN.

The first *WOX5* reporters used were created in the lab. It yielded expression in the OC, CEI and the vascular initials of the primary root. In lateral root primordia, *WOX5* is expressed throughout the whole primordium from stage I until emergence (Figure 4 A, Pre-Em.). Post-emergence, *WOX5* expression is the strongest in the CC and the CEI despite some remnant expression in the vasculature(Figure 4 A). As the expression of WOX5 occurred earlier and in a broader way than expected (Goh et al., 2016), another reporter was used to challenge the observed expression pattern in the growing LRP. The line created in the lab for WOX5 expression might not contain all the cis regulatory elements normally involved in WOX5 expression regulation. I thus used a translational reporter line in which the genomic WOX5 locus is tagged (*pWOX5::gWOX5:3xYFP*) and expressed in the *wox5-1* nul mutant background. This line, kindly provided by the lab of Thomas Laux, was shown to recapitulate the WOX5 expression pattern in the primary root and rescue the mutant phenotype. Interestingly, the same expression pattern was observed as with the transcriptional reporter. From stage I to stage VI WOX5 is expressed throughout the LRP, but the flanks (Figure 5).

The early and broad expression of WOX5 in lateral roots is unexpected. Indeed, it is first visible when no topological stem cell niche is present, then is much wider than the cells compositing the SCN. Those observations raise the point that WOX5 might play a different role in early lateral root development than it does in the primary root. It also suggests that in lateral roots, WOX5 might mark more than just the OC identity.

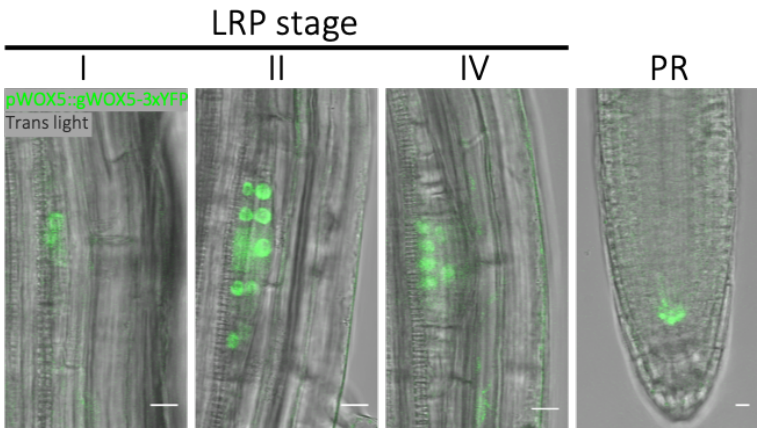


Figure 5 : Genomic WOX5 expression during early lateral root development. Confocal microscopy of the transverse section of LRP expressing the translational reporter *pWOX5::gWOX5:3-YFP*, in the *wox5-1* mutant background. Scale bar 10µm.

The SCARECROW (SCR) transcription factor establishes endodermis identity throughout the plant. It is also critical for the whole stem cell niche patterning. In the primary root SCR transcriptional reporter is visible in the CC, in the neighbouring CEI and in the endodermal cells (Figure 4.B, (Laurenzio et al., 1996)). It the context of lateral root, we assume that SCR expression represents the 3 above mentioned identities. SCR is first detected at stage II in the upper layer of the primordia, specifically in the small central cells of the upper layer (Figure 4.B, (Malamy & Benfey, 1997)). At stage III SCR is expressed in the 2 upper layers of the 3 layer primordium. As the LRP crosses the endodermis, the expression of SCR fades away from the outermost layer (where it still remains but at a low level). From stage VI of LRP development, SCR expression is mostly restricted to the layer under the outermost layer. Between stage VI or as soon as the T-divisions are distinguishable within the LRP, the SCR expression pattern is similar to the one observed in the primary root, and remains stable as LR grows further. Similarly to WOX5, the onset of SCR expression occurs prior the establishment of a topological stem cell niche, and could indicate a different role of SCR in LR SCN patterning compared to the PR. However this also suggests that very early on, the SCN might be established in the LR and that its patterning occurs in absence of the typical PR-like topology. Furthermore, the early SCR expression might as well reflect the specification of a layer of endodermis within the primordium instead of the onset of the stem cell niche.

In the proximal direction, vascular initials are another subset of the SCN. The two following reporters highlight the vasculature and/or the vascular stem cells in addition to the OC. BRAVO is a transcription factor regulating the proliferative behaviour of the OC in the PR. It is expressed in the OC itself and in a few vascular cells proximal to the OC (Figure 4. D (Vilarrasa-Blasi et al.,

2014a)). BRAVO is first detected as LR reach about 100µm. It is visible in the vascular initials, and to a lower intensity in the CC, and some CEI in a non-consistent manner (Figure 4. D). The observed expression pattern does not change as the LR grow longer (Figure 4. D), and in either case is reminiscent of the one observed in the primary root.

In addition to BRAVO, the enhancer trap line Q0990 also highlights the CC and CEI, but is expressed in the whole vascular cylinder : the initials, their daughters, and vascular cells above the meristem. In lateral root, cells between the T-divisions and Q0990 positive are considered as OC/CEI while all the other cells expressing the enhancer trap line are considered as vascular tissue. Q0990 expressing starts at stage IV. The expression is limited to the central part of the LRP, where the vasculature is expected to be. Up to emergence, Q0990 expression is excluded from the 2 outermost layers of the LRP and from the cells between the T-division. It is only post-emergence, when LR reach about 200µm that Q0990 expression starts to be visible in the CC and CEI, in addition to the vasculature (Figure 4. C). Both BRAVO transcriptional reporter and Q0990 enhancer trap line indicate that OC identity might arise post emergence, while a vascular domain is defined already within the LRP at stage IV.

On the other side, the distal patterning of the stem cell niche involves the establishment of the columella stem cells (CSC) and lateral root cap initials (LRCi). In the primary root the SOMBRERO transcription factor is strictly expressed in all the differentiated cells of the root cap but is excluded from the CSC (directly abutting the CC in the distal direction), and the LRCis (Figure 4.E, (Bennett et al., 2010; Fendrych et al., 2014; Kamiya et al., 2016)). Thus, the expression pattern of the SMB transcription reporter (*pSMB::H2B:GFPx3*) gives indirect evidences of the presence of distal stem cells. Indeed cells distal to the CC but not expressing SMB can be considered as CSC or LRCi. SMB is first detected at emergence (Figure 4. E). It is expressed in the outermost layer of the root cap, but the layer directly distal to the CC does not show the reporter. As LR grow longer, the number of columella layer increases and all additional layers express SMB (Figure 4. E). However the layer in direct contact with the CC still does not express SMB and is therefore assumed to be CSCs. This suggest that on the distal side, the SCN patterning might occur slightly earlier than in the proximal side, as CSCs seem to be present at emergence already.

Taken together, these observations suggest that the patterning of the different identities of the stem cell niche starts early during lateral root development (prior emergence for tissue identities proximal to the CC). However, OC establishment seems to occur only shortly after emergence.

To refined our understanding of the OC specific identity patterning, additional enhancer trap lines and transcriptional reporters were assessed. The enhancer trap line QC184 and QC25 driving a GUS reporter are both expressed in the CC and the CEIs of the PR (Figure 6). Their expression expands to the columella cells as the primary root grows older (from 5 days post germination)(Timilsina et al., 2019). The expression pattern of QC184 and QC25 was assessed in lines kindly provided by Thomas Laux (Pi et al., 2015). The first one is in the *wox5-1* mutant complemented by *pWOX5::gWOX5:YFP*, while the second one is in the Col-0 WT background. A GUS staining of 48h was performed on each of the line to reveal the expression of the enhancer traps. All three lines consistently show neither QC184 nor QC25 expression in lateral root primordia from stage I up to emergence (Figure 6 A). The transition toward QC25 and QC184 expression in the OC occurs post-emergence, in short LR. Together , the observations of those 2 markers supports the post-emergence acquisition of OC specific identity.

To further confirm this, I used a truncated version of the AGAMOUS-LIKE 42 (AGL42) gene fused to GFP as a translational reporter indicative of the OC identity in the PR. The AGL42 gene was found to drive the QC specific expression of an enhancer trap, and associated to genes which are QC specific (Nawy et al., 2005). It is mainly expressed in the CCs and CEIs, with a low residual expression in the vascular initials (Figure 6(Nawy et al., 2005)). Preliminary observations show that AGL42 is first expressed in lateral root of about 100µm length. The very low expression is localized in the CC and some of the CEI. As lateral root grow longer (up to about 1000µm), the expression of AGL42 remains restricted to the CC and CEI.

Altogether those observations indicate that stem cell niche patterning starts early during lateral root development, but is completed by the acquisition of OC specific identity only post-emergence. The next step is to understand how those identity translate into specific behaviours.

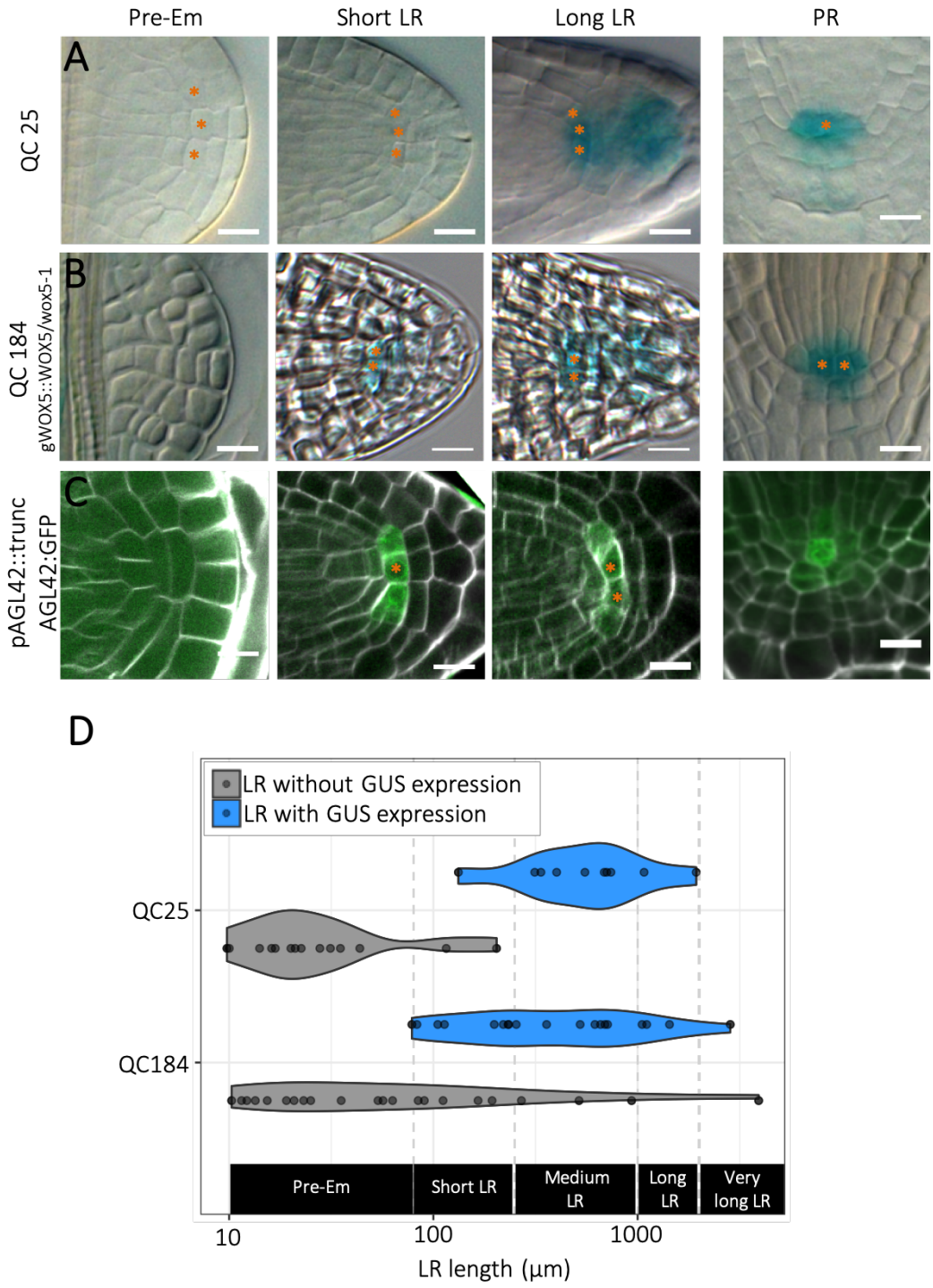


Figure 6 : Ontogeny of OC specific marker expression during LR development.
Expression of OC specific markers at the transverse section of LR and PR.
(A) Expression of the *QC25:GUS* enhancer trap line in the Col-0 background. (B) Expression of the *QC184:GUS* enhancer trap line in the *wox5-1* mutant complemented by *pWOX5::WOX5-YFP*. (C) Expression of the translational reporter of a truncated version of AGL42 transcription factor, in cell wall stained roots. Scale bar 10µm. (D) Violin plot of the length of lateral root showing GUS staining for QC25 or QC184 markers in the SCN. Scale bar : 10µm.

Chapter 2: Functional characterization of SCN during LR development.

Identity reflect the onset of transcriptional programs generating specific cell/ tissue behaviors in order to perform a function useful to the whole organism. So far the distribution of tissue identity within the growing lateral root indicates that very early on during LR development there seem to be an organizing centre (Figure 4 and 4. (Du & Scheres, 2017b; Goh et al., 2016)). However markers of identities do not inform about the actual behaviour and functionality of the cells expressing them.

Therefore the aim of this chapter is to address the proliferative behaviour of the CC as well as their functionality as stem cell niche organizer. As previously mentioned, a characteristic feature of the OC is to be quiescent compare to its neighbouring cells (L. Dolan et al., 1993). This led to determining when quiescence, or very slow cell cycling, is established in the central cells during lateral root development.

2.1 Central cells quiescence is established post emergence in short lateral roots.

To assess cell proliferation during the whole development of lateral roots, EdU incorporation assays were performed. EdU, 5-ethynyl-2'-deoxyuridine is an analogue of thymidine which is incorporated into DNA during the S phase of the cell cycle (Kotogány et al., 2010; Salic & Mitchison, 2008). The incorporation of EdU, over a given period of time, integrates all cells going through cell cycle during the incubation period. After incorporation, EdU is linked to the Alexa647nm fluorophore via the Click-iT reaction (Bourge et al., 2015; Salic & Mitchison, 2008) rendering proliferative cells detectable by confocal microscopy.

In the experimental set up that I used, lateral root formation is synchronized by gravistimulation. As described for in the identity patterning section, SCN topology and proliferative state are assessed at the transverse section of cell wall stained lateral roots. Cells containing EdU or the expression of the G2/S phase marker of Cytrap (Yin et al., 2014) are considered actively proliferative.

2.1.1 Organizing centre cells are proliferative prior emergence

LRPs at stages IV to emerging are induced by 30h, 36h or 42h of gravistimulation. EdU incubation occurred during the last 8h of the gravistimulation before fixation and EdU revelation. The SCR transcriptional reporter introduced in the previous chapter is used to mark OC and SCN identities. Therefore, within the same observation, identity distribution and proliferative behaviour are combined with topological information, based on cell wall staining.

To further narrow down on cells susceptible to be and behave as OC, I referred to the expression profile of the DR5 GUS reporter, published by Benkova et al. (Benkova et al., 2003). I defined a "High DR5 area" as a group of 4 centrally localized cells, with strong DR5-GUS signal, within the 2 uppermost cell layers, at the tip of the LRP (outlined area in the Calco Fluor white channel, Figure 7 A). Within the "High DR5 area", the proportion of proliferative OC is calculated as the number of cells which are SCR and EdU positive, out of all the cells being SCR positive only.

