INVESTIGATING THE ROLE OF SUCROSE IN CONTROLLING LATERAL ROOT FORMATION IN ARABIDOPSIS THALIANA

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Declaration

I declare that this thesis has been composed solely by myself. All the data discussed in this thesis were generated, analysed, and interpreted by me. The images of LRP stages shown in chapters 1, 2, and 4 were taken by Frank Machin (PhD), who is a post-doctoral research associate at the Doerner lab at the Institute of Molecular Plant Sciences—University of Edinburgh. And he has been duly acknowledged in the legends of those figures. All other images adapted from the literature have been duly acknowledged in the relevant figure legends.

This thesis has not been submitted in whole or in part for any other degree or professional qualification.

Georgina Pegu

March 2023

Abstract

Sucrose is the major transported carbon in plants and its dual role as both a nutrient and a signal, enables it to promote diverse physiological processes in plants, both directly and indirectly. In *Arabidopsis thaliana* (Arabidopsis), sucrose has been reported to promote lateral root formation, however mechanistic understanding of how sucrose promotes lateral root formation in Arabidopsis is lacking. Efforts aimed at studying the effect of sucrose on lateral root formation in Arabidopsis have been mostly focused on quantifying lateral root numbers, lengths, and densities. These parameters only give insights into the effect of sucrose on the emergence and outgrowth of lateral roots; not the primordia initiation and morphogenesis stages that underpin root system architecture and development in Arabidopsis. Those studies that have quantified lateral root primordia have not categorised them into the various developmental stages, therefore potential checkpoints of regulation by sucrose in the initiation and development of lateral root primordia cannot be determined. This thesis, therefore, aimed at understanding how sucrose might promote lateral root primordia initiation and morphogenesis, focusing on the effect of sucrose on the distribution of lateral root primordia among the various developmental stages. It did this by generating experimental methodologies that allowed the temporal dynamics of lateral root primordia development to be studied to identify potential checkpoints of regulation of lateral root primordia initiation and development by sucrose. Using a combination of tools from physiological and cell biology, this thesis shows that, sucrose promotes lateral root primordia formation by accelerating the rate of the developmental progression of lateral root primordia from the early stages to later stages. Sucrose does this by promoting nuclear migration of lateral root founder cells towards their common cell wall to signal the initiation of lateral root primordia and the initiation of lateral root primordia itself. This thesis also shows a new experimental system that allows the direct supply of exogenous sucrose to the shoot without the contact of the root with sucrose. This new system can be a valuable tool for studying the relationship between shoot-derived resources and root growth and development under *in vitro* conditions.

Lay summary

Plants use sunlight to make sugar by photosynthesis in the leaves, this sugar is transported from the leaves to the root where it provides energy and building blocks for root growth and development. The root is important for acquiring water and nutrients from the soil, and these water and nutrients are transported by the root to other parts of the plant for their growth and development. For the root to perform this function efficiently, it must undergo a process called branching. Root branching increases the surface area of the root, enabling it to acquire water and nutrients better—even ones at more distant locations. The sugar that is transported from the leaves to the root has been observed to promote root branching, however, exactly how the sugar does that is unknown. This project, therefore, aimed to understand how sugar promotes root branching by using different experimental procedures. The project has shown that sugar promotes root branching by affecting the early processes that underpin root branching and the whole root system—which the whole plant depends on for growth and survival. The understanding of how sugar promotes root branching will help to improve the efficiency of the root's function of acquiring water and nutrients from the soil. Improved efficiency of water and nutrient acquisition from the soil will increase plant yield, which means increased food to feed the growing global population.

Acknowledgements

Now unto him, that is able to do exceeding abundantly above all that we ask or think, according to the power that worketh in us. Unto him be glory in the church by Christ Jesus throughout all ages, world without end. Amen (Ephesians 3:20-21). I, first of all, want to acknowledge God almighty, the KING of the universe for being my source and sustainer, and for helping me and seeing me through this challenging, yet, fulfilling PhD journey.

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Finis coronat opus—the end crowns the work.

Abbreviations

PEO-IAA	(2-(1H-Indol-3-yl)-4-oxo-4-phenyl-butyric acid)
2,4-D	2,4-dichlorophenoxyacetic acid
alf4	*aberrant lateral root formation 4 mutant*
AFB1-3	AUXIN SIGNALLING F-BOX PROTEIN1–3
APL	ADP-glucose pyrophosphorylase large subunit
APS	ADP-glucose pyrophosphorylase small subunit
ARF	AUXIN RESPONSE FACTOR
AuxREs	Auxin Response Genes
AUX/IAA	AUXIN/INDOLE-3-ACETIC ACID
AUX1	AUXIN1
BDL	BODENLOS
CASP1	Casparian strip domain protein1
CO_2	Carbon dioxide
Col-0	Columbia
COP1	CONSTITUTIVE PHOTOMORPHOGENESIS1
CYCD	CYCLIN D
DAG	Days after germination
DIC	Differential Interference Contrast
DMSO	Dimethyl sulfoxide
DR5	DIRECT REPEAT 5

DR5:LUC	DR5:LUCIFERASE
AtNRT1	DUAL AFFINITY NITRATE TRANSPORTER
HY5	ELONGATED HYPOCOTYL
ER-GFP	Endoplasmic Reticulum-Targeted GFP
eir1	*ethylene insensitive 1*
F6P	Fructose 6-phophate
F1,6BP	Fructose 1,6-bisphosphate
G6P	Glucose 6-phosphate
GSA	Gravity Set point angle
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IBR	IBA-Resistant
IDA	INFLORESCENCE DEFICIENT IN ABSCISSION
LBD	LATERAL ORGAN BOUNDARIES DOMAINS
lrd	*lateral root development2*
LRs	Lateral roots
LAX3	LIKE AUX1 3
lhw	lonesome highway
LRFC	Lateral root founder cell
LRP	Lateral root primordia
MP	MONOPTEROS
NAA	1-Naphthaleneacetic acid
NPA	N-1-naphthylphthalamic acid

ANR1	NITRATE REGULATED1
OZ	Oscillation Zone
PEG	Polyethylene Glycol
PPFD	Photosynthetic photon flux density
PAR	Photosynthetically active radiation
PIN	PIN-formed
PR	Primary root
RuBP	Ribulose 1,5-bisphosphate
RSA	Root system architecture
SE/CC	Sieve element/companion cell
SAUR15	SMALL AUXIN UPREGULATED15
SCR	SCARECROW
SHY2	SHORT HYPOCOTYL2
slr	*solitary root*
smb	*sombrero*
SnRK1	Sucrose non-fermentation-1-protein kinase-1
SPS	Sucrose phosphate synthase
SPP	Sucrose phosphate phosphatase
SUC2	SUCROSE-PROTON SYMPORTER 2
TAA1	Tryptophan Aminotransferase1
TPR	Tetratricopeptide repeat
TSS	TPR-DOMAIN SUPPRESSOR OF STIMPY
TIFF	Tag Image File Format
TIR1	TRANSPORT INHIBITOR RESPONSE1

UDP-G	UDP-glucose
XPP	Xylem pole pericycle
YUC	YUCCA flavin monooxygenase

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Chapter 1 Introduction

1.1 *Arabidopsis thaliana*

*Arabidopsis thaliana (*Arabidopsis, hereafter*)* is a small angiosperm and a member of the mustard (Brassicaceae) family, alongside crops such as cabbage and broccoli. Arabidopsis has a very small and fully sequenced genome making it amenable to detailed genetic analyses (Meinke et al., 1998; Charlesworth and Vekemans 2005; Schmid et al., 2005; Koornneef and Meinke 2010; Fulgione and Hancock 2018). The Arabidopsis root has a simple radial organisation of the tissues around the vasculature. There are two levels of organisations of the tissues in the Arabidopsis root: a concentric organisation of the endodermis, the cortex, and the epidermis and a bilateral symmetry of the diarch vasculature consisting of two poles of the xylem and the phloem (Dolan et al.,1993; Parizot et al., 2008).

Concentric layers of the vasculature and the pericycle, the ground tissues (endodermis and cortex), and the dermal tissue (epidermis) converge into the stem cell niche which has the quiescent centre in the middle (Figure 1.1)—which contains the stem cells. These cells, also known as initials (Figure 1.1), are confined to cell files (Benfey et al., 2010), such that, the developmental history of a particular tissue can be determined through the observation of the cell files (that make up the tissue) along the longitudinal axis. This makes the Arabidopsis root excellent for developmental and ontological studies. Another major reason why Arabidopsis roots are attractive for developmental and physiological studies is that they are readily observed when grown on agar media, typically on vertically oriented Petri dishes (for ease of visualisation of roots) making physiological analyses such as gravitropic responses easy. This classic growth of the Arabidopsis root has made it an elegant model organ to study cell division and growth in a plant meristem (Rahni and Birnbaum, 2019).