At stage IV, before the LRP crosses the directly overlying endodermis, 4 LRPs out of 5 showed proliferation in 100% of their OC cells. At stage V, half of the LRP (n = 3 out of 6) show a fully proliferative OC. And at stage VI when the LRP starts crossing the cortex, half of the observed LRP show 100% of proliferative OC cells (n =5 out of 10). None of the LRP observed between stage IV and VI displayed fully quiescent OC. Only 1 out of 5 LRP showed less than 75% of proliferative OC cells at stage IV, against 1 out of 6 at stage V, and 5 out of 10 at stage VI (Figure 7 B).

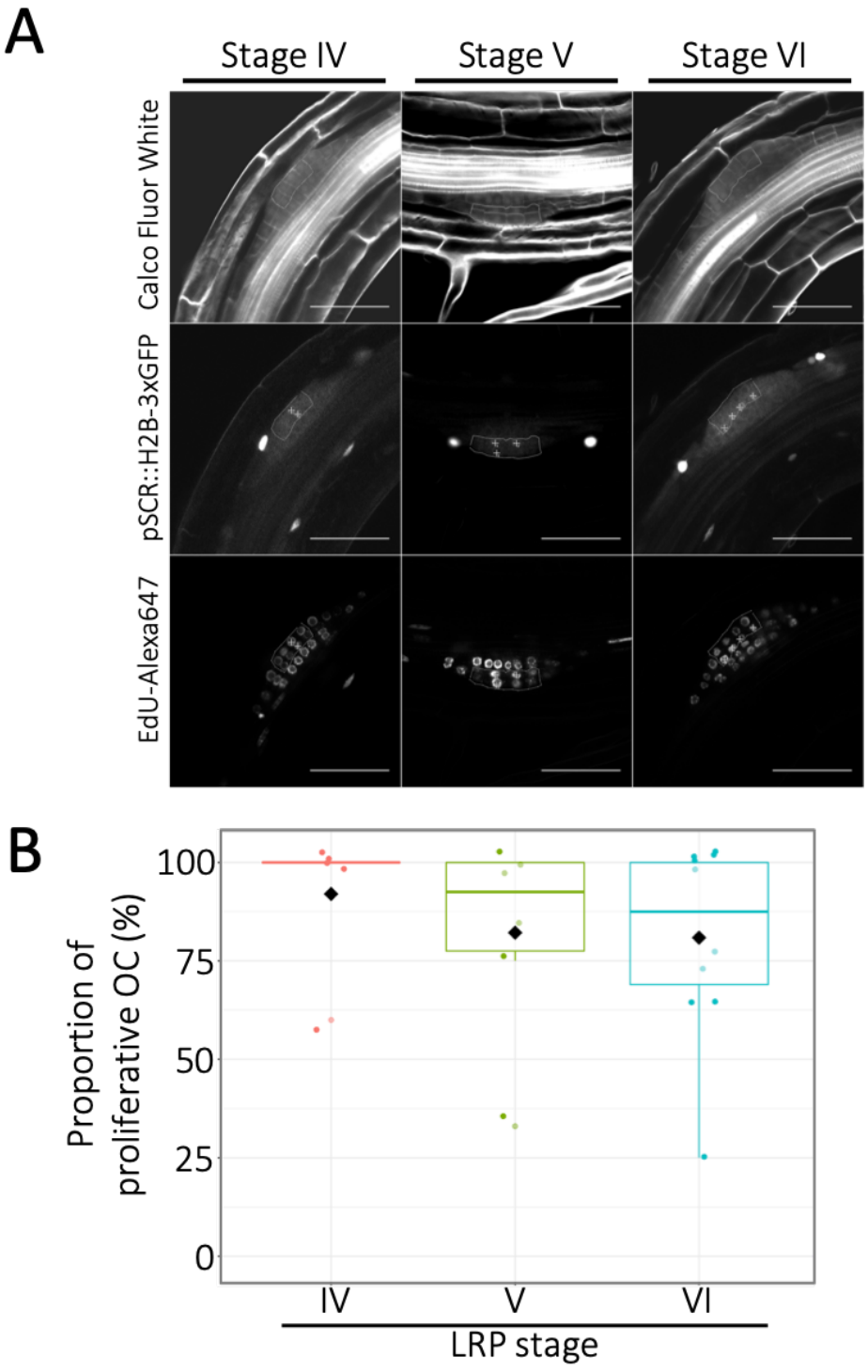


Figure 7 : Proliferative profile of LRP prior emergence based on EdU incorporation.
(A)Profile of EdU incorporation after 8h of EdU pulse, at the transverse section of LRPs expressing a SCR reporter. OC cells are assumed to be within the dotted white line. (B) Proportion of proliferative OC cells at indicated LRP stages. The average proportion of proliferative CC is indicated with a black diamond. Scale bar 50µm.

Taken together those results indicate that prior emergence, cells which already display OC identity are not quiescent.

Those evidences are further supported by qualitative live imaging observations.

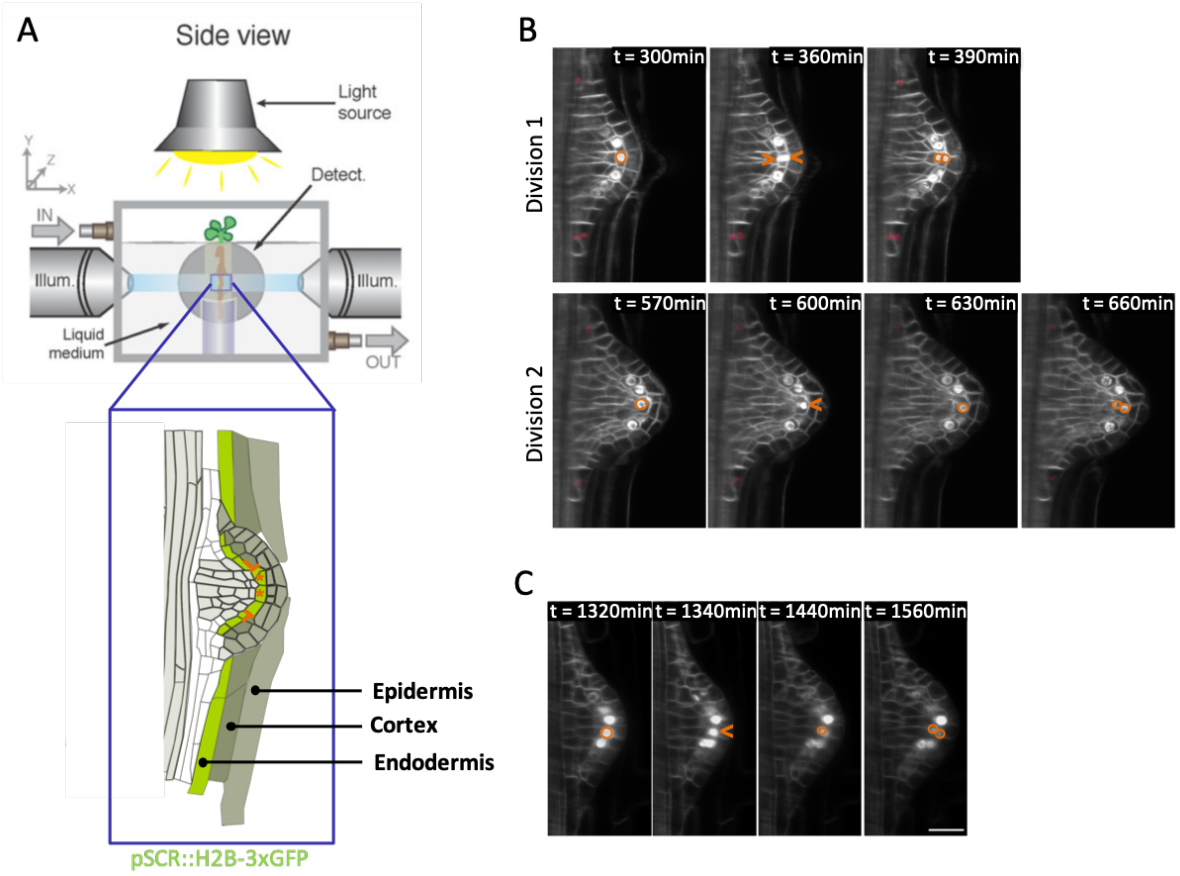


Figure 8 : Live imaging of cell division during LR emergence.
(A)LR growth is followed using light sheet microscopy in close to physiological conditions with liquid 1/2MS and LED lights (A). (B-C) Two time lapses of the transverse section of emerging LR expressing the SCR reporter. Nuclei before and after divisions are marked with an orange circle. Mitotic figures are indicated with chevrons. (A) Adapted from Berthet et al. 2016.

Using light sheet microscopy, lateral root growth can be followed in close to physiological condition for up to 40h (Figure 8 A) (Berthet & Maizel, 2016; von Wangenheim, 2014). LR development is followed in a line expressing the ubiquitous plasma-membrane marker (*pUBQ10::PIP1,4:GFPx3*) combine with a SCR transcriptional reporter (*pSCR::H2B:3xGFP*). A stage II LRP is followed up to emergence with a 30 min temporal resolution (Figure 8 B). While the LRP starts engaging through the epidermis of the primary root, a SCR expressing cell at the tip of the LRP, between the T-divisions, shows a mitotic figure (Figure 8 B, orange chevron), indicating central cell proliferation. About 4 hours later, cells at a similar position (SCR positive, between T-divisions) display a mitotic figure. CC proliferation could be observed in another recording (Figure 8 C). Thus during the late stages of emergence out of the primary root, the central cells are proliferating .

2.1.2 Quiescence is acquired in the central cells post-emergence.

Following the assumption that lateral root reproduce the tissue organization and behaviour of the primary root (Tian, Jia, et al., 2014; Waidmann et al., 2020), quiescence in the OC must be acquired at some point during the development. The previous results indicate that the OC of LR could acquire quiescence post emergence.

In the primary root, OC quiescence translates by the absence of EdU incorporation after 24h of incubation (Cruz-Ramírez et al., 2013; Timilsina et al., 2019). To test whether quiescence in LR´s OC is indeed acquired post emergence, LR of different length, expressing the GFP tagged histone H2B under SCR promoter, were incubated with EdU for 24h before revelation.

I classified the EdU incorporation in five profiles :

Type of EdU incorporation profile	Description
Type 0	occurs in lateral root primordia, all the cells have incorporated EdU over 24h (Figure 9 A, G)
Type 1	occurs in emerged LR with an average length of 341.6µm, no EdU incorporation is visible within the root (Figure 9 C, G).
Type 2	occurs in LRP or short emerged lateral, only the basis of the LR display EdU incorporation (Figure 9 B, D, G).
Type 3	occurs in short, medium and long LR, with an average length of 1128µm, EdU incorporation takes place in the whole LR but in the CC, the columella and the transit amplifying compartment of the meristem (Figure 9 E, G).
Type 4	occurs in long and very long LR, with an average length of 2975µm, EdU incorporation is visible in the whole root but the CC (Figure 9 F, G).

Type 3 and 4 incorporation profiles suggest that quiescence is indeed established at some point, post-emergence in the CC. Furthermore, CC quiescence combined with the stabilized SCN topology and the OC identity, indicate that indeed, at some point during the post-emergence development of LR, a SCN organized around a quiescent centre similar to the PR is being set.

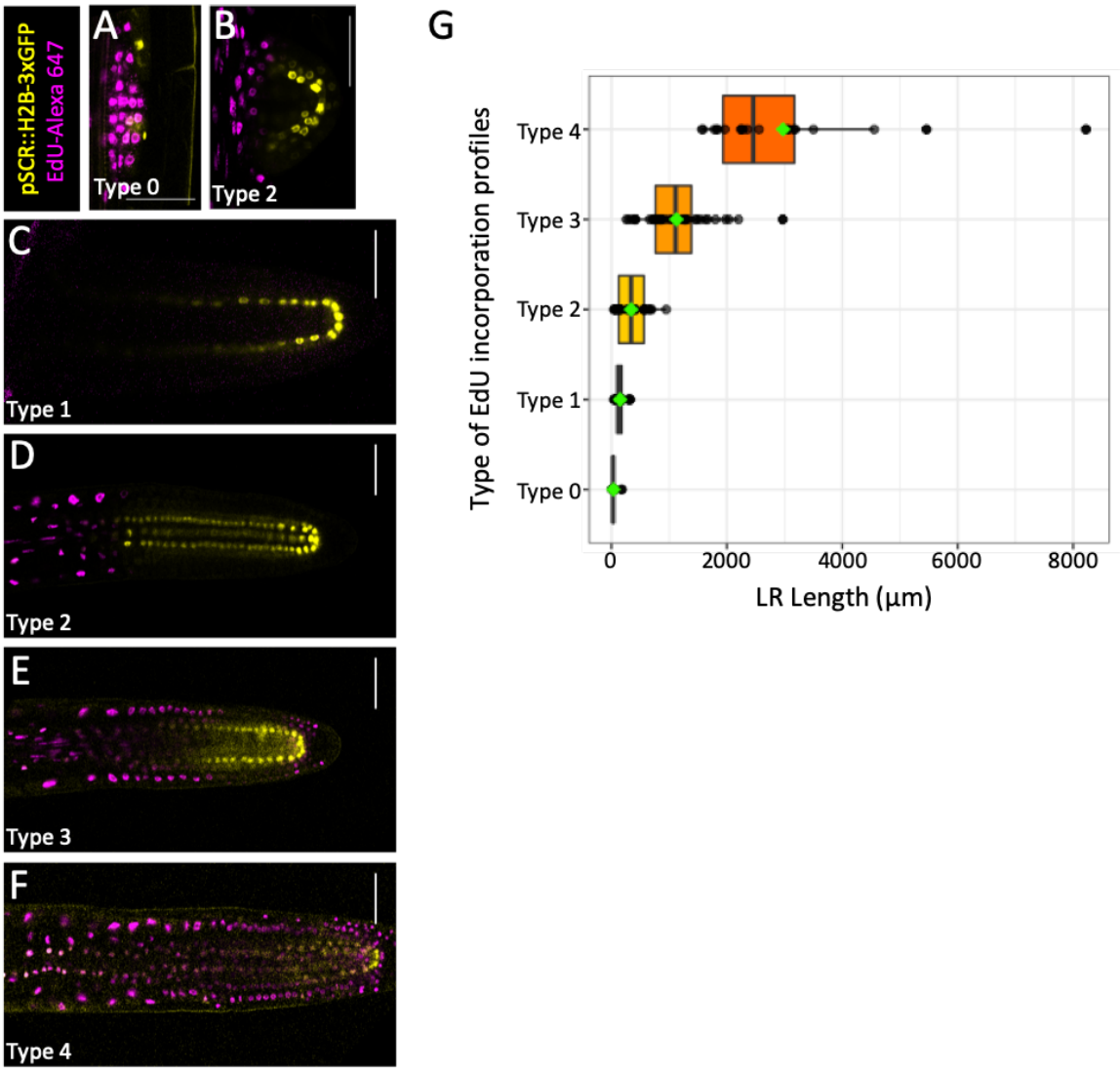


Figure 9 : EdU incorporation profile in LR during and after emergence.
(A-F) Transverse section of LR expressing a SCR reporter, after 24h of EdU incorporation. Five different types of EdU incorporation profiles were observed. (A) EdU incorporation profile Type 0 : in LR prior emergence, whole cells incorporated EdU. (B and D) Type 2 : Only the basis of LR or differentiated cells from the LR, incorporated EdU. The Tip remained EdU free. (C) Type 1 : No EdU incorporation is visible within the LR. (E) Type 3 : EdU incorporation is visible at the basis of the LR, in differentiated cells, and around the SCN. (F) Type 4 : EdU is incorporated all along the root. (G) LR length distribution for each type of EdU incorporation profile. The average root length for each profile is indicated with a green diamond. Scale bar : 50µm.

However, it still remains unclear when exactly, at which lateral root length, quiescence is established. The Type 1 EdU incorporation profile prompted the hypothesis that CC could already be quiescent at emergence and LR growth during and shortly after emergence, could be supported by cell elongation rather than cell proliferation (Figure 9 B,C (Malamy & Benfey, 1997). However

longer LR belonging to the same plant could take up EdU in the differentiation zone, or around the stem cell niche (Figure 9 D,E,F). Additionally, LRP (Figure 10 A, A´) and short emerged lateral roots (Figure 10 B-C´) clearly displayed mitotic figures (Figure 10 A´-C´, white arrow heads). Furthermore, LRPs from the same plants could also incorporate EdU. This inconsistent EdU incorporation lead to doubt the ability of short emerged lateral roots to take up EdU.

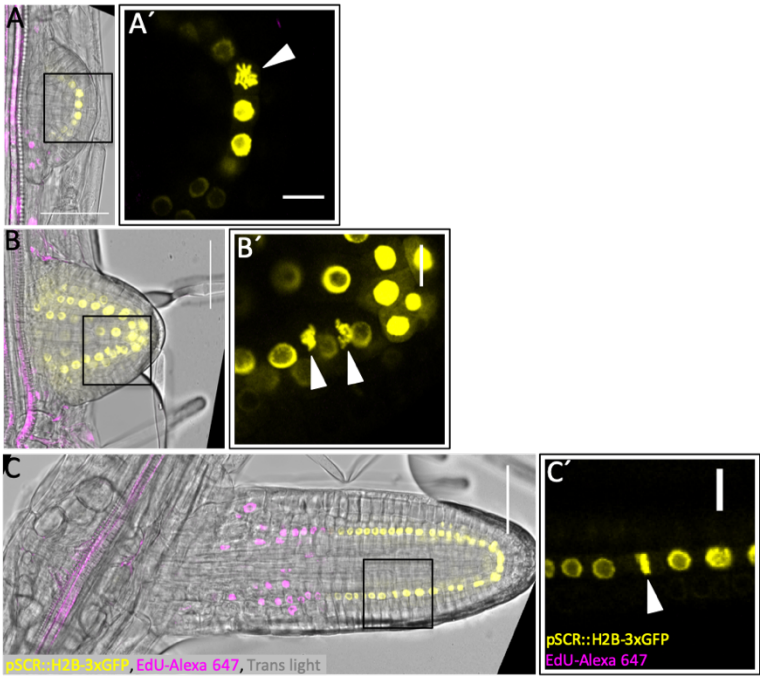


Figure 10 : Evidences of cell proliferation in LR despite the absence of EdU incorporation. (A-C) Transverse section of LR expressing a GFP-tagged-histone reporter in SCR domain, after 24h of EdU incorporation. (A´-C´)Close up of visible mitotic figures. White arrow head point at the different phase of cell divisions. (A-C)Scale bar : 50µm, (A´-C´)Scale bar : 10µm

To test whether the absence of EdU in short LR is linked to the absence of cell proliferation, or is a technical artefact, I used BCECF-AM (a vacuolar dye,(Krebs et al., 2010)), and Hoechst 33342 (a DNA staining, (Bucevičius et al., 2018)) to check the ability of LR to take up chemicals. Interestingly the incorporation pattern of BCECF-AM in short and medium lateral roots is similar to the one observed with EdU (Figure 11 A, B). Lateral root shorter than 300µm do not take up the vacuolar dye, while longer ones show incorporation in elongating cells at the basis of the LR (Figure 11 B). A similar profile is observed with Hoechst 33342 (Figure 11 C). Short emerged lateral roots, and even LRPs do not take up the staining, while longer LR do take up the DNA dye in a similar way the primary root does e.g. from the columella cells all along the meristem (Figure 11 C). This strongly suggests that the EdU pattern observed in the short and medium lateral root is most likely due to difficulties in EdU intake. Hence, it is not possible to conclude on whether emergence and post-emergence growth is supported by cell proliferation, or by cell elongation.

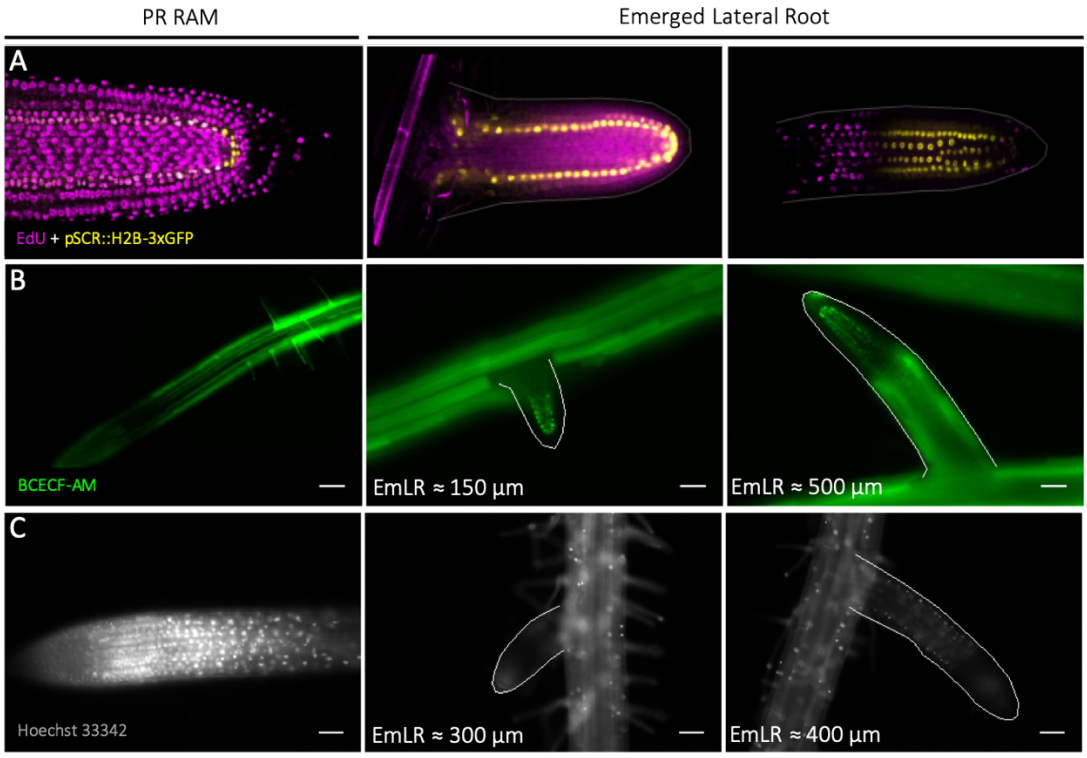


Figure 11 : Chemicals incorporation in short and medium lateral roots.
Incorporation profile of : EdU after 24h incubation (A), the vacuolar dye BCECF-EF (B) and the DNA staining Hoechst33342(C) in the primary root meristem and emerged lateral roots. Scale bar : 50µm.

Short emerged lateral root seem to not take up chemicals. A possible rational is that early lateral roots are coated with compound like a cuticle, preventing chemical incorporation as supported by the limited propidium take up in shortly emerged LR and the cellular tracer fluorescein diacetate (Berhin et al., 2019; Reinhardt et al., 2016)). In order to circumvent EdU uptake limitations, I used a mutant line with enhanced permeability : the *dcr* mutant (Berhin et al., 2019). The DEFECTIVE IN CUTICULAR RIDGES (DCR) mutant presents altered cutin biogenesis (Panikashvili et al., 2009). As a consequence, the cuticule-like structures which form the diffusion barrier covering the LRP/short LR, is more permeable in the *dcr* mutant compared to the wild type (Berhin et al., 2019). Supposedly, EdU uptake in short LR could be facilitated. Lateral root formation is induced in the *dcr* mutant by 48h, 72h, 96h and 120h of gravistimulation (GT), and the proliferation profile is assessed with a 24h pulse of EdU, during the last 24h of GT. Cell proliferation and stem cell niche topology are assessed as previously described. Consistently with the observations made in the WT background, prior LR emergence, the proportion of proliferative CC is 100% (LR shorter than 50µm, n = 2, corresponding to a stage VI) (Figure 12

A Pre-Em, B). After emergence, the proportion of proliferative CC decreases to 30% (n = 1) or 0% (n = 3) in short LR. In longer LR (from medium to very long), the proportion of proliferative CC remains at 0% (n =9), with a few exception (n = 1, with 30% of proliferative CC) (Figure 12). Those observations suggest that quiescence in the CC is established post-emergence in short LR. This roughly coincides with the onset of OC specific identity within the SCN.

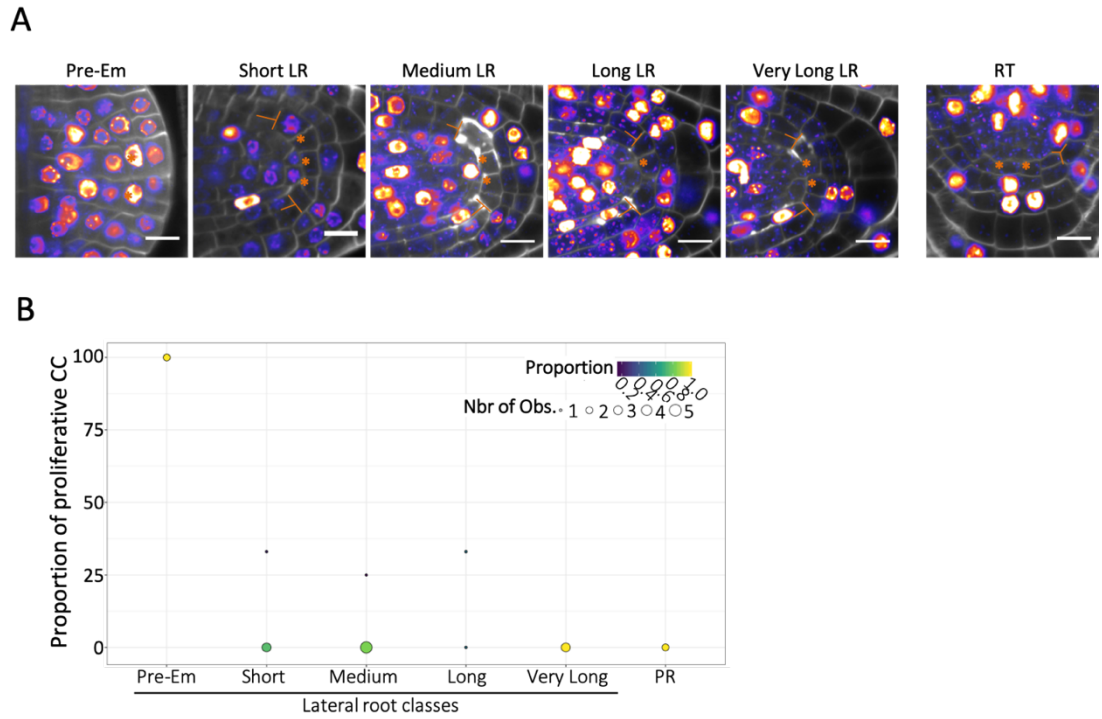


Figure 12 : EdU based stem cell niche proliferation profile in *dcr* mutant roots.
(A) Stem cell niche proliferation profile at the transverse section of LR and PR after 24h of EdU incubation in the *dcr* mutant. (B) Distribution of the proportion of proliferative CC in the different LR classes. The proportion of observations at a given values is colour coded. Scale bar : 10µm. Nbr of Obs. : number of observations.

To further supports and validate the previous observation, I used live imaging of the cell cycle progression reporter Cytrap (Yin et al., 2014).

The cell cycle progression reporter, Cytrap (Yin et al., 2014) consists of two reporters. The first, *pHTR2::CDT1a (C3)-RFP*, highlights the S/G2 phases, therefore giving an endogenous, genetically encoded, read out for cell proliferation, similar to the one provided by EdU incorporation. The second, *pCYCB1::CYCB1;1-GFP*, marks the G2/M phases. A key difference being that contrarily to EdU incorporation, Cytrap reporter provides a snapshot of the cell proliferation taking place in the root, at a given time point and not an integration of all replication events that occurred during the labelling phase.

I computed the proportion of proliferative CC as follow : CC proliferation % = (Nbr of proliferative CC / Total Nbr of CC)x 100. Consistent with the observations based on EdU incorporation, not quiescence is observed prior emergence (Figure 13, Pre-Em). In short LR the transition to quiescence occurs with about a quarter of the root observed showing 100% of CC proliferation, and a third of the observed roots showing no proliferative CC (Figure 13). Past the 250µm length, longer LR most frequently show fully quiescent CC (Figure 13. The plot needs verification.).

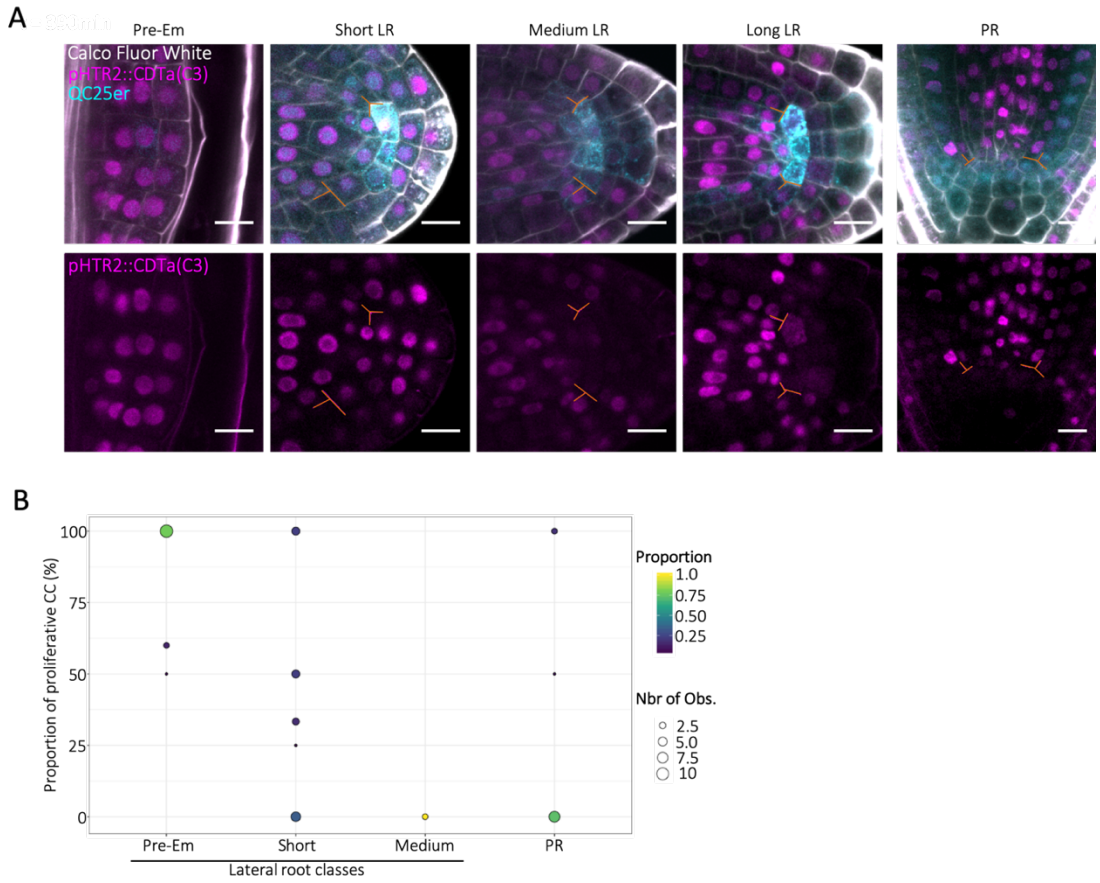


Figure 13 : Cytrap based stem cell niche proliferation profile in LR.
(A) Expression pattern of the S-G2 phases reporter (Magenta), and the OC marker QC25erCFP (Cyan) at the transverse section of cell wall stained (White) LR. (B) Distribution of the proportion of proliferative CC (CC expressing the S-G2 marker) in the different root classes. Scale bar : 10µm. Nbr of Obs. : number of observations.