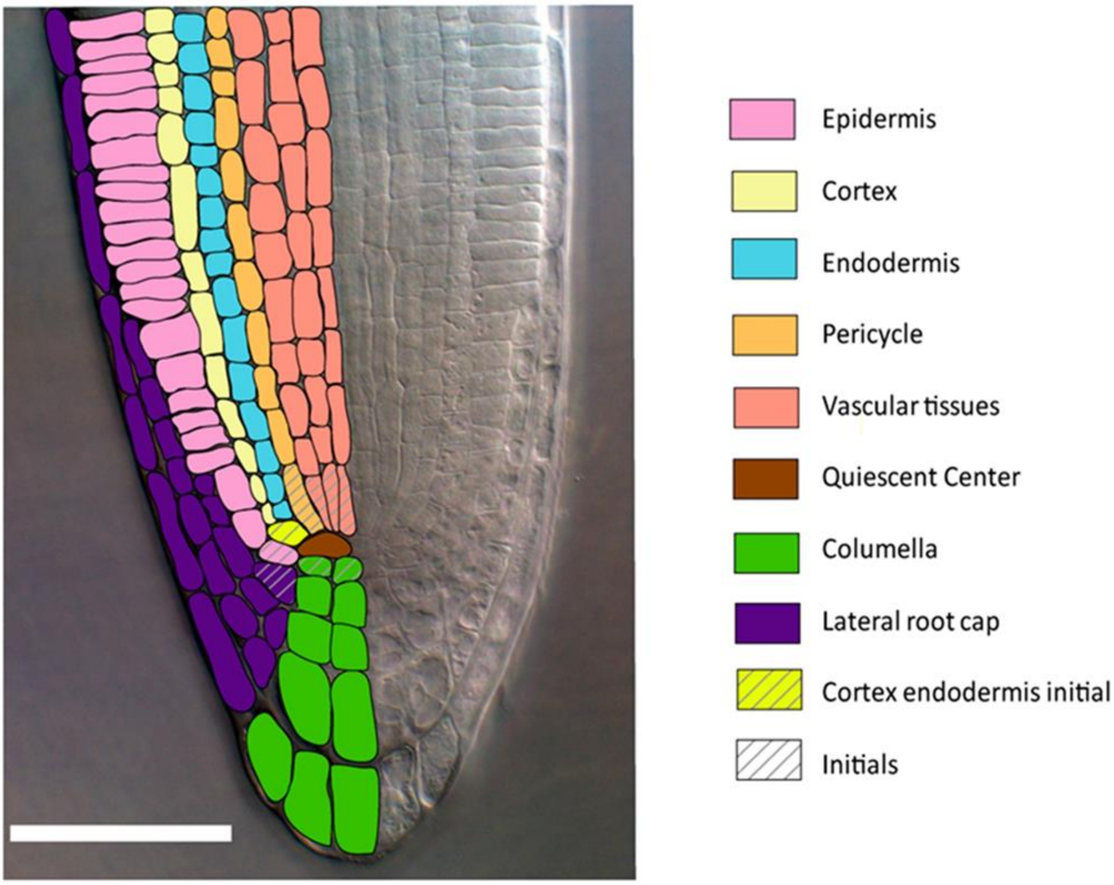

Figure 1.1 **A longitudinal axis of the Arabidopsis primary root tip.**

Differential interference contrast micrograph of the Arabidopsis primary root tip. Concentric layers of tissues surround the vascular tissues and converge at the quiescent center. Stem cells from the quiescent center give rise to progeny known as initials. The columella and the lateral root cap are additional layers beneath the quiescent center. Different colours represent different tissues. Bar is 50 µm. Image was taken from (Barrada et al., 2015).

1.2 Root system architecture.

The distribution of most resources within the soil environment is heterogeneous. For example, the topsoil tends to be drier than the deeper zones of the soil profile, which is caused by evaporation and percolation of water, consequently, water availability in the soil increases with depth (Concha and Doerner, 2020; Morris et al., 2017). The decomposition of organic materials via microbial activities creates nutrient-rich patches in the topsoil, causing non-uniform availability of nutrients (Giehl and von Wirén, 2014). The different properties of mineral nutrients contribute enormously to their spatial availability. For example, phosphorous, a limiting nutrient for plant growth and development forms insoluble complexes with ions such as calcium, iron, and aluminium within the topsoil, making it concentrated in the surface layer, hence, it becomes unavailable for acquisition by deep roots (Lynch and Brown, 2001; Vance, 2001; Giehl and von Wirén 2014; Morris et al., 2017).

Nitrate, another limiting nutrient is soluble and mobile, and, therefore, is readily leached beyond the root zone, rendering it unavailable for uptake by the root (Lynch, 2013). Taken together, the heterogeneity in the distribution of resources within the soil and the inability of the root to acquire these resources negatively impacts growth and development. In addition, resource distribution within the soil and their availability for uptake shape root system development (Lynch and Brown, 2001; Morris et al., 2017).

The root system refers to the overall network of plant roots, which functions to acquire essential resources and provides anchorage (Concha and Doerner ,2020). The three-dimensional arrangement of the root system is referred to as root system architecture (RSA) (Morris et al., 2017). RSA in *Arabidopsis* consists of the primary root (PR), lateral roots (LR), and root hairs (Giehl and von Wirén, 2014; Concha and Doerner, 2020).

Resources in the soil serve as signals that interact with the plant's genetics to influence RSA (Lynch and Brown, 2001; Lynch, 2013; Giehl and von Wirén, 2014; Morris et al. ,2017). The different spatial distribution of various resources necessitates that plants use different strategies to acquire them (Kembel and Cahill, 2005; García-Palacios et al., 2012; Tian and Doerner, 2013; Giehl and von Wirén, 2014; Weiser et al., 2016). In most cases, plants make architectural trade-offs when multiple resources with different patterns of distribution within the soil are to be acquired (Ho et al., 2005). For example, deep roots favouring primary root growth over root branching are formed for water uptake, while shallow roots favouring root branching over primary root growth are formed to forage phosphate (Uga et al., 2013; Concha and Doerner, 2020).

Similarly, under combined resource starvation (e.g., low phosphate and drought), the same architectural trade-offs acquire resources often of different spatial distribution patterns (Ho et al., 2005). The various resource acquisition strategies used by plants to acquire multiple resources are underpinned by the phenotypic plasticity of the root system, which enables the plant to integrate environmental cues into the endogenous developmental mechanism to optimise growth (Kembel and Cahill, 2005; García-Palacios et al.,2012; Tian and Doerner, 2013; Giehl and von Wirén, 2014; Weiser et al., 2016).

Root growth development is often measured as root length, angle, and density under experimental systems (Giehl and von Wirén, 2014). Although these parameters affect all components of RSA (primary root (PR), lateral roots (LRs), and root hairs) and impact their nutrient foraging capacity, those that affect LRs are more important because LRs underpin the root system function of resource acquisition by increasing its total surface area—this increases the volume of soil the root can explore to optimise resource acquisition. LRs are, therefore, considered the building blocks of RSA. Most importantly, the extent of root branching impacts the efficiency of resource acquisition, which is crucial for plant growth and development. Therefore, understanding the formation and regulation of LRs is of agronomic significance.

1.3 Lateral root formation

LRs are postembryonically formed from the PR and they originate from the pericycle cells that are adjacent to the xylem known as the xylem pole pericycle (XPP) cells (Figure 1.2). LR formation in Arabidopsis is a complex multi-step process consisting of priming, LR founder cell (LRFC) identity specification, nuclear migration, LR primordia (LRP) initiation, LRP morphogenesis, LR emergence, and meristem activation (Malamy and Benfey, 1997; Santos Teixeira and Ten Tusscher, 2019). The following sub-sections review the various processes that comprise LR formation in Arabidopsis.

1.3.1 Processes involved in lateral root formation.

1.3.1.1 LR patterning.

LR patterning consists of a series of successive processes that lead to the positioning of future LRs (De Smet et al., 2007). The patterning processes involved in LR formation are: (i) priming, and (ii) LRFC identity specification (De Smet et al., 2007; De Rybel et al., 2010; Moreno-Risueno et al., 2010). The subsequent sub-sections are aimed at discussing these processes in detail.

1.3.1.1.1 LR priming.

An endogenous mechanism for LR patterning known as priming confers on a subset of XPP cells the ability to form future LRs (De Smet et al., 2007; Moreno-Risueno et al., 2010;). This mechanism is based on an oscillation in the expression of the synthetic auxin reporter, *DIRECT REPEAT 5* (*DR5)* (De Smet et al., 2007; Moreno-Risueno et al., 2010) (Figures 1.3B & 1.3C). *DR5:GUS* reports a repeated generation of an auxin maximum in the protoxylem files of the basal meristem (located between the apical meristem and the elongation zone) which is transduced to the overlaying pericycle cells (De Smet et al., 2007).