Thus the Cytrap reporter independently confirms and validates observations obtained with EdU in WT and in the *dcr* mutant: quiescence in CC is acquired shortly after emergence, around the developmental time OC specific identities are established.

To investigate in more details whether the phase of CC quiescence coincides with the activation of OC markers, I performed live imaging on a line expressing both the OC identity marker

pQC25::erCFP (Goh, Toyokura, Wells, Swarup, Yamamoto, Mimura, Weijers, Fukaki, Laplaze, Bennett, & Guyomarc'h, 2016)(Figure 14 A) and the Cytrap cell cycle reporter (Figure 14 B). Lateral root development was triggered by gravistimulation, and recorded from 74h post GT, with a temporal of 2h.

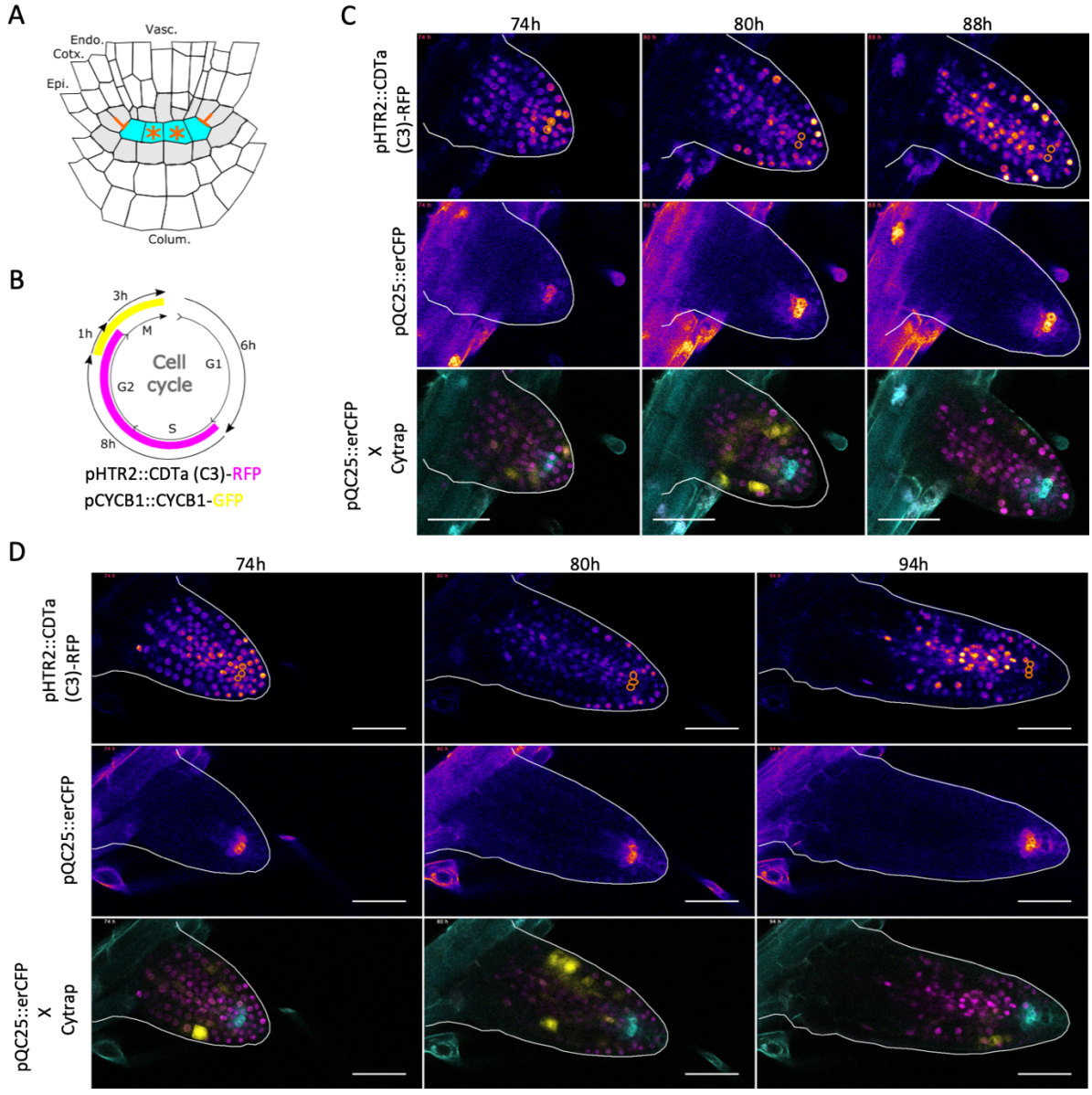


Figure 14 : Live imaging of organizing centre cells acquiring quiescence after emergence. (A)Schematic of the OC marker QC25erCFP expression in the stem cell niche. (B) Schematic of the components of the Cytrap cell cycle reporter. (C-D)Two confocal microscopy time lapses of post-emergence growth of lateral root. OC cells are circled in orange in the pHTR2::CDTa(C3)-RFP channel. Scale bar : 50µm.

The Cytrap reporter is clearly visible in the OC, at the beginning of the movie (Figure 14 C, 74h time point). As the lateral root reached 134µm long the expression of the S/G2 marker is lost in the OC cells (Figure 14 C, 80h time point). In another recording, cells expressing the OC

identity marker also expressed the S/G2 marker of Cytrap (Figure 14 D, 74h time point). However as the LR reached 170µm, the Cytrap expression is lost in the OC cells (Figure 14 D, 80h time point). This supports the acquisition of quiescence in the OC cells shortly after lateral root emergence.

Taken together all those observations confirms that quiescence in the OC is acquired shortly after emergence (within the 24h following the LRP tip pointing out of the primary root epidermis) while LR reach about 250 to 300µm.

2.2 Central cells only partially function as organizing centre during lateral root development.

While the quiescence is coincident with the expression of OC identity, it still does not inform about the actual functioning as an organizing centre. In the primary root, characteristics of a functional OC are : its ability to generate tissues in both distal and proximal directions (Rahni et al., 2016); its capacity to maintain cells in its direct contact in a stem cell state (Bennett et al., 2014; Berg et al., 1997; Pi et al., 2015; Wink et al., 2009), its ability to resist genotoxic stresses (Fulcher & Sablowski, 2009; Hong et al., 2017) and finally its capacity to regenerate damaged stem cells (Berg et al., 1995; Fulcher & Sablowski, 2009). In this section, I assess when the 3 first characteristics of a functional OC are established during LR development.

2.2.1 Prior quiescence acquisition, the organizing centre supports growth only in the proximal direction.

In order to determine if and when the central cells contribute to tissue growth in both proximal and distal direction, I used a lineage tracing approach. Under dexamethasone induction, a line expressing the CRE-GR construct under the SCR promoter, creates a inheritable genomic editing of cells containing the *p35S::lox-Ter-lox:erCFP* construct (Efroni et al., 2016). Edited cells are therefore CFP fluorescent and transmit the CFP expression to their daughters.

LRP stage II-III are induced with dexamethasone for 38h before confocal imaging. LR transverse section show CFP signal in the CC, the CEIs, and some endodermal cells (Figure 15 B). Interestingly, vascular cells directly abutting to the CC also showed CFP expression (Figure 15). This observation suggests that CC proliferation, post emergence, before quiescence acquisition, contributes to vascular tissue formation. In this preliminary experiment no sign of CC

contribution to the distal growth direction. Indeed, no CFP signal could be distinguished in columella cells.

This suggests that no OC supporting bidirectional growth is present before quiescence acquisition in CC.

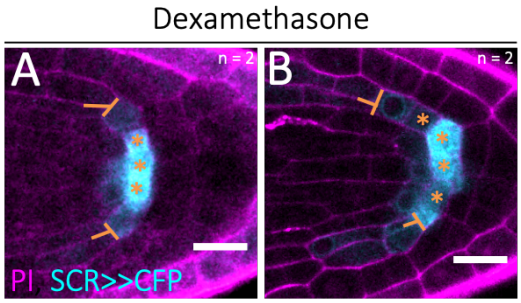


Figure 15 : Central cells contribution to tissue growth in LR by lineage tracing.
Confocal microscopy of PI stained lateral roots after 72h of gravistimulation, including 38h of Dex treatment. Cells induced for lineage tracing show erCFP signal in CC, CEI and vascular initials (A). Cells induced can also show erCFP expression in CC, vascular initials and CEI and their daughters (B). n : number of observations. Scale bar : 10µm.

2.2.2 Cells in the position of columella stem cells show signs of differentiation.

To assess when the CC start to maintain the cells in their direct neighbourhood in an undifferentiated state, I used the modified Pseudo-Schiff propidium iodide (mPS-PI) starch staining (Truernit et al., 2008) and the SMB transcriptional reporter. I focused my analysis on the columella. In the columella, stem cells and differentiated cells are spatially close (Barlow, 2002). Additionally the state of differentiated columella cells are clearly identifiable by the presence of statholithes – starch granules, and expression the SMB gene (Bennett et al., 2010; Willemsen et al., 2008).

In the primary root the layer of cells directly distal to the CC does not show statholiths nor SMB expression (Figure 16 , Figure 4 E), this implies that those cells are CSC. The first visible statholiths are detected in 100µm long LR (Figure 16). They are present in both layers of columella, including the one directly distal to the CC. Up to 2mm length, small statholiths are visible in the columella layer in direct contact with the CC (Figure 16). Interestingly, SMB expression is not visible in the columella layer abutting the CC, independently of LR length (Figure 4 E).

This suggest that, at least in the distal direction, CC do not function as organizing centre from emergence up until 2000µm. Indeed the cells directly neighbouring the CC are not maintained undifferentiated.

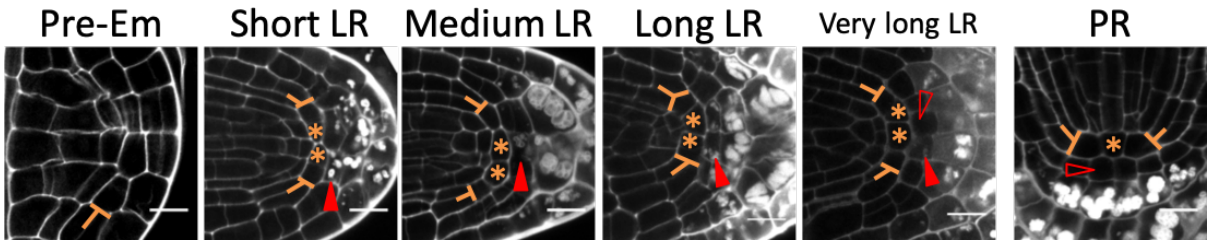


Figure 16 : Columella differentiation during LR development.
Confocal microscopy of the transverse section of LR stained with mPS-PI. Cells containing statholiths directly distal to the CC are marked with a red arrow head, while cells without statholiths -undifferentiated columella cells, are marked with empty arrow head. Scale bar : 10µm.

2.2.3 An organizing centre resistant to genotoxic stress is present prior lateral root emergence.

In the primary root the central cells act as organizing centre of the stem cell niche. A characteristic of the OC, as a pool of back up cells, is increased resistance to DNA damages compared to their neighbouring stem cells.

To test whether the LR central cells are resistant to genotoxic stresses and if so, from which stage, I used phleomycin treatment combined with propidium iodide (PI) staining. Phleomycin is an antibiotic inducing DNA double strand breaks in the stem cells of the primary root (Fulcher & Sablowski, 2009; Timilsina et al., 2019). Therefore, cells sensitive to phleomycin die and incorporate the PI staining (Truernit & Haseloff, 2008). LR are treated with phleomycin for 24h before PI staining and confocal microscopy.

Up to 1000µm length, most LR show PI incorporation at the position of vascular initials (Figure 17). However in some medium length LR, PI staining is also present in the CSC. Before emergence and in medium LR CC sensitivity can sometime be observed. As LR reach the medium size, the profile of sensitivity to phleomycin is similar to the one observed in the PR (Figure 17, (Timilsina et al., 2019)).

Taken together, the profile of phleomycin sensitivity suggest that CC are resistant to genotoxic stress already before emergence. Additionally cells directly proximal to the CC show sensitivity to phleomycin all along LR development, suggesting that vascular initials are present early on in LR.

Altogether, those results show that the CCs only progressively function as a PR-like organizing centre.

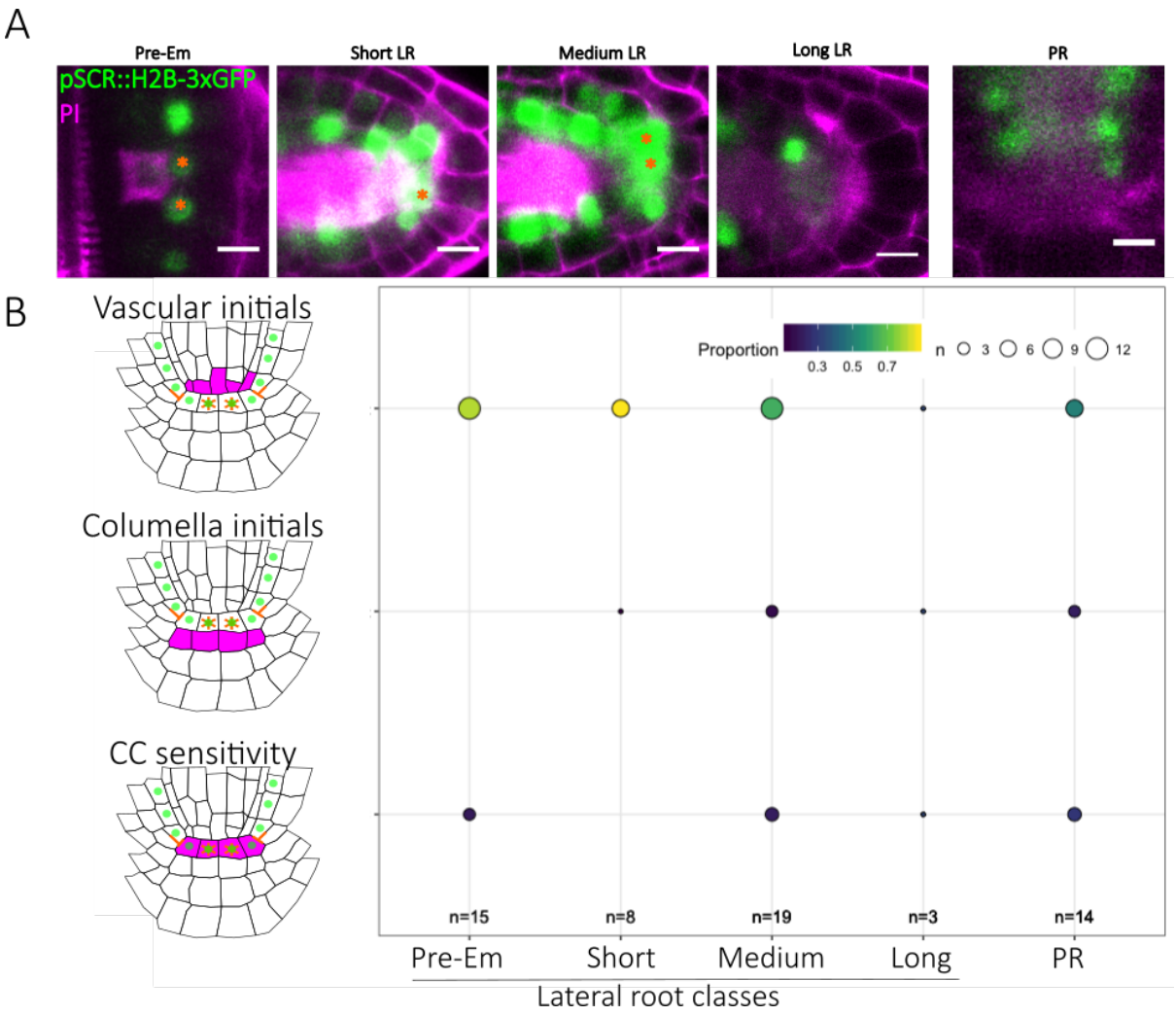


Figure 17 : Stem cell niche sensitivity to genotoxic stresses during LR development.
(A) Transverse section of PI stained lateral root after 24h of treatment with phleomycine. (B) Quantification of observed LR showing sensitivity in either the vascular initials, the columella initials or the central cells. Pre-Em : pre-emergence LR. CC are marked with orange asterisks. n : number of observations. Scale bar : 10µm.