To complement these observations, Moreno-Risueno and colleagues reported oscillations in the expression of genes such as the LR formation regulator, AUXIN RESPONSE FACTOR7 (ARF7) (Okushima et al., 2005; Okushima et al., 2007; Moreno-Risueno et al., 2010)*,* which is regulated by auxin through the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALLING F-BOX PROTEIN1–3 (TIR1/AFB1–3) signalling pathway (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005).

The oscillation in auxin response and the concomitant oscillatory gene expression leads to the formation of sites of stably maintained auxin response in the so-called oscillatory zone (OZ) (Figure 1.3B). These sites are referred to as prebranch sites, which are memories of auxin maximum during oscillation as reported by *DR5: Luciferase* (*DR5:LUC*) (Moreno-Risueno et al., 2010; Kircher and Schopfer, 2016; Laskowski and Ten Tusscher, 2017). In their study, LUC was used as a reporter in place of the frequently used GUS reporter because, GUS has a low turnover compared to LUC, rendering it unsuitable for observing temporally regulated events such as oscillation in auxin response and gene expression (Jefferson et al., 1987). Additionally, LUC is useful in non-destructive reporter assays compared to the destructive nature of histochemical staining that the use of GUS requires, making it impossible to be used in living tissues (Koo et al., 2007; Moreno-Risueno et al., 2010).

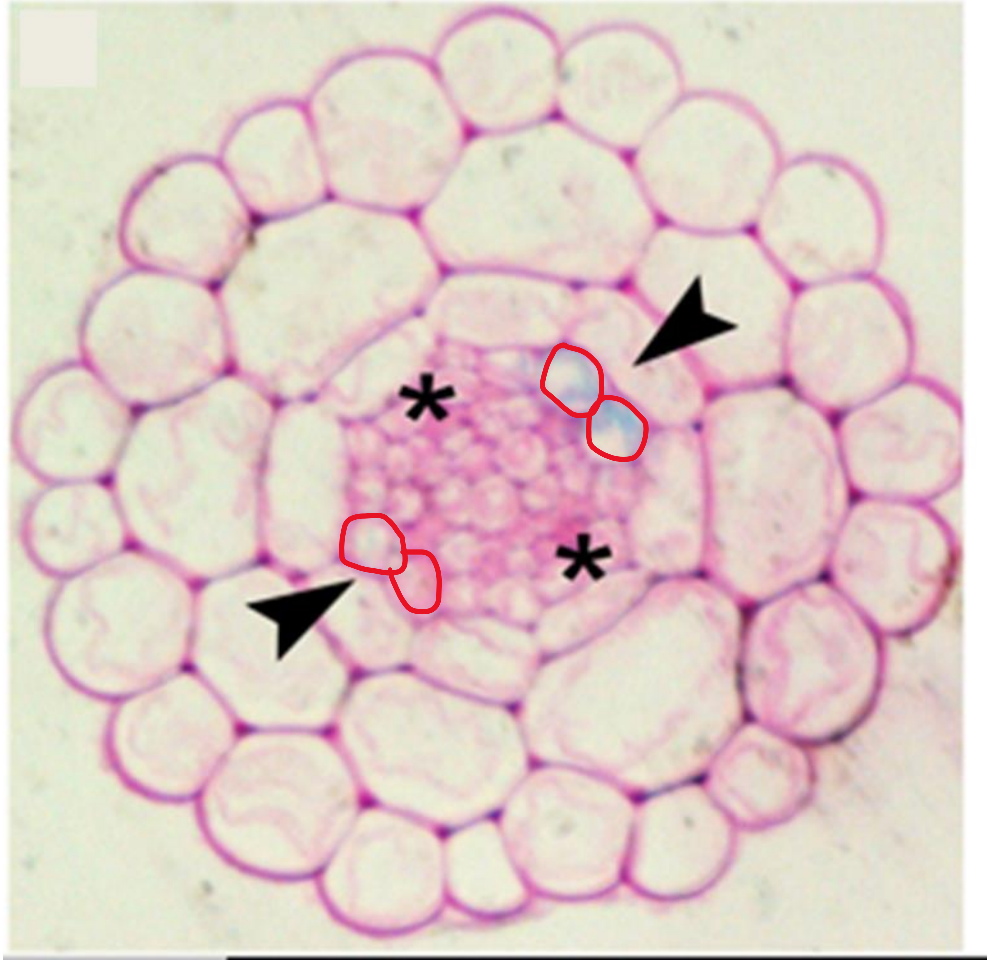

Figure 1.2 Section through an LRP showing *pGATA23::GUS* expression at one side of the xylem pole pericycle (XPP). Asterisks and arrowheads indicate phloem pole and XPP cells, respectively. Red circles show XPP cells. Image was adapted from (De Rybel et al., 2010).

In a subsequent study, Xuan et al (2015) showed that the oscillation in DR5 expression is dependent on auxin perception and signalling (Xuan et al., 2015). Using the *tir1afb2* (TIR1/AFB2 is the auxin response receptor) double mutant, they showed that, prebranch site formation is inhibited in the *tir1afb2* double mutant (Xuan et al., 2015). This observation was evidenced by a reduction in the number of prebranch sites in the *tir1afb2* double mutant (Xuan et al., 2015). The reduction in the number of prebranch sites was reflected in a similar reduction in LRs and LRPs in addition to the reduction in the intensity of *DR5:LUC* expression, additionally, the reduced *DR5:LUC* signal was not maintained but diminished

(Xuan et al., 2015). Taken together, these data show that the oscillation in DR5 is dependent on auxin response and signalling, and this is required to translate the oscillation signal to prime XPP cells to form future LRs (Xuan et al., 2015).

Additionally, Xuan et al (2015) showed that mutations in genes involved in the conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA) have reduced prebranch sites, suggesting a potential role in prebranch site formation (Xuan et al., 2015). The authors showed that mutants of IBA-Resistant1(IBR1), IBR3, and IBR10 and ENOYL-COA HYDRATASE2(ECH2), *ibr1ibr3ibr10* and *ech2ibr1ibr3ibr10*, mutants that are defective in IBA-to-IAA conversion pathway and had reduced prebranch sites, LRs, and LRP numbers, suggesting a relationship between IBA-to-IAA conversion and prebranch site formation (Xuan et al., 2015). Additionally, a decrease in DR5 signal intensity in the OZ of the *ibr1ibr3ibr10* and *ech2ibr1ibr3ibr10* mutant plants was observed, similar to the tir1afb2 mutant, justifying the previously observed reduction in prebranch, LR, and LRP numbers. Treatment with 1 µM exogenous IBA restored the DR5 signal and, prebranch site, LR, and LRP phenotypes (Xuan et al., 2015).

IBA-to-IAA conversion genes are expressed in the root cap cells, suggesting, a possible role of IBA-to-IAA conversion in creating a local auxin source in the root cap (De Rybel et al., 2012). Observation of DR5:GUS expression patterns in response to exogenously supplied IAA and IBA showed that, unlike IAA, exogenous IBA specifically induced an auxin response maximum in the root cap and the OZ that is dependent on IBA-to-IAA conversion enzymes (Xuan et al. ,2015). Based on the aforementioned hypothesis, the potential role of different root cap tissues in IBA response and LR priming was investigated, A GAL4-based transactivation approach was applied for targeted expression of IBR3 in different root cap tissues in the *ibr3* mutant background.

The *ibr3* mutant was resistant to IBA or naxillin, a synthetic compound that promotes IBA-to-IAA conversion (De Rybel et al., 2012; Xuan et al., 2015). An XPP driver line (J0121) was also used to determine whether a potential IBA-to-IAA conversion occurs in the XPP as well. Reduced prebranch site, LR, and LRP phenotype in the *ibr3* mutant was restored by the expression of IBR3 under the control of the native promoter or transactivating IBR3 in the expression domain of J3411 (lateral root cap) and J0951 (outer lateral root cap cells, epidermis, and pericycle in the differentiation zone), but not by the expression of IBR3 under the native promoters of J1092 (lateral root cap initials), J0121 (XPP cells), or GOLVEN5 promoter (columella).

Further analyses by the application of IBA to isolated regions of the PR tip including the meristem and the OZ led to an intense induction of DR5 response in the root cap and the OZ, thus promoting prebranch site and LR formation in an *ibr1ibr3ibr10*-dependent manner, however, no prebranch site or LR were observed under control conditions. Additionally, IBA did not induce prebranch site formation in the regions of the PR tip lacking the root meristem and OZ (Xuan et al., 2015). Altogether, these data suggest the IBA-to-IAA conversion pathway is active specifically in the outer lateral root cap cells and it produces a root cap-derived auxin, and that the perception of this auxin in the OZ is crucial for LR priming and prebranch site formation (Xuan et al., 2015).