Chapter 3: Is the timing of OC quiescence acquisition during lateral root development relevant?

So far, I have gathered evidences that SCN identity patterning, OC quiescent behaviour and SCN organizer functions are established progressively during lateral root development.

In this section, I question the importance of the timing of the OC quiescence acquisition. This can be achieved by either forcing CC to become quiescent earlier than they would naturally do or by preventing quiescence to establish.

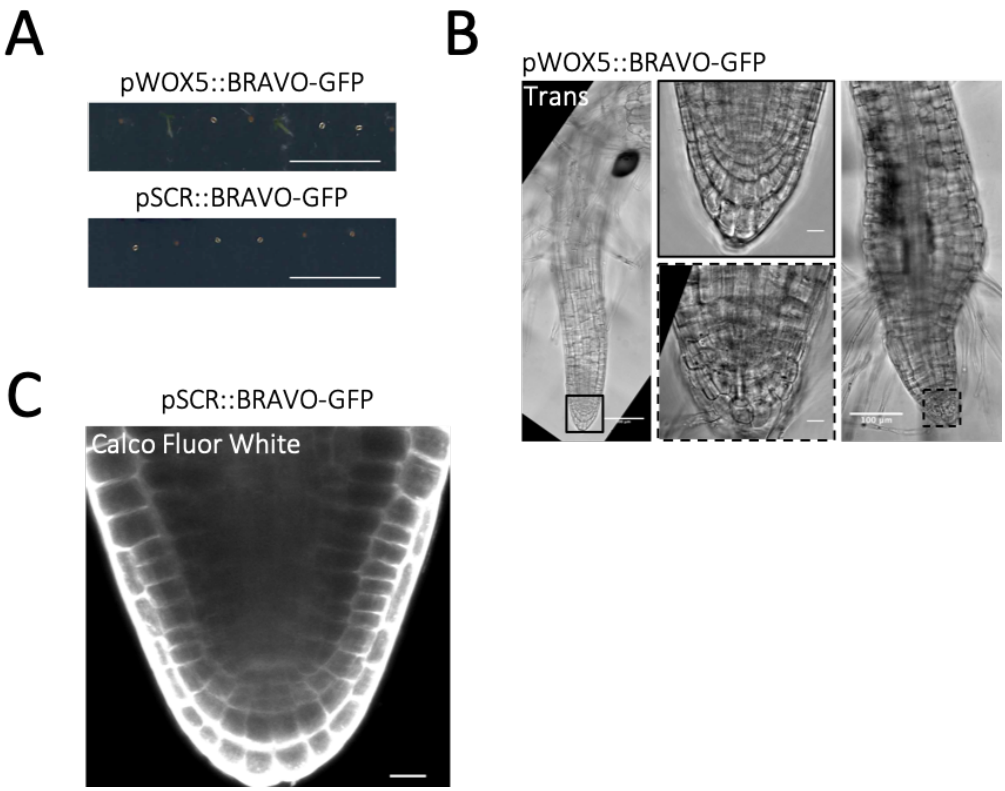
3.1 Forced early quiescence in the central cells of LR

In order to induce quiescence in CC before they would naturally stop proliferating, I used the ectopic expression of the BRAVO transcription factor. BRAVO regulates the maintenance of quiescence in the OC of the PR (Vilarrasa-Blasi et al., 2014b). Therefore we hypothesized that expressing BRAVO in the CC prior emergence could lead to their pre-mature quiescence. To this aim, I used both constitutive and inducible expression.

3.1.1 Constitutive expression of BRAVO in SCR and WOX5 domains

In order to prevent proliferation in the OC of LR, as early as possible, BRAVO tagged with GFP was expressed constitutively under either WOX5 or SCR promoter.

Figure 18 : Primary root phenotype of the constitutive ectopic expression of BRAVO in WOX5 and SCR domains.
(A) T1 seed germination of transformants expressing pWOX5::BRAVO-GFP or pSCR::BRAVO-GFP after 9 and 10 days post germination respectively. (B) Primary root of pWOX5::BRAVO-GFP T1 transformant, at 9 day post germination. Left, representative picture of a PR with a wild type aspect, and a typical SCN arrangement (upper close up)(n = 2). Right, PR with limited growth post radicle emergence and altered SCN arrangement (lower close up)(n = 2) (C)Topology of the radicle of cell wall stained embryos expressing BRAVO in the SCR domain. Scale bar : 1cm (A), Scale bar : 100µm (B), Scale bar : 10µm (C) and close up (B).



T1 transformant expressing either *pWOX5::BRAVO:GFP* or *pSCR::BRAVO:GFP* had a low germination rate (Figure 18 A). I examined these non-germinating seed expressing BRAVO in the SCR domain contain and observed a developed embryo, with a WT like radicle topology (Figure 18 C). This suggest that seedling development stopped before radicle emergence from the seed coat. In germinating seeds expressing *pWOX5::BRAVO:GFP*, the primary root growth stops shortly after radicle emergence(Figure 18 B).Additionally, LR ranging from primordium to medium length were visible in the seedlings expressing the *pWOX5::BRAVO:GFP* construct (Figure 19 A). In 2 of the observed LR, the expression of the Cytrap reporter suggests that cell proliferation is still occurring in cells containing in the stem cell niche (Figure 19 B). Furthermore, the SCN of emerged lateral roots does not show topological alterations (Figure 19 B).

Taken together those observations suggest that the constitutive ectopic expression of BRAVO in the WOX5 or SCR domain affect seed germination and primary root growth. Studying lateral root development in this context is difficult.

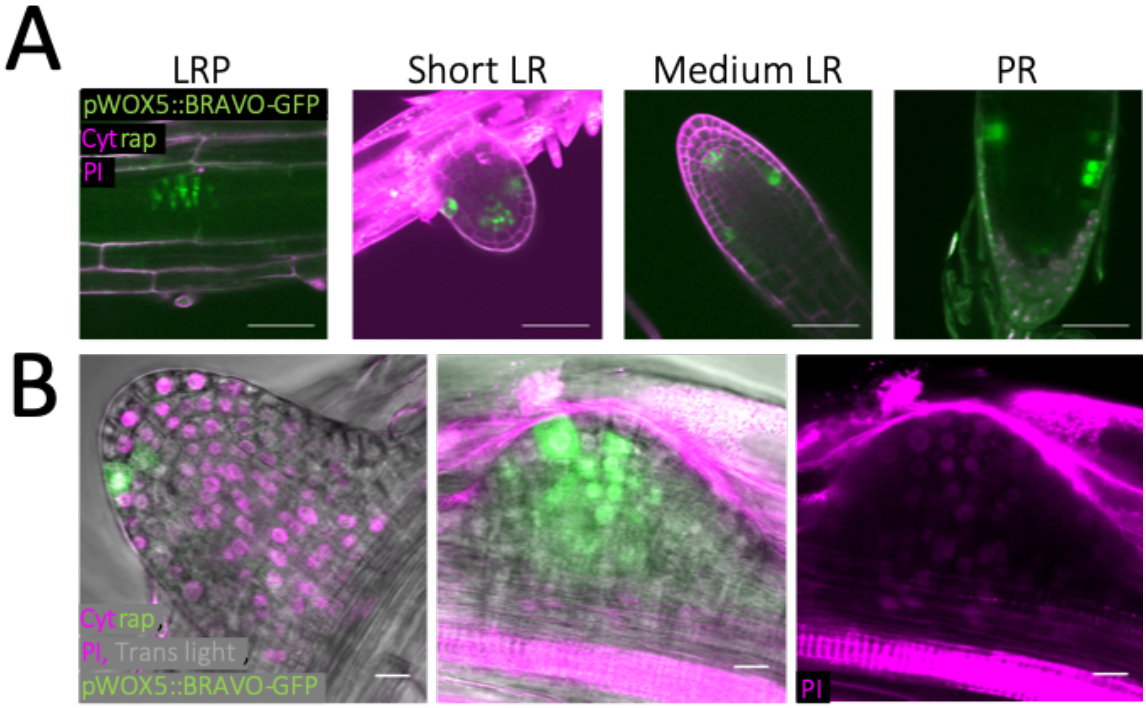


Figure 19 : The constitutive expression of BRAVO in WOX5 domains does not prevent lateral root development.
(A) Confocal microscopy of PI stained (Magenta) LR and PR from T1 seedlings expressing pWOX5::BRAVO-GFP (green, nuclear signal) in the Cytrap background (Magenta nuclei and green entire cells). (B) LRP and short LR showing expression of the S-G2 marker of cytrap (Magenta nuclei) within the SCN. Scale bar : 50µm (A), Scale bar : 10 µm (B)

3.1.2 Inducible expression of BRAVO in SCR domain

In order to bypass the limitations of the constitutive expression of BRAVO, I used a two component dexamethasone inducible system (Schürholz et al., 2018). The first component of the system is the driver *pSCR::LhG4-GR*. The expression of LhG4-GR can be mostly restricted to the CC and CEI by adjusting the concentration of dexamethasone (Dex) and the duration of incubation. The second component of the system is a response element, the *p4Op::BRAVO* construct. Additionally, the driver line contains the *p6Op::mTurquoise2* response element leading to ER-localized mTurquoise signal. Upon dexamethasone treatment, the expression of both mTurquoise2 and BRAVO are induced in the SCR domain.

To test whether BRAVO expression in LRPs would affect lateral root development, I treated stage II primordia with Dex for 24h. mTurquoise2 expression was clearly visible in the SCR domain of Dex treated LRP while expressed at very low level or absent of the DMSO control, suggesting that the induction worked (Figure 20 A). I measured the length of the LRP at the transverse section in 4 independent lines. In 3 of them, Dex and DMSO conditions showed no significant length difference. In both cases, LRP emerge with an average length of (63.87µm for Dex condition and 69.93µm for DMSO). However in the line #2, Dex treated LRP are significatively ($p = 0.00603$) shorter (50.47µm in average) than their DMSO counterpart (122.21µm in average) (Figure 20 B). This suggests that BRAVO expression across the LRP did not affect their growth dynamic. Additionally this indicates that BRAVO early expression did not prevent cell proliferation in the SCR expressing cells. Furthermore, Dex treated LRP do not show aberrant topology (Figure 20 A), suggesting that BRAVO ectopic expression did not alter early SCN development.

To further assess the effects of ectopic BRAVO expression in LR, I treated stage II primordia with 72h of Dex. At the end of the treatment I measured the length of LR at the transverse section. No significative length difference could be observed between Dex and DMSO for the lines #1 and #7. However, the average root length of Dex treated seedlings is significatively higher (1576.9µm) in line #11, than in the DMSO control (567.37µm, $p = 0.00745$)(Figure 20 C).

Taken together, these preliminary experiments suggest that ectopic expression of BRAVO in the SCR domain does not affect lateral root development. In addition BRAVO expression does not seem to prevent cell proliferation. Therefore this approach is not suited to force quiescence in CC before they would naturally acquire it.

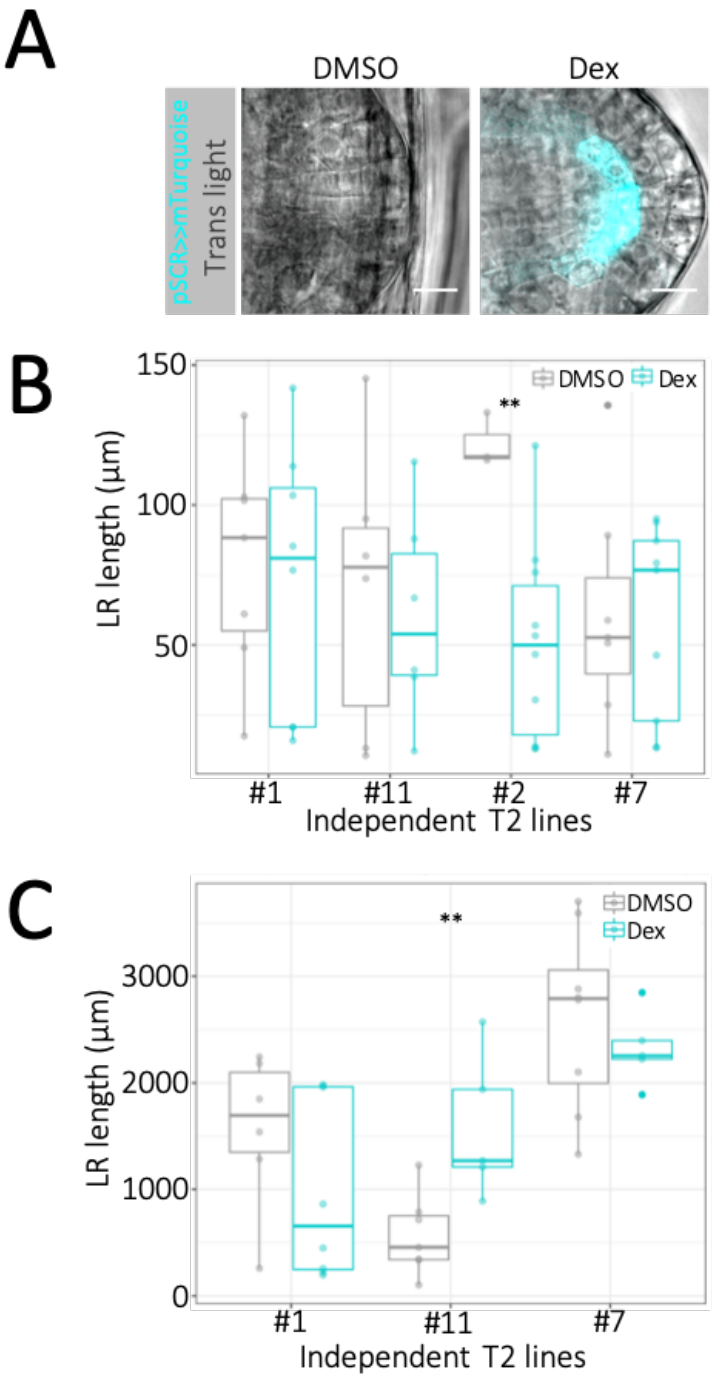


Figure 20 : Phenotype of line expressing BRAVO in the SCR domain upon Dex induction.
(A) Confocal microscopy of an LRP expressing the SCR driver line inducing mTurquoise (Cyan) and BRAVO expression at 48h after gravistimulation and 24h of dex induction. (B) Quantification of lateral root length in 4 independent T2 lines after 48h of gravistimulation and 24h of Dex induction. (C) Graph of lateral root length in 3 independent T2 lines after 96h of gravistimulation and 72h of Dex induction. Scale bar : 10µm (A). t test with linear regression analysis using Col-0 as reference. Significance codes : ***: p-value <0.001;**: p-value<0.01;*: p- value<0.05.