In a follow-up study, Xuan et al (2016) investigated the auxin dynamics in the lateral root cap and surrounding regions. They observed a frequent disappearance of *DR5rev: VENUS-N7* expression in cells of the lateral root cap when these cells reach the basal meristem before their apoptosis. When the location in which this *DR5rev: VENUS-N7* expression disappears in the root cap was marked, LRs were later observed to arise from XPP cells in the PR close to the location of the fixed marker. In the *sombrero* (*smb*) mutant, in which delayed apoptosis of the root cap is associated with an abnormally long root cap, the formation of prebranch sites is significantly slower and less regular. Based on these data, the authors concluded that the periodic apoptosis of cells in the root cap influences the auxin fluxes that lead to the formation of prebranch sites (Xuan et al., 2016).

1.3.1.1.2 LR founder cell (LRFC) identity specification.

The Arabidopsis PR has been divided into developmental zones, namely: the meristematic, elongation, and differentiation zones (Dolan et al., 1993; Verbelen et al., 2006) (Figure 1.3A). Growth-induced displacement of cells in the meristematic zone, causes these cells to move through each of these developmental zones during their lifetime. As primed cells traverse through later developmental zones, some attain founder cell identities to become future LRs (Malamy and Benfey, 1997; De Rybel et al., 2010). Organogenesis in plants is known to begin with founder cell identity specification, followed by the activation of the founder cells to go through a series of divisions to form an organ (Chandler, 2011; Dubrovsky et al., 2008).

LR founder cell (LRFC) identity specification is a process whereby primed XPP cells acquire the competence to respond to activation signals to undergo coordinated cell divisions. LRFC identity specification confers on a subset of primed XPP cells the fate to form future LRs (Dubrovsky et al., 2006; Dubrovsky et al., 2008; Santos Teixeira and Ten Tusscher, 2019).

The expression of the transcription factor, GATA23, which is a member of the GATA-type family of transcription factors is reported to coincide with LRFC identity specification (De Rybel et al., 2010). *pGATA23::GUS* activity was observed to be absent from the root apical meristem (RAM), but was observed in the early stages of LRP morphogenesis, an intense expression of GATA23 was found in the first two developmental stages of LRP morphogenesis, with a gradual reduction in expression in subsequent stages, until its expression eventually diminishes (De Rybel et al., 2010). *pGATA23::GUS* activity was also observed in XPP cells close to the basal meristem, which is a region lacking LRP, suggesting that GATA23 has a role in a process before LRP initiation and morphogenesis (De Rybel et al., 2010).

To investigate the role of GATA23, De Rybel et al (2010), used RNA interference (RNAi) lines based on the specific tag for GATA23 (GATA23 RNAi) (De Rybel et al., 2010). Using this line, they observed a reduction in the number of early-stage LRP and an overall decrease in the number of emerged and unemerged LRPs. Conversely, the use of a GATA23 overexpression line, generated by using the XPP-specific J0121 GAL4 driver line (J0121>>UAS::GATA23) led to significant increases in unemerged LRPs compared to the J0121>>Col-0 control, in-depth analyses of the LRPs in J0121>>UAS::GATA23 plants revealed an increase in the first two developmental stages of LRP formation (De Rybel et al., 2010). Additional analyses of the LRPs in the GATA23 overexpression line revealed that a significant number of these LRPs were close to each other, which later resulted in emerged LRs that were close to each other, compared to those in the control plants (De Rybel et al., 2010). The authors, therefore, concluded that the observed ectopic LRPs in the GATA23 overexpression line indicate increased LRFC identity specification events and that GATA23 promotes LRFC identity specification (De Rybel et al., 2010).

LRFC identity specification was later found to be controlled by an auxin maximum in the basal meristem, as auxin was observed to accumulate in single or two neighbouring XPP cells as reported by *pDR5::GUS* and *DR5rev::GFP* with a periodicity of 15h (De Rybel et al., 2010). *pGATA23:GUS* promoter activity was observed in specific patches along PR before the first developmental stage that initiate LRP, these patches increased in number over time, increasing by an average of one patch per root about 10 hr after each *pDR5::GUS* pulse, showing a correlation between the auxin response in the basal meristem and GATA23 expression (De Rybel et al., 2010).

A 24h incubation of 3-day-old seedlings of *pDR5::GUS* and *pGATA23::GUS* reporter lines, independently on a media containing 25 mM (2-(1H-Indol-3-yl)-4-oxo-4-phenyl-butyric acid (PEO-IAA) (an anti-auxin that inhibits TIR1/AFB auxin receptors and therefore, auxin perception and signalling), revealed that newly grown parts of the PR no longer expressed *pGATA23::GUS* compared to control PRs, suggesting the dependence of GATA23 activity on auxin perception and signalling (De Rybel et al., 2010). Taken together, the above data show that

LRFC identity specification is mediated by GATA23 in an auxin-dependent manner.

1.3.1.2 Nuclear migration

After the specification of founder cell identity, the nuclei of two longitudinally abutted founder cells migrate towards their common cell wall, in a process known as nuclear migration (De Smet et al., 2007; De Rybel et al., 2010). LR formation relies on asymmetric cell division that generates cells of different sizes, placement, and fate (Blakely and Evans, 1979; Blakely et al., 1982; Malamy and Benfey, 1997; Beeckman et al., 2001; Cartwright et al., 2009; Ashraf and Facette, 2020; Muroyama et al., 2020).
To generate cells of different sizes, the nucleus moves to a polar position nearer the division site (Ashraf and Facette, 2020). The migration of nuclei of two adjacent founder cells towards their common cell wall serves as a signal for the asymmetric division that underpins LR formation (Figure 1.3D) (De Smet et al., 2007; De Rybel et al., 2010).

In the same study by De Rybel et al (2010), as described in section 1.3.1.1, the authors investigated whether the nuclear migration event is dependent on the auxin signalling co-receptor SOLITARY ROOT (SLR)/ IAA4 (SLR/IAA4), which is an established regulator of LR formation (Fukaki et al., 2002; Fukaki et al., 2005) to do this, they subjected *slr-1* mutant plants to prolonged auxin treatment which led to the polar movement of nuclei and induction of asymmetric cell divisions, compared to control conditions where no polar nuclei movement could be observed (De Rybel et al., 2010).

It is noteworthy that in response to auxin treatment asymmetric divisions could only be observed in just single XPP cells, never in a pair of XPP cells, and did not result in the anticlinal cell division that is a prerequisite for the initiation of LRPs (De Rybel et al., 2010). Similar observations were made in the double mutant of the downstream target of SLR/IAA4, AUXIN RESPONSE FACTOR7;19 (ARF7;19), *arf7arf19*. These observations suggest that the nuclear migration event is dependent on Aux/IAA and ARF-dependent signaling and it is required for the initiation of LRPs (De Rybel et al., 2010).

Additionally, De Rybel and co-authors investigated whether the nuclear migration which (as already alluded to is the subsequent event to the LRFC identity specification process) is mediated by GATA23, to do this, *in vivo* analyses of a GATA23 reporter line with a nuclear localisation signal (NLS), named, *pGATA23::NLS-GFP* was used, and the reporter activity revealed simultaneous migration of two nuclei of XPP cells toward their common cell wall in GATA23 expression patches right before the first asymmetric division (De Rybel et al., 2010). This observation was not made in the *pGATA23::GATA23-GFP* translational fusion, suggesting that GATA23 acts in a non-cell autonomous manner. Altogether, the aforementioned observations show that GATA23 is expressed before nuclear migration and may play a role in the nuclear migration process (De Rybel et al., 2010).