3.2 Preventing quiescence in the central cells of LR

In order to prevent quiescence in the central cells, I used the knockout mutant line *bravo-2* and the over expressor line ERF115-OE. ERF115 is a transcription factor that positively regulates OC proliferation. Its over-expression in the primary root leads to an increase in QC division (Heyman et al., 2013). Similarly, the *bravo-2* mutant line shows increased QC proliferation compared to WT in the primary root (Vilarrasa-Blasi et al., 2014b). Therefore, we assumed that in the *bravo-2* mutant and the ERF115-OE lines, CC quiescence could be prevented in LR, possibly affecting SCN topology and growth dynamic. To test this, I measured LR length after 72h or 96h of gravistimulation and counted the number of central cells, at the transverse section of cell wall stained LR.

LR from ERF115-OE and *bravo-2* lines do not show aberrant stem cell niche topology (Figure 21 A). To determine whether either the over-expression of ERF115 or the disruption of BRAVO gene, lead to more proliferative CC, I counted the number of CC and the number of CC dividing perpendicular to the proximo-distal axis of the LR. In WT, after 72h of gravistimulation, most LR showed 3 central cells, and in half of the root observed 1 CC divided perpendicular to the proximo-distal axis. After 96h of gravistimulation, most LR show 1 CC and no perpendicular division. The *bravo-2* mutant showed most frequently 2 CC both after 72h and 96h of gravistimulation, with no perpendicular division in most roots. The ERF115-OE shows a similar behaviour with mostly no CC dividing perpendicularly (Figure 21 B). This suggests that in ERF115-OE and in *bravo-2*, the CC of LR are not more proliferative than their WT counterparts. The average LR length after 72h of gravistimulation is similar in the WT and the *bravo-2* mutant (257.1µm and 292.5µm respectively), while in the ERF115-OE, LR reach 453µm in average, indicating a faster growth. After 96h of gravistimulation, the average LR lengths are 1335µm in the WT, 1231µm in ERF115-OE and 1770µm in *bravo-2*, showing a faster growth of the *bravo-2* mutant at later stages of LR development(Figure 21 C).

Taken together, those observations show that preventing quiescence in the CC of lateral roots does not strongly affect SCN topology. Additionally they suggest that ERF115 might play a role in regulating LR growth during the first 24h post emergence, when quiescence is acquired in the CC. In the other hand, BRAVO seems to play a more prominent role only after LR grow past 300µm.

While the approach used to force early quiescence in the CC was not conclusive, preventing CC quiescence post emergence seems to have only moderate effect of LR development and SCN topology. Those experiments hint that the timing of quiescence acquisition in the CC of LR might not be critical to the proper development of lateral roots.

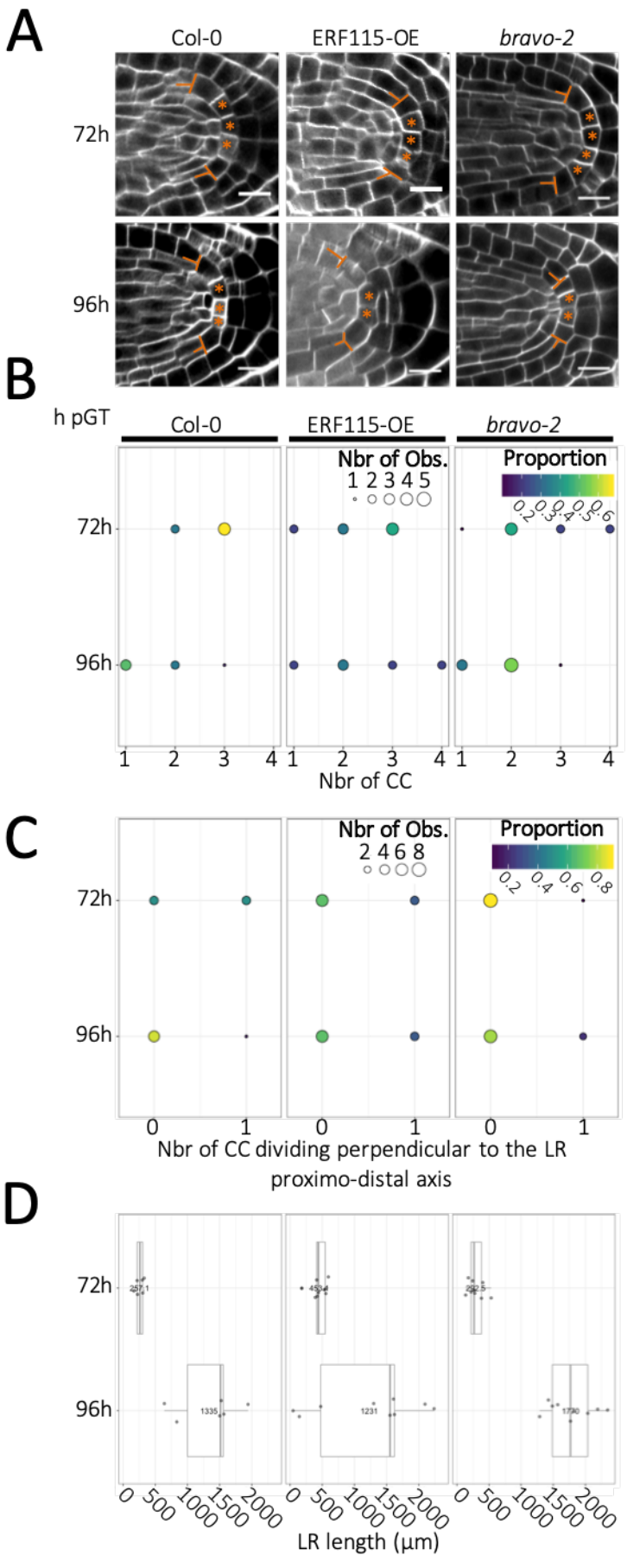


Figure 21 : Phenotype of the *bravo-2* mutant and the ERF115 over-expressor line. (A) Confocal microscopy of the transverse section of LR SCN after cell wall staining at 72h and 96h post gravistimulation (hpGT). (B) Quantification of the number of central cells (CC) after 72h and 96h or gravistimulation. (C) Quantification of the number of CC dividing perpendicularly to the proximo-distal axis of LR. (D) Quantification of lateral root length after 72h and 96h post gravistimulation. Scale bar : 10µm.

Discussions and Conclusions

1. Tissue identity patterning during early stages of lateral root development provides an incomplete picture.

The first step in the investigation of the ontogeny of the LR stem cell niche was to assess identity patterning.

Patterning of the stem cell niche was found to start early during lateral root development, with SCR, SHR, WOX5, PLETHORAs genes and a transcriptional reporter of QC25 being first expressed between stage II and V (Du & Scheres, 2017b; Goh, Toyokura, Wells, Swarup, Yamamoto, Mimura, Weijers, Fukaki, Laplaze, Bennett, & Guyomarc'h, 2016). Consistently the reporters for transcriptional readout of auxin and cytokinin (Figure and (Benkova et al., 2003)), as well as stem cell niche identities (Figure 4) also indicate early patterning.

However, the expression pattern of these reporters in early stages of LR development is slightly different from the ones of the primary root tip. For instance the WOX5 expression observed in this study starts earlier and is much wider than that of the PR. Additionally other QC specific reporters showed expression only post emergence, when the SCN is similar to the one of the PR (Figure 6). While not in direct contradiction with observations of very early patterning, this suggests that stem cell niche patterning and specification of the stem cell niche organizer itself might occur at different times. The early stages of LR development are dynamic, the expression of identity reporter might be more representative of the process of patterning itself, and less representative of the final identity distribution of the established SCN.

Transcriptional reporters and enhancer trap lines are genes selected because they are expressed in restricted domains. However, this approach is limited. Because the regulation of these genes might be context dependant and because these genes are single genes, they might not accurately represent fate commitment, and do not inform about cell behaviour. For instance, the enhancer trap lines QC25 and QC184, specifically expressed in the QC of the primary root, are already expressed in globular and hear stage of embryogenesis (), while the PR stem cell niche is being pattern. At the contrary, in lateral roots they are expressed only after the SCN patterning occurred. This suggest that despite similarities between fully developed and functional lateral roots, and the PR, the reporters used might be regulated differently in the two contexts.

Additionally, WOX5 is expressed in the QC of the PR (Sarkar et al., 2007), Figure) and controls QC quiescence by downregulating CYCD1;1 and CYCD3;3 (Forzani et al., 2014b). However in lateral rootsWOX5 expression is wider than in the PR (Figure) and does not appear to be associated to quiescence. It would be interesting to assess when and where CYCD1;1 and CYCD3;3 are expressed within the growing LRs, and whether they are down regulated in CC just before quiescence acquisition.

To go beyond the expression of specific reporters, it would be interesting to use single cell sequencing approaches at different stages of LR development to unravel gene regulatory networks involved in tissues and SCN patterning (Drapek et al., 2018; Gala et al., 2020; Kortz et al., 2019; Shahan et al., 2020; Voß et al. 2015). This would provide insight into the developmental trajectories of LRP cells as they become assigned to a specific tissue, similar to what has been done in the PR (Shahan et al., 2020). Additionally, in the PR, gene regulatory networks involved either in the general patterning of the stem cell niche patterning or in the specification of the individual type of stem cells within the SCN, have been identified (Clark et al., 2019). It would be interesting to determine whether the same gene networks are at play during lateral root development.

2. Post-emergence acquisition of quiescence in lateral root central cells.

2.1 Cell proliferation assessment

In this study cell proliferation was assessed using EdU incorporation, qualitative analysis of live imaging of growing LR, and analysis of the expression pattern of a cell cycle progression reporter. These approaches were complementing one another in covering different parts of LR development, and congruently support the post-emergence establishment of quiescence in the central cells of LRs.

While EdU incorporation allows to integrate cell proliferation over a given period of time, this approach is limited by the capacity of cells to take up EdU from the medium. Short lateral root are entirely covered with a cuticle-like structure which can be stained with the fluorol yellow dye. Longer emerged lateral roots show fluorol yellow staining only around the root cap, excluding the elongation zone formed as the root grew longer (Berhin et al., 2019). Interestingly, the profile of incorporation of chemicals observed in lateral roots (Figure 11) correspond to the portion of LR unstained by fluorol yellow.

The *dcr* mutant shows improved EdU intake thanks to its discontinuous cuticle-like structure therefore provides insights into cell proliferation during and shortly after emergence. However, it grows slower than the WT (The average LR length is 165µm after 72h post gravistimulation for the *dcr* mutant, in contrast to 200µm for the WT). The expression pattern of the Cytrap reporter provides a snap shot of the proliferation profile at a given time instead of integrating proliferation over a period of time. Cytrap expression also indicates a transition toward CCs quiescence as LR grow from 100 to 300µm. This suggests that the acquisition of quiescence in the CCs might not depend on the growth rate of the emerging LR.

Taking advantage of the developing technologies for automated cell segmentation and tracking in large data set (Wolny et al., 2020), it would be really interesting to use light-sheet based long terms live imaging to follow growing LR from early stages up to post-emergence. In this way, a 3D map could be constructed, with high temporal resolution of the evolution of the proliferative behaviour of the stem cell niche as LR development occurs.

2.2 Relevance of the timing of OC quiescence acquisition

The OC of LR becomes quiescent shortly after emergence from the primary root. In order to understand the relevance of that specific timing, two approaches were used : the first one was aimed at inducing OC quiescence prior emergence, while the second one was aimed at forcing OC proliferation after it would normally had stopped dividing.

In the primary root, the transcription factor BRASSINOSTEROIDS AT VASCULAR AND ORGANIZING CENTER (BRAVO) prevents QC cells division (Vilarrasa-Blasi et al., 2014). In this study the ectopic expression of BRAVO in WOX5 and SCR domains was used as an attempt to prevent OC proliferation during early LR development. While the constitutive expression of BRAVO in either WOX5 or SCR domain did not prevent embryo development, it strongly affected germination and further growth of the PR (Figure 18), this phenotype seems consistent with previous reports that BRAVO over-expression affects PR growth (Vilarrasa-Blasi et al., 2014). Similar to the constitutive expression of BRAVO in the WOX5 domain in LR (Figure 19), LR growth or topology seemed unaffected by the post-embryonic induction of BRAVO within the SCR domain (Figure 20). This suggests that BRAVO might play a different role during early LR development than it does in the PR. Further investigations are required to determine whether the ectopic expression of BRAVO alters cell proliferation in LR. Alternative approaches to prevent OC cells proliferation during early LR development could rely on inducible disturbance

of the OC cells´ cytoskeleton, therefore preventing cell division (Fujita et al., 2013; Planchais et al., 2000).

Mutant for BRAVO, *bravo-1* and *bravo-2*, show an increase rate of QC division in the PR (Vilarrasa-Blasi et al., 2014b). Similarly, the over-expression of the transcription factor ERF115 leads to increased QC proliferation (Heyman et al., 2013). Neither the over-expression of ERF115 (ERF115-OE) nor the mutant *bravo-2* show strong effect on lateral root SCN topology, suggesting that prolonged CC proliferation may not affect SCN organization. This observation is consistent with evidences that in the PR, QC quiescence is not strictly required for SCN organization (Cruz-Ramírez et al., 2013). However, further experiments are required to verify that CCs of ERF115-OE and *bravo-2* are proliferative in root longer that 300µm.

In the embryonic PR, the ectopic expression of the cyclin CYCD3;3 in the QC was shown to induce QC division (Forzani et al., 2014a). An alternative approach to force proliferation in the CC of emerged lateral root, could rely on the inducible expression of CYCD3;3 in the CCs. Additionally, the PR of the ABA-deficient mutants *aba1-1, aba2-1, aba2-4* and *aba3-2* was shown to have a proliferative QC (Zhang et al., 2010). It would be interesting to investigate whether lateral roots of these mutants also show CCs proliferation after LR reached 300µm length.

3. Establishment of the organizing centre function in lateral root

3.1 OC resistance to genotoxic stress

In the PR the quiescent centre is more resistant to genotoxic stresses than the surrounding stem cells (Fulcher & Sablowski, 2009; Horvath et al., 2017). This is in part due to the low mitotic activity of these cells, but also due to the higher expression of DNA repair machinery (Fulcher & Sablowski, 2009; Horvath et al., 2017). In LR, OC cells show resistance to genotoxic stresses already prior emergence, while still proliferative (Figure16). This suggest that similar to the PR (Cruz-Ramírez et al., 2013), in LR, the function of OC might not require quiescence. It would be interesting to test whether in LR, the cells resistant to genotoxic stresses show higher expression of the kinases ATAXIA-TELANGIECTASIA MUTATED (ATM) and ATM/RAD3-RELATED (ATR), involved in DNA repair induced by DNA double -stranded breaks (Cimprich & Cortez, 2008; Shiloh, 2006).

Furthermore, the OC is assumed to be able to regenerate stem cells along both the proximal and the distal axis (Cruz-Ramírez et al., 2013; Sanchez-Corrionero et al. 2019) and therefore contributing to tissue growth in a bidirectional manner. It would be interesting to test when the CCs can assume the same contribution to tissue growth in LR. This could be analysed by ablating CSCs or vascular initials and following the behaviour of the CC during the recovery period.

In the PR, the rarely dividing QC cells were shown to contribute to the columella under normal growth conditions (Cruz-Ramírez et al., 2013). It would be interesting to use the lineage tracing approach and determine to which tissue the proliferative CC contribute before they acquire quiescence, and whether, similar to the PR, they contribute to the columella after quiescence was established.