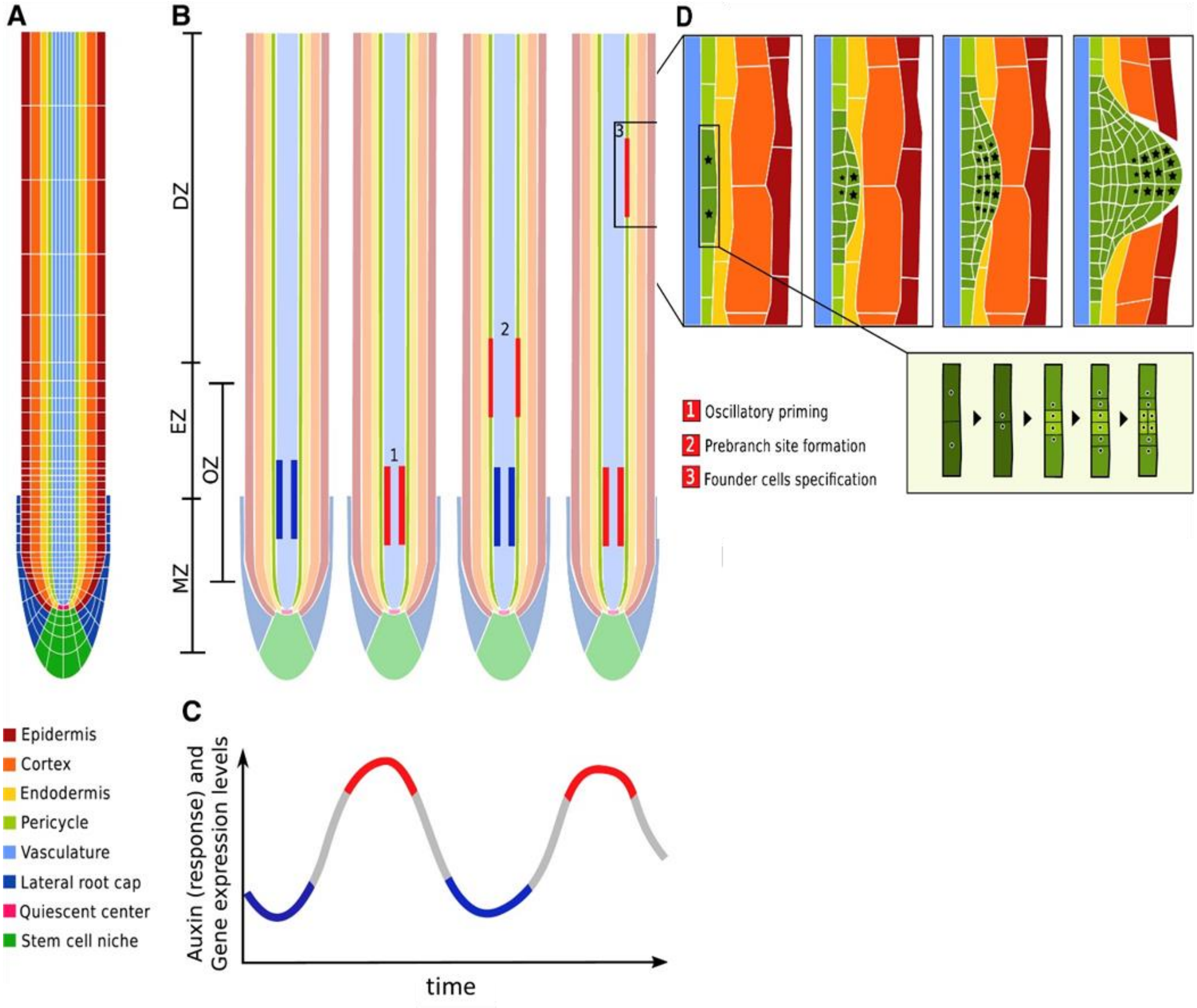

Figure 1.3 **Schematic overview of LR formation**

(A) Anatomy of the primary root with distinct cells and tissues in various colours. (B) Schematic of LR priming with oscillations of auxin (response) and gene expression in the oscillatory zone (blue, low levels; red, high levels), stable memorization of the high phase of the oscillation (1) into pre-branch sites (2), and subsequently founder cells (3). EZ, elongation zone; DZ, differentiation zone; MZ, meristematic zone; OZ, oscillation zone. (C) Temporal dynamics of oscillatory auxin response and gene expression in the oscillatory zone, with low and high levels indicated as in (B). (D) Stages of lateral root development, from left to right, founder cell identity specification, initiation, primordium formation, and emergence. Inset shows the nuclear migration and asymmetric divisions during lateral root initiation. Stars indicate auxin accumulation. Adapted from (Santos Teixeira and Ten Tusscher 2019).

1.3.1.3 LR initiation

The formation of LRs from a subset of founder cells requires a tightly-coordinated asymmetric cell division to generate diverse cells and tissues of distinct patterns (De Smet, 2012). Cell lineage analyses have shown that in *Arabidopsis,* LRs initiate from three XPP cell files (Dubrovsky et al., 2000). In Arabidopsis, LRs can initiate from either of the two xylem poles (diarch organisation), but not on either of the phloem poles (Beeckman et al., 2001; Dubrovsky et al., 2000; De Smet et al., 2006; Parizot et al., 2008) . The emanation of LRs on XPP cells is supported by analyses of LR development in *lonesome highway* (*lhw*) mutants, the *lhw* mutants have lost the diarch organisation of the vasculature, and therefore are only able to form one pole of the vasculature (Parizot et al., 2008). In the *lhw* mutants, LRs emerge solely from pericycle cells associated with the one pole of the xylem and not the phloem, suggesting that LRs exclusively form on XPP cells and not phloem pole pericycle cells (Parizot et al., 2008).

LRP formation, as already mentioned in section 1.3.1.2 is underpinned by a series of asymmetric cell divisions (Malamy and Benfey, 1997). An LRP is said to be initiated after the first anticlinal division occurs after nuclear migration (Malamy and Benfey 1997; De Smet et al., 2007; De Rybel et al., 2010). Pericycle cells are entrapped between the xylem and endodermis, the endodermis, has a highly lignified cell wall called the Casparian strip. The lignin constituent of the Casparian strip is a phenolic compound that is resistant to chemical and enzymatic degradation (Naseer et al., 2012; Lee et al., 2013). Prior to the first anticlinal division that leads to the initiation of an LRP, the endodermis which overlays the pericycle (XPP) must lose volume to allow for the radial growth of the founder cells (Vermeer et al., 2014; Marhavý et al., 2016). 4D imaging using light-sheet microscopy revealed that in association with nuclear migration, LRFCs increase in volume before LRP initiation and this is accompanied by the volume loss of the overlaying endodermis (Vermeer et al., 2014).

Marhavý et al (2016) showed that the ablation of the endodermal cells overlaying the XPP cells was sufficient to induce the division of XPP cells that had not been specified as founder cells (the so-called naïve pericycle cells) (Marhavý et al., 2016). It is worth noting that these naïve pericycle cells are not auxin responsive, and are, therefore unable to divide (due to auxin's prominent role in driving cell division in plants (Rayle and Cleland, 1992; Mockaitis and Estelle, 2008), however, the elimination of endodermal cells that hitherto placed mechanical restraints on them was sufficient to induce their division (Marhavý et al., 2016). This endodermal cell ablation, however, altered the cell division plane from anticlinal to periclinal, thereby preventing the initiation of LRPs (Marhavý et al., 2016).

The exchange of auxin flux between the pericycle and the endodermis is crucial for the formation of LRPs from LRFCs (Marhavý et al., 2013). This reflux is mediated by the auxin efflux carrier PIN-formed3 (PIN3); *pin3* mutants showed a delay in the initiation of LRPs from LRFC (Marhavý et al. 2013). An expression of the short hypocotyl 2-2 (*shy2-2*) gain-of-function mutant of AUX/IAA3 specifically in elongating endodermal cells to strongly reduce its ability for auxin response, revealed that a SHORT HYPOCOTYL2 (SHY2)-mediated auxin signaling module is essential for the role of the endodermis in LRP initiation (Vermeer et al., 2014). LRFC specification was observed in CASP1pro::shy2-2 plants, generated from the ectopic expression of *shy2-2* under the promoter for Casparian strip domain proteins1; CASP1 mediates Casparian strip formation in the endodermis, and as such its expression is endodermis-specific (Roppolo et al., 2014).

Although LRFC identity specification was observed in *CASP1pro::shy2-2* plants, they displayed a total block of formative divisions, resulting in plants with no LRs (Vermeer et al. 2014). The authors, therefore, hypothesised that SHY2 is involved in LRP initiation, and that auxin derived from lateral root founder cells is perceived by the endodermis through a SHY2-dependent-auxin signaling module, the endodermis would then signal back to the LRFCs to allow them to increase their cell volume and divide (Vermeer et al., 2014). It was also predicted that the LRFCs in CASP1pro::shy2-2 plants perceive the non-responding endodermis as increased resistance to their expansion growth, resulting in a total inhibition of their ability to enter mitosis and initiate LRPs (Vermeer et al., 2014). Altogether, these studies have demonstrated that the endodermis has an essential role in LRP initiation by accommodating the expanding pericycle cells and mediating the auxin dynamics in LRFCs during LRP initiation. These insights are vital for mechanistic studies that are aimed at establishing a causal relationship between a given treatment or molecule and LRP initiation to have an understanding of the spatial dynamics of already established regulators of LRP formation such as auxin.

1.3.1.3.1 The cell cycle and LRP initiation

Tightly coordinated cell cycle progression is crucial for asymmetric divisions in a subset of LRFCs for LRP initiation to occur (De Smet, 2012). Several studies have shown that an inaccurate cell cycle progression either leads to completely no divisions in founder cells or an uncontrolled cell proliferation instead of forming an LRP (Himanen et al., 2002; DiDonato et al., 2004; Vanneste et al., 2005; De Smet et al., 2010). For example, the phenotype of the *aberrant lateral root formation 4* mutant (*alf4*) does not have any LRs, because the XPP cells in the *alf4* mutant plants are unable to undergo the cell divisions required to initiate LRPs that will later become LRs (DiDonato et al., 2004).