3.2 Root cap formation during LR development

After lateral root induction by gravistimulation, LR growth is exponential up to 72h after gravistimulation, and then stabilizes at 100µm/h (Guyomarc'h et al., 2012). In the experimental set up used in this study, this transition toward a more stable LR growth rate corresponds approximately to the timing of quiescence acquisition in the CCs (LR induced by 72h of gravistimulation reach about 150-250 µm, while the CCs transition to quiescence occurs post-emergence, up to 300µm length). Furthermore, the authors detected the presence of starch granules in the root cap of LR, up from 12h after emergence (corresponding to 60h post-gravistimulation (Guyomarc'h et al., 2012), concordantly the mPS-PI staining used in this study highlighted the presence of statoliths in LR columella, shortly after emergence. However the cellular resolution given by the mPS-PI staining revealed the presence of statoliths in the columella cell layer directly abutting the CCs (Figure 16). In the primary root, these cells are maintained undifferentiated via the movement of WOX5 transcription factor from the QC to the CSCs (Pi et al., 2015) while the transcription factor FEZ regulates CSCs division (Willemsen et al., 2008). CSCs and their first daughter were shown to proliferate over 24h of EdU treatment, while starch granules were found only in the daughters of CSCs, and in differentiated columella cells (Hong et al., 2015). Interestingly, in lateral roots, EdU incorporation was observed in some columella cells directly abutting the CCs from emergence on (Figure 12). This suggests that despite showing signs of differentiation those cells might play the role of CSCs. However further investigations are to be done to confirm that cells directly distal to the CCs are CSCs. Reporters for the ACR4 receptor-like kinase (ARABIDOPSIS CRINKLY4) are strongly expressed in the CSCs and to a lower

intensity throughout the SCN of the PR (Gifford et al., 2003). These reporters could inform about the fate of the cell layer directly distal to the CCs in LR. It would also be interesting to investigate WOX5 mobility during columella formation in LR and whether the transcription factor CYCLING DOF FACTOR 4 (CDF4) is expressed in the distal cell layer abutting the CCs. Following the establishment and evolution of the gradient of CDF4 expression in the columella of LR could inform about the distal stem cells functioning.

In the primary root, the size of the columella is maintained to five layers from the fourth day after germination (Dubreuil et al., 2018). In this study, a five layer columella was observed only in very long lateral roots, suggesting that columella growth might occur with a different timing in lateral roots. The columella size of the PR is kept constant up to ten days after germination by the auxin-regulated coordination of CSCs division and sloughing off of the outermost layer of the root cap (Dubreuil et al., 2018). The transcription factors BEARSKIN1 and BEARSKIN2, regulate differentiation and sloughing off of the outermost layer of the root cap (Bennett et al., 2010; Kamiya et al., 2016). It would be interesting to determine when and where, they are expressed during LR growth. Furthermore, determining when the first outermost layer of root cap is sloughed off and whether this is associated to CSCs division, would bring additional insights into the establishment of the distal growth axis and the functioning of the distal stem cells.

3.3 Post-emergence meristem activation

Observations based on the topology and sensitivity to hormone treatment suggests that LR emergence from the primary root occurs mostly through cell elongation. Emergence would then be followed by a phase of meristem activation (Malamy & Benfey, 1997). Cytrap expression and EdU incorporation profile indicate that cell proliferation also contribute to emergence of LR from the primary root tissues. This suggests that emerging LR could already contain an active meristem. While the central cells are proliferative during emergence, a thorough analysis of the pattern of division within the whole SCN would indicate whether the activity of the stem cell niche drives the growth occurring during emergence.

The phenotype of several mutants support the idea of post-emergence meristem activation in both the primary and lateral roots. In the *root meristemless 1 and 2* (*rml1* and *rml2)* mutants, the primary root growth stops after reaching about one to two millimetres length, cell proliferation does not occur at the tip of the root and the meristems differentiates (Cheng Jin-Chen et al., 1995). Interestingly, growth seemed to stop at the same developmental time point in both the lateral and

primary root, once the root contained about seventeen epidermal cells per cell file (Cheng Jin-Chen et al., 1995). While growth arrest shortly after emergence could indicate a failed meristem activation, it could also indicate a failed transition toward meristem maintenance, which in the case of the *rml1* and *rml2* mutants leads to meristem termination as meristematic cells differentiate (Cheng Jin-Chen et al., 1995). It would be interesting to assess cell proliferation in lateral roots of the *rml1* and *rml2* mutants, and determine whether LR growth arrest is due to the lack of activation of cell proliferation, or whether it is due to the loss of cell proliferation. The *aberrant lateral root formation 3* (*alf3-1*), mutant shows arrested LR growth shortly after emergence which can be reverted by exogenous auxin application, suggesting that auxin also takes a part in post-emergence meristem activation (Celenza et al., 1995). Additionally, abscisic acid (ABA) reversibly inhibit LR growth shortly after emergence (De Smet et al., 2003), while it promotes quiescence in the QC and prevents differentiation of the surrounding stem cells in the PR (H. Zhang et al., 2010). It would be interesting to investigate how ABA signalling evolves during the formation of the LR stem cell niche, specifically during CC quiescence acquisition.

Different growth phases can be distinguished during root growth : root meristem formation, indeterminate growth phase and termination growth phase. During the indeterminate growth phase the meristem is actively producing cells and supports root growth. At contrary, during termination growth phase, the root meristem irreversibly becomes exhausted, all cells differentiate, no further growth takes place (Shishkova et al., 2008). It would be interesting to further investigates whether the post-emergence acquisition of quiescence in the CC is a tipping point for meristem activation and/or transition toward indeterminate growth.

4. Conclusion

In the context of root development, stem cell niches are critical for maintaining the tissue organisation of organs, and sustain growth. During the primary root development, the stem cell niche is established first, during embryogenesis, and maintains tissue organisation post-embryonically. On the contrary, the stem cell niche of lateral roots seems to be an emergent property of LR development, as cells dividing in a non-stereotypic manner (Wangenheim et al., 2016), robustly arrange into the typical PR-like topology. This study proposes evidences that the establishment of the PR-like stem cell niche is a progressive process leading to the formation of a functional stem cell niche only post-emergence.

Materiel and methods

1.Transgenic material

1.1 Receive plant materiel.

Line name	Composition	Description	Background	Reference
sR491	*pTCSn::2xVenus-NLS*	Cytokinin signalling read out.	Col-0	Kindly provided by Jan Lohmann lab.
sC111	*DR5v2::3xYFP:NLS, pRPS5A::dtTomato-NLS*	Auxin signalling read out.	Col-0 *pUBQ10::GRP-3xPIP1,4, pGATA23::H2B:3xmCherry*	
sR456	Q0990 enhancer trap line	Vasculature and QC reporter	Col-0	Radoeva et al. 2016
sR640	*pWOX5::gWOX5:3xYFP*	WOX5 expression in a context where cell to cell mobility is prevented	wox5-1, also containing QC184::GUS enhancer trap line	Pi et al. 2015
sR530	*pBRAVO::BRAVO:GFP*	QC and vascular initials reporter		Vilarrasa-Blasi et al., 2014
sR640	*QC184::GUS*	QC specific identity reporter	Wox5-1 complemented with *pWOX5::gWOX5:3xYFP*	Pi et al. 2015
sR436	*QC25::GUS*	QC specific identity reporter	Col-0	
sR518	*pAGAMOUS-LIKE42::AGAMOUS-LIKE 42:GFP*	QC specific identity reporter	Col-0	Nawy et al., 2005
sR355	Cytrap, *pHTR2::CDT1a (C3)-RFP, pCYCB1::CYCB1;1-GFP*	Cell cycle progression reporter	Col-0	Yin et al. 2014
sR522	*dcr*	Mutant in DEFECTIVE IN CUTICULAR RIDGES gene	Col-0	Panikashvili et al. 2009

sR517	*QC25er::CFP*	Cloned QC marker from the QC25 enhancer trap line	Col-0	Goh et al. 2016
sR612	*p35S::lox-Ter-lox:erCFP, pSCR::CRE-GR pWOX5::GFP*	Lineage tracing experiments	Col-0	Efroni et al. 2016
sR611 #3	*pSCR::LhG4-GR, p6Op::mTurquoise2*	Driver line inducing expression in the SCR domain	Col-0	Schürholz et al. 2018
sR529	*pCaMV 53S ::ERF115*	Over expressor of ER115	Col-0	Heyman et al. 2013
sR532	*Bravo-2*	Mutant line in the BRAVO genes	Col-0, SALK_062413	Vilarrasa-Blasi et al. 2014

1.2 Transgenic lines generation.

Transgenic lines were generated by floral dip into a suspension of *Agrobacterium tumefaciens* previously transformed by plasmid cloned using the Green Gate method. Wild type plants used for transformation or as control are the Columbia (Col-0) ecotype of *Arabidopsis thaliana*.

Line name	Construct	Background
stLB014	*pSCR::H2B:GFPx3*	Col-0
sC184	*pSCR::H2B:GFPx3*	*pUBQ10::PIP1,4:GFP3x, pGATA23::H2B:mCherryx3,* Col-0
stLB018	*pWOX5::H2B:GFPx3*	Col-0
stLB017	*pSOMBRERO::H2B:GFPx3*	Col-0
stBB018	*pBRAVO::WOX5:GFP*	Col-0
stBB019	*pBRAVO::SCR:GFP*	Col-0
stBB022	*pBRAVO::WOX5:GFP*	Cytrap reporter line, Col-0
stBB023	*p4Op::BRAVO*	*pSCR::LhG4-GR, p6Op::mTurquoise2,* Col-0

stLB lines were generated by Lotte Bald.
stBB lines were generated by me.

1.2.1 Green gate cloning.

1.2.1.1 Principle

The GreenGate method was used to build all the constructs cloned in this study (Lampropoulos et al., 2013). Briefly genomic fragments of interest were amplified by PCR with the Q5® High-

Fidelity DNA Polymerase (NEB). The primer used for amplification add specific overhangs to the fragments. Those overhangs are restriction site for the Eco31I fast digest restriction enzyme (ThermoFisher). The sticky-ends generated by digestion are design so that they create a specific sequence of overhang which allow for a modular combination of digestion product. T-DNA can therefore be generated using a standard assembly method in a one-tube reaction containing all the fragments necessary for expression (Lampropoulos et al., 2013).

1.2.1.2 Construct composition

The pGGZ backbone contains a resistance cassette to spectinomycin.

Construct name	A module	B module	C module	D module	E module	F module	Z module	Used for
pLB006	SCR promoter pLB003	H2B sequence pGGB024	3xGFP pGGC025	C-Dummy pGGD002	UBQ10 terminator pGGE009	Kanamicin resistance pGGF007	Plasmid backbone pGGZ001	SCR transcriptional reporter expression
pLB007	SMB promoter pLB004	H2B sequence pGGB024	3xGFP pGGC025	C-Dummy pGGD002	UBQ10 terminator pGGE009	Kanamicin resistance pGGF007	Plasmid backbone pGGZ001	SMB transcriptional reporter expression
pLB008	WOX5 promoter pLB005	H2B sequence pGGB024	3xGFP pGGC025	C-Dummy pGGD002	UBQ10 terminator pGGE009	Kanamicin resistance pGGF007	Plasmid backbone pGGZ001	WOX5 transcriptional reporter expression
pKK004	SCR promoter pLB003	N-Dummy pGGB003	BRAVO coding sequence pKK001	Linker GFP pGGD001	UBQ10 terminator pGGE009	OLE1 Fast red selection pLB012	Plasmid backbone pGGZ001	Constitutive expression of BRAVO in WOX5 domain
pKK005	WOX5 promoter pLB005	N-Dummy pGGB003	BRAVO coding sequence pKK001	Linker GFP pGGD001	UBQ10 terminator pGGE009	OLE1 Fast red selection pLB012	Plasmid backbone pGGZ001	Constitutive expression of BRAVO in SCR domain
pKK006	p4Op pGGA016 Dex responsive promoter	N-Dummy pGGB003	BRAVO coding sequence pKK001	C- Dummy pGGD017	UBQ10 terminator pGGE009	Basta resistance pGGF-BarR	Plasmid backbone pGGZ003	Inducible expression of BRAVO

pLB plasmid were cloned by Lotte Balk.
pKK plasmid were cloned by Kristin Konopatzki.

1.2.1.3 Bacteria transformation

Bacteria transformation was performed using electroporation with a MicroPulser (Bio-Rad).

1.2.1.4 E. Coli strain

Plasmid amplification during cloning was performed using the Invitrogen TOP10 electrocompetent bacteria, grown at 37°C on lysogeny broth (LB, (Bertani, 2004; BERTANI, 1951)) medium, solidified by 15g/L of agarose, when appropriate and complemented by the suitable antibiotics.

1.2.2 Plant transformation.

Electrocompetent ASE pSOUP+ (Pruss et al., 2008) *Agrobacterium tumefaciens*, were transformed by electroporation and grown on LB medium supplemented with the appropriate antibiotics. *Agrobacterium tumefaciens* were then pre-cultures in about 5mL of LR supplemented with antibiotics at 28°C overnight. The following day, 500µL of pre-culture were added to 250mL of LR-antibiotics and grown overnight at 28°C. The day after, bacteria were pelleted then resuspended in a solution containing ½ MS, 5% of sucrose and 50µL/L of Silwet L-77. Inflorescences were dipped for about 3 minutes in the bacteria containing solution.

1.2.3 Transgenic line selection.

Transformant seeds were selected on ½ MS medium supplemented with antibiotic or herbicide depending on the resistance cassette present on the T-DNA. Lines used in this study were selected for single insertion in T2 generation and homozygoty in T3 generation.

2. Growth conditions.

2.1 Seed sterilization

Seeds were sterilized for 10 min with 1 mL of solution composed of 70% ethanol and 0.05% Triton X-100. Seeds were then washed 3 times with 1 mL of 70% ethanol then further incubated for 10 min with 1mL of 100% of ethanol. Seeds were then left to dry under the sterile bench.

Due to the specific sensitivity of the *dcr* mutant, seeds were stored at 4°C, and sterilized for 2 minutes with a solution containing : 10 mL of Bleach (Chemsolute Natriumhypochloritlösung, 6-14% active chlore), 20 mL of autoclaved miliQ water, 0.05% Tween 20. Seeds were then washed 6 x 1 min with sterile miliQ water, then sown.

2.2 Growth conditions

2.2.1 Seedling sterile-growth on plate

Seedlings were grown vertically, on plates, in an Adaptis Conviron A1000, with long day conditions (16h light / 8h dark) under white light, at 22°C. The plant medium used on plate is

½ Murashigo and Skoog medium (Duchefa, Murashige et al., 1962) buffered to pH 5.8 with 0.5g/L of MES, adjusted with KOH, and solidified by 0.8% phyto-agar (Duchefa). When needed lateral root formation was induced by gravistimulation (Ditengou et al., 2008; Oa et al., 2009).

2.2.2 Plant growth on soil

Plants were grown on soil (Einheitserde CLT SM fein), in growth chamber (Klimakammer Johnson Controls) at 22°C in long day (16h light / 8h dark), under blue and red LEDs , with 65% humidity.

2.2.3 Seeds imbibition for radicle imaging

Seeds were sterilized with ethanol as described above. Seeds were then placed on a sterile filter paper soaked with sterile miliQ water in a petri dish. The sealed petri dish is then placed in darkness at 22°C for the given time of imbibition. Before imaging, seeds were dissected with a needle and tweezer and under a dissection microscope, fixed with 4% PFA under vacuum for 15 min, then cell wall stained as described under, and mounted into 50% glycerol for imaging.

2.3 Treatments with chemicals

2.3.1 Dexamethazone inductions.

Dexamethazone (Dex, Sigma-Aldrich Chemie GmbH, PHR 1525) induction of BRAVO expression relies on Dex dependant translocation of the chimeric transcription factor LhG4 fused to the ligand binding domain of the rat glucocorticoid receptor GR (LhG4-GR) into the nucleus. Once in the nucleus, it binds the p4Op promoter and activates transcription (Craft et al., 2005).

Dex treatment was performed using plate with solid ½ MS medium supplemented with 15µM and 30µM of Dex dissolved in dimethyl sulfoxide (DMSO) for the induction of the CRE recombinase in SCR domain, and BRAVO expression respectively.

2.3.2 Phleomycin treatment.

The phleomycin (Sigma-Aldrich Chemie GmbH, P9564-5mg, dissolved at 5mg/mL in sterile miliQ water) treatment was performed for 24h in liquid ½ MS medium on small petri dishes (20µg/mL final concentration). After growth on regular ½ MS plates and gravistimulations,

seedlings were transferred in the liquid medium. The petri dishes were then sealed and returned to the Conviron until staining with PI and imaging.

2.3.3 BCECF-AM and Hoechst 33342 incorporation in LR.

After growth on vertical ½ MS plates, seedlings were transferred in liquid ½ MS containing 10µM BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (B-1170), Molecular probes ™, Invitrogen™) dissolved in DMSO, for 3h, or 5µg/mL Hoechst 33342 (Invitrogen, Thermo Fischer Scientific™, C10340) for 30 min, then rinsed with sterile miliQ water, before mounting for imaging at the epifluorescence microscope. The BCECF-AM dye was imaged using the GFP filter, while the Hoechst 33342 was imaged using blue filter of the epifluorescence microscope.

3. Microscopy

3.1 Microscope used in this study.

Type of microscope	Microsocpe references	Objectives used for imaging
Dissecting microscope	Nikon SMZ18 with Sola light engine Lumencor light	Nikon SHR Plan Apo 1X WD 60.
Epifluorescence microscope	Axio Imager. M1 (Carl Zeiss, Oberkochen, Germany)	Camera : AxioCamHR3_552, Objectives : Plan-Apochromat 20x/0.8 M27 and EC Plan-Neofluar 40x/0.75 M27
Conforcal microscopes	TCS SP8 inverted Confocal Laser Scanning Microscope (Leica)	Objective : APO HC PL 40X/1.30, oil immersion
	TCS SP8 DMi8 inverted Confocal Laser Scanning Microscope (Leica)	Visible-Argon laser (used at 20%) Objectives : 63X/1.40 APO PL HC, oil immersion
Light sheet microscope	MuViSPIM (Luxendo, Bruker, Germany)	Excitation : Nikon Plan Fluor 10X / 0,30W OFN 25 DIC N1 Detection : Nikon NIR Apo, 40X/0,80W N2 WD 3,5

3.2 Laser lines used for excitation of fluorescent molecules and detection ranges used in this study.

Fluorescent molecule	Laser line used for excitation	Detection range
Calco Fluor white	405nm	425-475 nm
GFP	488 nm	500-550 nm
Alexa fluor 647	638 nm	650-695nm
Venus	488 nm	521-550 nm
RFP	552 nm	600-650 nm
PI	488nm	600-700 nm
mPS-PI	488nm	600-700 nm
mTurquoise	405 nm	460-500 nm
CFP	405nm	443- 488 nm

3.3 Imaging of fixed transcriptional reporters.

Seedlings expressing fluorescent reporters were grown vertically on ½ MS plates then fixed for 30 min under vacuum in 4% paraformaldehyde (PFA), in PBS. After PBS washes, seedlings were submerged into ClearSee solution (5g Xylitol (Sigma-Aldrich), 7.5g Sodium deoxycholate (Sigma Aldrich, D6750 SLBT 3408), 12.5g Urea, 50 mL of miliQ water (Kurihara et al., 2015)) for 4 days before cell wall staining. Seedling were mounted in ClearSee solution for imaging. Imaging was performed using Leica TCS SP8 confocal microscope, with the 40X objective with oil immersion.

3.4 Live imaging with confocal microscopy.

Seedlings were gravistimulated for 52h, 2 days after germination, then transferred in an imaging chamber (Lab-Tek Chambered #1.0, Borosilicate coverglass system REF 155 361, Thermo Fisher Scientific) and covered with a piece of solidified ½ MS from the plate as described in (Marhavi & Benkova, 2015). Seedlings were imaged with the TCS SP8 Leica confocal, 20X objective with oil immersion. Imaging was performed with a temporal resolution of 2h. Movies were analysed and annotated using Fiji.

3.5 Live imaging with light sheet microscopy.

After sterilization, seeds were sown on the top of glass capillaries (intraMARK Micropipette BLAUBRANDR 100µL, REF 7027 44) filled with ½ MS jellified with 1%Phytagel (Sigma-Aldrich P8169), taped in petri dishes (Figure 22). After 2 days of stratification at 4°C in the dark, the petri dishes were transferred to the plant growth chamber for 3 to 4 days of growth. Capillaries were removed from the petri dish, and about 5mm of the seedling´s root surrounded by Phytagel was extruded out of the glass capillary before being placed in the MuViSPIM (LUXENDO, Germany) imaging chambers. The imaging chamber was filled with ½ MS filtered through a syringe filter sterile with a pore size of 0.22µm (Berrytec). Imaging was performed with a 0.250 µm z-step, with a temporal resolution of 30 min. Between stack scanning, seedling were illuminated by blue and red LED (ThorLABS SM1CP2M). The MuVISPIM contains 2 sets of objectives, one pair of objectives for the illumination of the sample (Nikon Plan Fluor 10X / 0,30W OFN 25 DIC N1) and a pair of objectives for the detection (Nikon NIR Apo, 40X/0,80W N2 WD 3,5). The detection objectives are coupled to Hamamatsu digital camera C11440 Orca-flash 4.0 cameras. While the MuViSPIM set up allow for the simultaneous recording of opposite view thanks to its 2 cameras, the movies presented in this studies are taken from the camera providing the best image quality. Movies were analysed and annotated using Fiji.

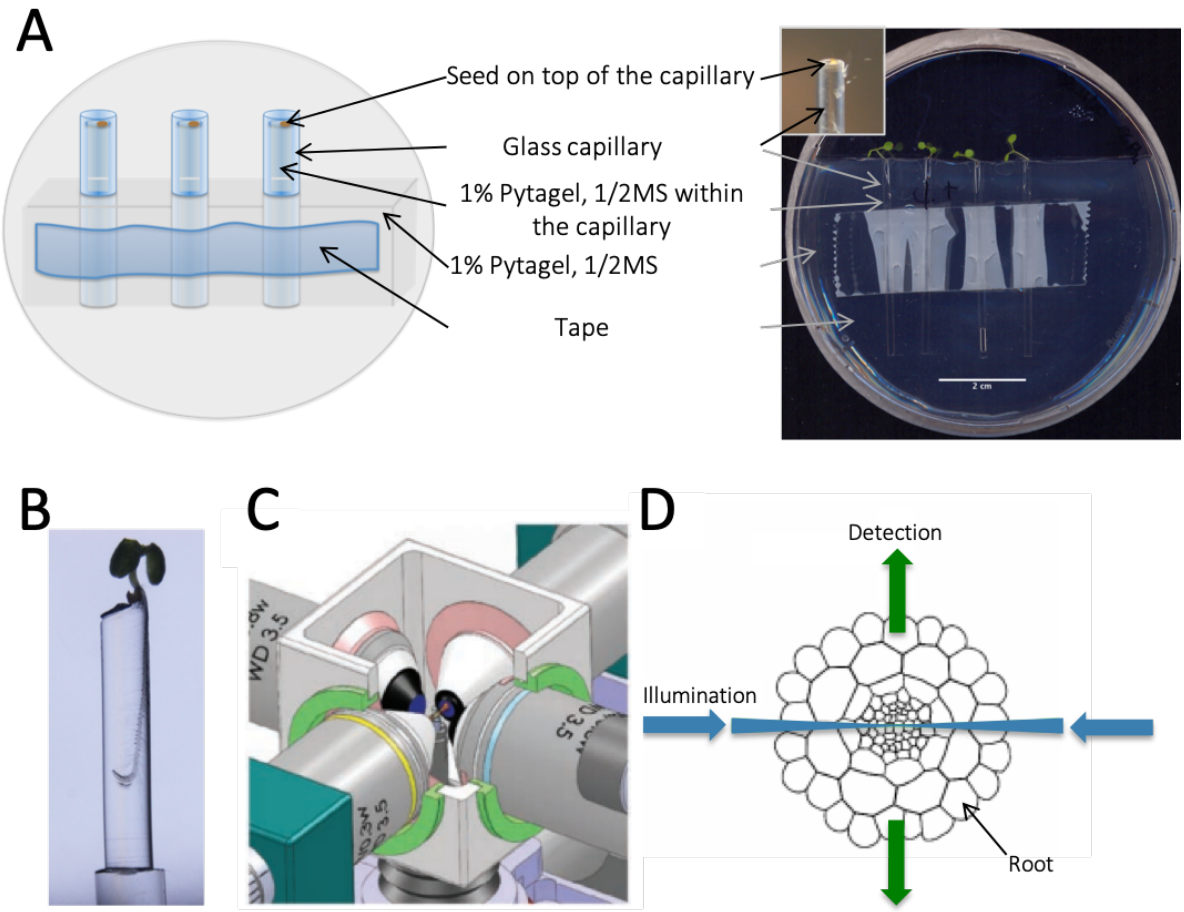


Figure 22 : MuViSPIM Light sheet microscopy for live imaging.
Sterilized seeds were sown on top of glass capillaries filled with ½ MS medium solidified with 1% of Phytagel (A). Before imaging, the capillaries are removed from the petri dish, and the seedling´s root, surrounded by a cylinder of medium, is extruded out of the glass capillary (B). Both the extruded seedling with the capillary are placed within the imaging chamber of a multi view selective-plane illumination microscope (MuViSPIM), filled with sterile liquid ½ MS medium (C). In this set up, two opposite objectives create an illumination beam, scanning the field of view, and across the whole thickness of the root. The emitted fluorescence is detected at a 90° angle by a different set of objectives (D). (C) Adapted from Krzic et al. 2012 (D) Adapted from von Wangenheim dissertation, 2014.

4. Cell proliferation assessment

4.1 Cytrap line, and cell proliferation assessment.

Cytrap expressing seedlings were grown vertically for 4 days before being gravistimulated for 120h, 96h, 72h, 48h. Seedlings were then fixed in 4%PFA in PBS for 30 min, then cell walls were stained and confocal imaging using the 40X objective of the Leica TCS SP8 confocal microscope.

4.2 EdU staining.

EdU incorporation was performed by transferring seedlings first grown on ½ MS plates to ½ MS plates containing EdU.

Experiments	EdU concentration used	Period of incubation
Cell proliferation in LRP	1 µM	8h
Cell proliferation in WT LR	0.5 to 10 µM	24h
Cell proliferation in *dcr* LR	0.5 µM	24h

At the end of the incubation period, incorporated EdU was revealed by Click iT ™ coupling of the fluorophore Alexa647nm to the EdU molecules, following a modified version of the protocol from the manufacturer (Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647, Invitrogen, Thermofisher™, C10340). Seedlings were first fixed in 4%PFA in PBS for 15 min followed by 2 times 1 min wash with 3% BSA (Bovine serum albumin) in PBS. Seedlings were then incubated for 20 min with 0.5% Triton X-100 in PBS and washed with 3% BSA 2 times for 1min, then incubated in the dark for 30 min with the Click-iT™ reaction cocktail. After the Click-iT™ reaction, seedlings were rinsed with PBS and washed with 3% BSA in PBS 3 times for 1 min, then 2 times with PBS before cell wall staining. The Click-iT™ reaction cocktail was assembled according to the manufacturer ´s instructions. Stained seedlings were imaged using the 40X objective of the TCS Leica SP8 confocal microscope, or the Axio Imager. M1 (Carl Zeiss, Oberkochen, Germany).

5. Cell wall staining and topology assessment.

5.1 Calco Fluor white.

Cell walls were stained with the dye Calco Fluor white (Fluorescent brightener 28 F3543-1G Sigma-Aldrich).

Cell walls of lines expressing fluorescent reporters were stained with 0.1% Calco Fluor white in ClearSee solution for 30 min, followed by 3x5 min wash in ClearSee (Ursache et al., 2018).

Cell wall staining, after Click iT reaction to reveal EdU incorporation, was performed using 0.1 % Calco Fluor white in PBS, for 30 min in the dark.

Wild type radicle and ERF115-OE and *bravo-2* seedlings were stained in the same ways seedlings treated with EdU were.

5.2 mPS-PI staining.

mPS-PI staining was performed according to (Truernit et al., 2008).

5.3 Propidium staining.

Seedlings were stained for 5 to 15 min in 10 to 45 µg/mL propidium iodide (Truernit & Haseloff, 2008).

5.4 Root topology assessment.

Seedlings were grown vertically for 3 to 5 days before being gravistimulated for 48h, 72h and 96h to induce lateral root formation. After cell wall staining, the transverse section of roots was imaged. The T-divisions formed by the CEI, and the first cells of cortex and endodermis was identified and used as a reference to determine the position of the central cells (cells between the T-divisions, but not directly contributing to it). The number of columella cell layers was counted from the first cells directly distal to the CC, up to the last layer of cells still attached to the PR. As soon as a periclinal division was visible in a columella cell, this was considered as an additional layer.

LR and PR topology was assessed on the same seedlings, therefore the PR root apical meristem observed were 7 to 9 days post germination.

6. GUS staining and clearing.

Seedlings were fixed for 20 min in 90% acetone at room temperature, before being placed in the staining solution, at 37°C for 48h. The staining was then stopped by replacing the staining solution by 70% ethanol for 30 min. The GUS staining solution contains : 10% of Triton X-100, 0.5 M of $NaPO_4$ at pH 7.2, 100mM of $K_3(Fe(CN)_6)$, 100mM of $K_4(Fe(CN)_6)$, and 100mM of X-Gluc (ROTH, 0018,3) solubilized in dimethylformamide.

Following the GUS staining, seedlings were cleared using a modified version of the protocol from (Malamy et al. 1997). Seedlings were incubated for 15 min at 70°C in a solution of 4% HCl and 20% methanol. Next they were incubated for 15 min in a solution of 7% NaOH and 60% ethanol at room temperature. This step is followed by a series of 10 min long incubations in 40%, then 20% then 10% ethanol. Seedlings were then incubated with 25% glycerol and 5% ethanol for 10 min before being placed in 50% glycerol, and stored at 4°C.

7. Software

7.1 R studio and R.

R studio was used to generate graphs and statistical analysis. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/ and R studio (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/).

7.2 Fiji.

Fiji was used for image analysis. (Schindelin *et al.*, 2012)

7.3 Filemaker pro.

References to the transgenic material are stored on the lab database FileMaker Pro (URL http://www.filemaker.com/).

7.4 Microsoft Office.

Text writing, spreadsheets for data acquisition, and figure mounting was performed using Microsoft Office for Mac.

7.5 Geneious.

Cloning relied on the Geneious software for in silico molecular biology ({Formatting Citation}).

7.6 Mendeley.

Mendeley was used as reference manager. (https://www.mendeley.com/)

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Publications

Research article

Escamez, S., Andre, D., Sztojka, B., Bollhöner, B., Hall, H., Berthet, B., Voß, U., Lers, A., Maizel, A., Andersson, M., Bennett, M., and Tuominen, H. (2020). Cell Death in Cells Overlying Lateral Root Primordia Facilitates Organ Growth in Arabidopsis. Current Biology 30, 455–464 https://doi.org/10.1016/j.cub.2019.11.078

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