CYCLIN B1 (CYCB1), which is essential for cell cycle activation is induced very early in the LRP initiation (Okushima et al., 2005; Vanneste et al., 2005). Commonly, the *CYCB1;1:GUS reporter* (Ferreira et al., 1994) is used for studying LRP initiation in Arabidopsis, as such, a lack of *CYCB1;1::GUS* activity in the pericycle of *alf4* mutants demonstrated the inability of their XPP cells to undergo cell division (DiDonato et al., 2004). The observation of a lack of *CYCB1;1:GUS* activity in *alf4* mutants therefore, led to the hypothesis that ALF4-encoded proteins enable pericycle cells to maintain the ability to divide in the differentiation zone where LRs initiate, and where most cells have lost their ability to divide (DiDonato et al., 2004; Dubrovsky et al., 2008).

The progression of XPP cells through the cell cycle during LR initiation is dependent on auxin response (De Smet, 2012). Progression of pericycle cells at the beginning of the G1-S transition is inhibited when polar auxin transport is blocked by NPA or when auxin response is inhibited in the gain-of-function *solitary root* (*slr-1*) mutant, this results in an inhibition of LR initiation, as no LRs are observed on the PRs of *slr-1* mutants (Himanen et al., 2002; Fukaki et al., 2002; Fukaki et al., 2005; Vanneste et al., 2005). Treatment of 4-day-old *slr-1* seedlings with 1 µM IAA, 1 µM 1-Naphthaleneacetic acid (NAA), or 0.1 µM 2,4-Dichlorophenoxyacetic acid (2, 4-D) (and incubated for 3 days) did not restore the LR formation, however, the same treatments induced the formation of many LRs in wildtype Columbia (Col-0) (Fukaki et al., 2002).

In addition*, CycB1;1::GUS* activity was not detected in young *slr-1* seedlings but could be detected in a small number of LRP initiating sites in older seedlings, however, the number of LRP initiation sites with *CycB1;1::GUS* activity was still reduced compared to the wildtype (Fukaki et al., 2002). The enhancer trap line End199 has the GUS reporter gene fused upstream of the SCARECROW (SCR) gene (that is required for radial patterning) and is expressed in the daughter cells of periclinal divisions during LR formation. No End199 activity was detected in the pericycle cells of 5-day-old *slr-1* seedlings*,* End199 activity was, however, found in the pericycle cells of the wild type (Fukaki et al., 2002). This indicates that sites of periclinal divisions are lacking in *slr-1* seedlings. SLR was later shown to encode *IAA14*, a member of the Aux/IAA protein which is involved in auxin response (Fukaki et al., 2002).

Taken together, these data suggest that anticlinal division is partially inhibited in the *slr-1* mutant, while periclinal division is completely blocked, and that defects in SLR-mediated auxin response inhibit the cell divisions necessary for LR initiation. Downstream targets of *SLR/IAA14, AUXIN RESPONSE FACTOR7 (ARF7),* and ARF19, as well as the direct downstream targets of *ARF7* and ARF19, LATERAL ORGAN BOUNDARIES DOMAINS16 (LBD16), LBD18 and

LBD33, were identified and are extensively discussed and reviewed elsewhere (Fukaki et al., 2005; Okushima et al., 2005; Okushima et al., 2007; Santos Teixeira and Ten Tusscher, 2019).

The asymmetric cell division that marks LRP initiation is also regulated by the E2Fa transcription factor (Berckmans et al., 2011). E2Fa is a transcriptional activator, and its overexpression has been shown to lead to increased cell proliferation (De Veylder et al., 2002; Sozzani et al., 2006). Berckmans et al (2011), showed strong transcription of E2Fa after auxin treatment, which coincided with the initiation of the G1/S and S phases of the cell cycle (Berckmans et al., 2011). Additionally, microarray data generated from green fluorescent protein (GFP)–based sorted cells from the J0121 enhancer trap line, showed that E2Fa is transcriptionally activated in the pericycle cells, suggesting it is involved in activating the cell cycle in XPP cells (Berckmans et al., 2011).

Moreover, a *ProE2Fa:E2Fa-GFP* translation protein fusion line was shown to be expressed in the pericycle cell nuclei upon auxin treatment, further corroborating the role of E2Fa in cell cycle activation to induce cell division in XPP cells. The *ProE2Fa:E2Fa-GFP* was also observed in the nuclei of two XPP cells undergoing nuclear migration, suggesting E2Fa activity before LRP initiation (Berckmans et al. 2011). Analyses of two independent E2Fa TDNA insertional lines revealed a reduction in early developmental stages of LRP morphogenesis, including the LRP initiation stage, confirming the role of E2Fa in LRP initiation (Berckmans et al., 2011). Auxin-dependent E2Fa expression was abolished in the *slr-1* mutant, as discussed in earlier sections, the wild type, SLR is a major regulator of LRP initiation, suggesting that E2Fa-mediated cell cycle activation during LRP initiation is SLR-dependent (Berckmans et al., 2011). Taken together, these results demonstrate the role of E2Fa in cell cycle activation that characterise LRP initiation.

1.3.1.4 LRP morphogenesis

After LR initiation, a series of anticlinal and periclinal cell divisions continue to give rise to a growing LR. Our understanding of LRP formation stems from the classic categorisation of LRPs into stages by Malamy and Benfey (1997) (Malamy and Benfey, 1997). Using a combination of Normaski Differential Interference Contrast (DIC) microscopy and histochemical approaches (i.e., root clearing), the authors show that LRP formation consists of a series of cell divisions that gives rise to a structure with a similar radial organisation as the primary root from which it originates (Figure 1.3; (Malamy and Benfey, 1997). It is noteworthy that the classification of LRPs into stages is artificial and is to aid in research data sampling and analyses, and even though the LRP development has been categorised into stages, the process itself is one continuous fluid process. Malamy and Benfey (1997) categorised the developmental stages of LRP formation into 8 stages, these characterisation and classification of LRPs have been the rubric on which most, if not all, breakthroughs in LRP research have been based (Dubrovsky et al., 2001; Fukaki et al., 2002; Dubrovsky et al., 2006; De Smet et al., 2007; Okushima et al., 2007; Dubrovsky et al., 2009; De Rybel et al., 2010; Goh et al., 2012).

The authors reported that stage I LRP is characterised by closely spaced cell walls in the pericycle layer in perpendicular orientation to the root axis (arrows, Figure 1.4A). An increased frequency of anticlinal divisions is seen as compared to the pericycle cells at the opposite side of the stele. In the longitudinal plane, approximately 8-10 ‘short’ pericycle cells are formed, which enlarge in a radial direction. At stage II, a periclinal division occurs which divides the LRP into an outer layer (OL) and an inner layer (IL)) (Figure 1.4B). At stage III, the OL undergoes periclinal divisions, thereby generating a three-cell-layer primordium comprising OL1, OL2, and IL (Figure 1.4C). Some peripheral cells do not divide, creating outer regions that are one and two-cell layers thick. This further emphasizes the domed shape of the LRP.

At stage IV, the IL divides periclinally, creating a total of four cell layers (OL1, OL2, IL1, IL2) (Figure 1.4D). At this stage, the LRP penetrates the overlaying endodermal layer. At stage V, a central cell in OL1 and OL2 undergoes anticlinal divisions to form four small cuboidal cells (Figure 1.4E, arrow; cells 5 and 6 in Figure 1.4F). The cells adjacent to these two cells in the OL1 and OL2 also divide (arrows, Figure 1.4F), creating an outer layer (OL1) that contains 10-12 cells (Figure 1.4F). Cells in IL2 undergo radial enlargement and divide (short arrow, Figure 1.3F), pushing the overlying layers up, thus compressing the cells in IL1 and OL2. The LRP at this stage is midway through the parent cortex but has not penetrated it.

Many events occur at stage VI: (i) Cells of OL2 undergo a periclinal division, creating a new internal layer (Figure 1.4G, arrows). These layers are designated OL2a and OL2b. (ii) The four central cells of OL1 divide periclinally (Figure 1.4H, arrow). After this division, OL1 at the tip of the primordium is considered to be the inner of these two layers. At this point there are a total of twelve cells in OL1, four in the middle that have undergone the periclinal division and four on either side (Figures 1.4G & 1.4H). The LRP passes through the parent cortex layer and has penetrated the epidermis. It is noteworthy that the central cells of the LRP, apparently derived from IL2, have a distinctive elongated shape characteristic of vascular elements (Figure 1.4H, arrowhead). At this point, the stage VI LRP begins to resemble the PR tip, containing 3 layers that could correspond to the epidermis, cortex, endodermis surrounding a core of presumptive stelar tissue, and a potential root cap at the tip of the LRP.

At stage VII, it is difficult to characterise the divisions in the internal layers as the LRP enlarges. However, many of the cells of the LRP continue to undergo anticlinal divisions. In the OL1, this results in 8-10 cells on either side of 8-10 central cells, referred to as the 8-8-8 cell pattern (Malamy and Benfey, 1997) (Figure 1.4I). The LRP emerges out of the PR at this point (Malamy and Benfey, 1997).

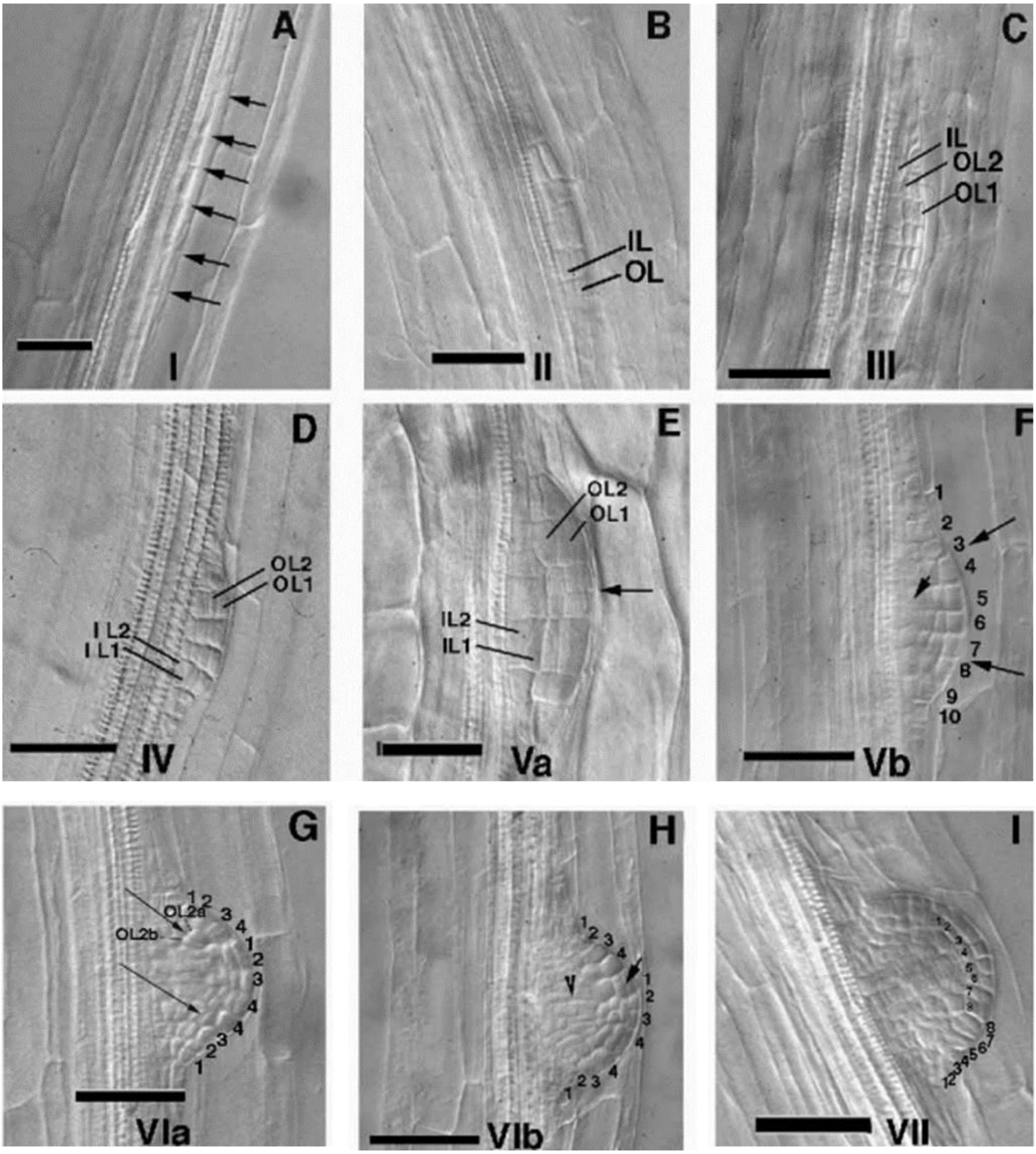
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Figure 1.4 **Stages of LRP formation**

(A) Stage I. Arrows point to new cell walls indicating anticlinal divisions in the pericycle. (B) Stage II. A periclinal division has divided the LRP into two layers, the outer layer (OL) and inner layer (IL). (C) Stage III. A periclinal division in OL has created a total of three layers. (D) Stage IV. A periclinal division in IL creates the fourth layer. (E) Stage Va. Arrow indicates a single anticlinal division in the center cells of OL1 and OL2. (F) Stage Vb. Arrows indicate two additional anticlinal divisions in OL1 and OL2. The short arrow indicates the region in which cells of the IL2 undergo expansion and division, distorting the shape of IL1 and OL2. Cells in the outermost layer are numbered to indicate the constant organization at this stage. (G) Stage VIa. A periclinal division in all but the center cells of OL2 creates a new tier of cells (arrows). The two tiers are designated OL2a and OL2b. (H) Stage VIb. Four central cells in OL1 divide periclinally to create another new tier at the tip of the primordium (arrow). The numbering shows that there are now four cells in OL1 on either side of four divided central cells. The OL1 is the inner of these two layers of divided central cells. The arrowhead points to the elongated cells reminiscent of vascular elements. The events shown in G and H may not necessarily occur sequentially; they are shown in two panels for clarity. (I) Stage VII. All the cells in the OL1 have undergone anticlinal divisions, as shown by the cell shape and the increased number of cells in this layer. This gives the characteristic 8-8-8 cell pattern, as indicated by cell numbering. In the central region of the tip of the primordium, numbers are on top of the relevant cells. Bar, 50 μm. Image taken from (Malamy and Benfey,1997).

1.3.2 The auxin signalling modules that regulate LR formation.

The nuclear auxin signalling pathway regulates several auxin response modules identified to modulate LR formation (Santos Teixeira and Ten Tusscher, 2019): In the absence of, or at low auxin concentrations, AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressor proteins form dimers with ARF transcription factors, thereby blocking their activation. Intracellular auxins are sensed by the TRANSPORT INHIBITOR RESPONSE1/ AUXIN SIGNALLING F-BOX PROTEIN1–3 (TIR1/AFB1–3) which targets Aux/IAA proteins for proteolytic degradation, freeing the ARFs (Figure 1.5) (Parry et al., 2009; De Smet et al. 2010). ARFs once freed from Aux/IAA proteins regulate the expression of auxin–responsive genes (Korasick et al., 2014) which regulate LR formation.

Different auxin response modules (Aux/IAA-ARF pairs) regulate the different stages of LR formation (Figure 1.6); four auxin response modules are currently known to regulate the various stages of LR formation individually or co-ordinately: (1) IAA28 and ARF5;6;7;8;19 (De Rybel et al., 2010) (2) SLR/ IAA14 and ARF7/19 (Fukaki et al., 2002; Fukaki et al., 2005; Okushima et al. 2005 Okushima et al. 2007) (3) IAA12/BODENLOS (BDL) and ARF5/ MONOPTEROS (MP) (De Smet et al. 2010) (4) SHY2 /IAA3-ARF7 (Figure 1.6A). (1) Module 1 regulates LRFC specification by activating GATA23, which has a downstream effect on LRFC specification (Figure 1.6B). GATA23 is specifically expressed in xylem pole pericycle cells to mark LRFC specification (De Rybel et al., 2010; De Smet et al., 2010). Although we know LRFC specification occurs downstream of GATA23, the mechanistic role of GATA23 is unknown (De Rybel et al., 2010).

(2) Modules 2, 3, and 4 regulate nuclear migration and asymmetric cell divisions during LR initiation (Figure 1.6C). LBDs are direct downstream targets of ARFs (Okushima et al., 2005). LBD16;18 and LBD33 act downstream of ARF7;19 to directly regulate nuclear migration and asymmetric cell divisions (Fukaki et al., 2002; Fukaki et al., 2005; Santos Teixeira and Ten Tusscher, 2019) (Figure 1.6C).

(3) Modules 2 and 3 regulate LRP morphogenesis (Fukaki et al., 2002; Okushima et al., 2007; De Smet et al., 2010; Santos Teixeira and Ten Tusscher, 2019) (Figure 1.6D). (4) Module 4 regulates LRP emergence through the endodermis (Figure 1.6E). Module 3 regulates LRP emergence through the cortex and epidermis, which involves cell wall remodelling. The auxin influx-carrier gene LIKE AUX1 3 (LAX3) is induced by ARF7 and ARF19 which causes auxin influx in cells nearest the emerging LRP (Swarup et al., 2008). Auxin induces genes encoding cell wall remodelling enzymes such as subtilisin-like protease in a *LAX3*-dependent manner to promote cell separation in the cortex and the epidermis (Swarup et al., 2008; Lavenus et al., 2013; Santos Teixeira and Ten Tusscher, 2019) (Figure 1.6E).

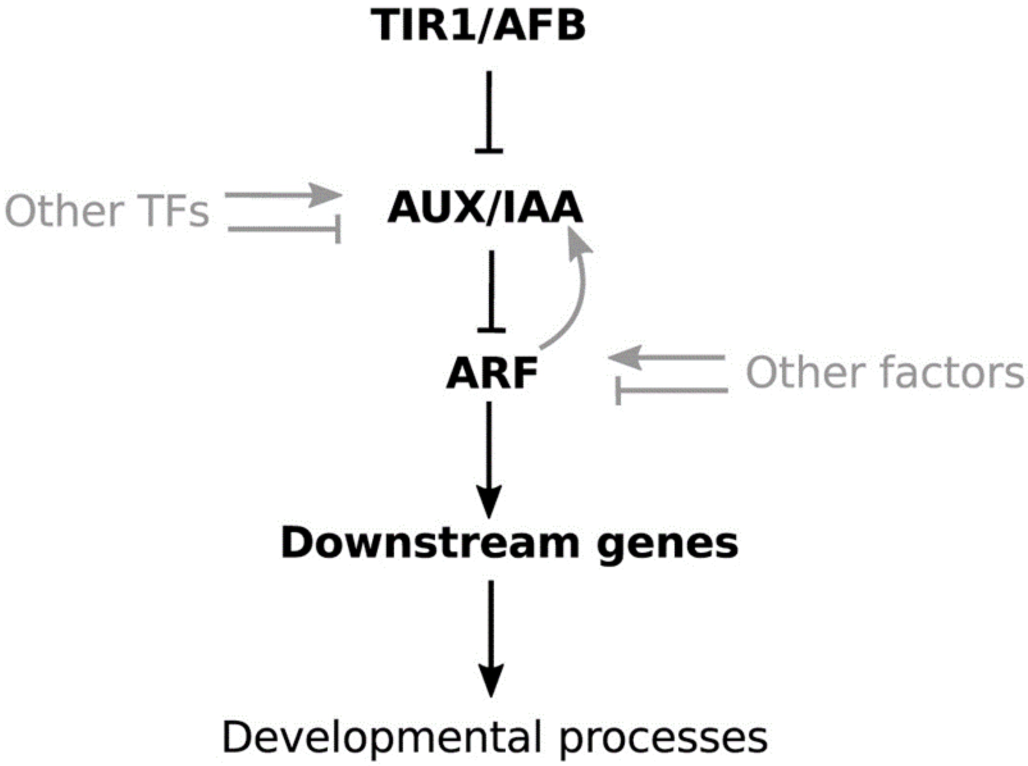


Figure 1.5 **Layout of the *TIR1/AFB* or the nuclear auxin signaling pathway through which auxin affects gene expression**.

In the absence or low auxin concentrations, *AUX/IAA* repressor proteins form dimers with *AUXIN RESPONSE FACTOR* (*ARF*) transcription factors, thereby blocking their activation, In the presence of auxin, intracellular auxins are sensed by the *TIR1/AFB1–3* which targets *Aux/IAA* proteins for proteolytic degradation, freeing the ARFs. In light grey, additional regulatory interactions often found in specific auxin signaling modules. Image taken from (Santos Teixeira and Ten Tusscher, 2019).

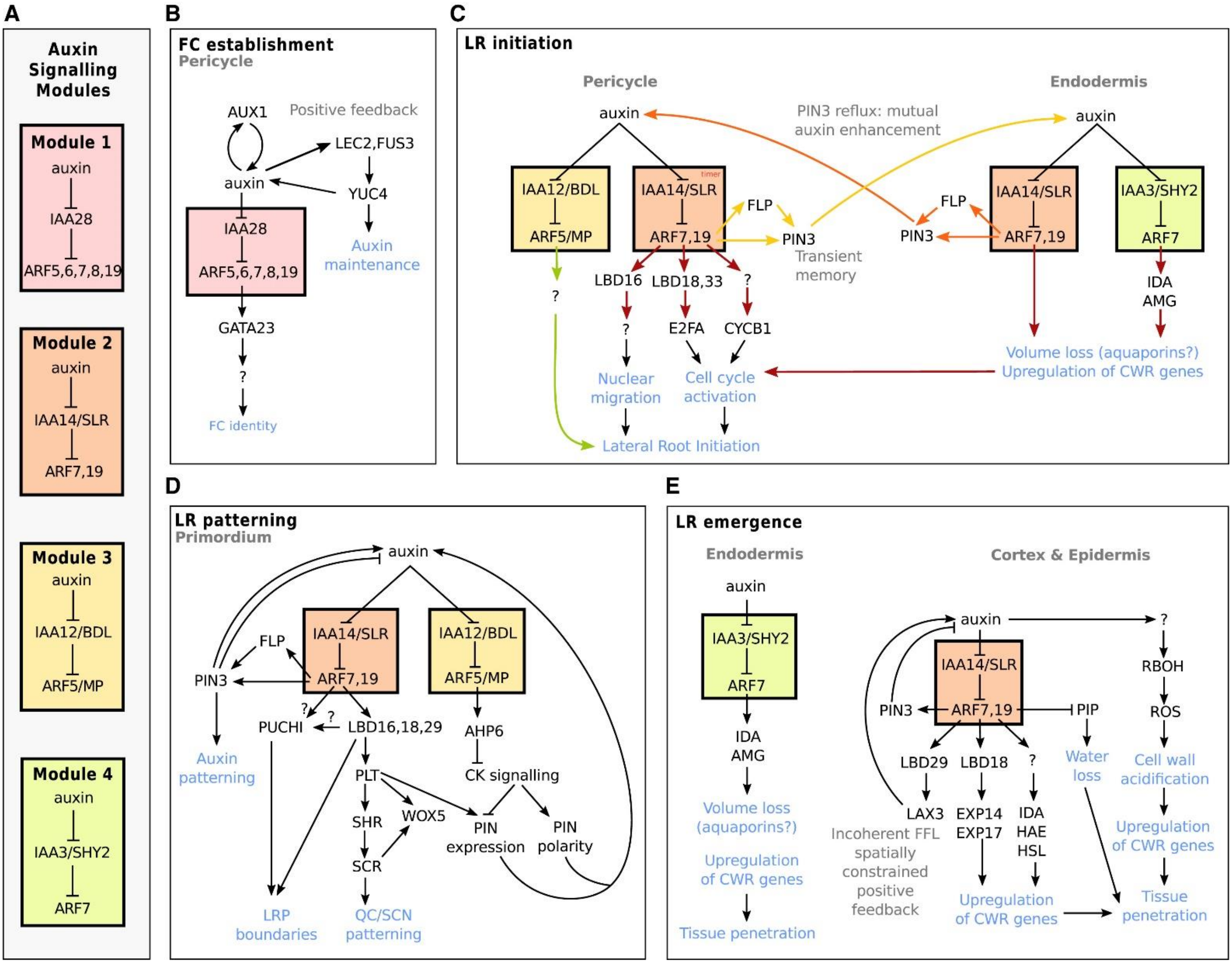


Figure 1.6 **An overview of the auxin signalling regulatory modules involved in lateral root development.**

(A) Layout of auxin signalling modules 1–4, with their specific Aux/IAA and ARFs. (B) Pericycle activation of Module 1 involved in founder cell identity establishment. (C) Lateral root initiation involves the activation of Modules 2 and 3 in the pericycle founder cells, and of Modules 2 and 4 in the overlaying endodermal cells. Shown are other indirect regulators such as *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*). (D) Further divisions and patterning of the growing primordium continue to be directly regulated by modules 2 and 3. (E) Lateral root emergence involves: first, passage through the endodermis, then, the cortex and epidermis. Emergence through the endodermis involves auxin signalling module 4, whereas emergence through the cortex and epidermis involves the activation of Module 2. In these tissues, Module 2 causes the sequential activation of *LAX3*, which enables a localized increase in auxin levels that subsequently enables the activation of expansins and cell wall remodelling enzymes. This enables the separation and pushing aside of cortical and epidermal cells by the primordium. Image was taken from (Santos Teixeira and Ten Tusscher, 2019).

1.3.3 Mechanical induction of LRPs

Lateral root initiation correlates with root bending (root waving), LRs stereotypically emerge at the convex side of the bends on the PR—this is evident in the left-right orientation of LRs along the waving in PRs in experimental systems (Figure 1.7) (De Smet et al., 2007; Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008; Richter et al., 2009; De Smet, 2012). Manual bending by forming J hooks on the PR using sterile forceps or the application of a gravity stimulus (gravistimulus, hereafter) by rotating growth plates against the gravity vector typically by a 90° induce LR initiation (Ditengou et al. 2008; Lucas et al. 2008; Xun et al. 2020; Qian et al. 2022).

The agravitropic *aux1* mutant (mutant of AUXIN1 (AUX1)) defies the stereotypical left-right alternating pattern of LR formation in Arabidopsis*,* and thus LRs do not form on the convex side of every curvature on the PR (De Smet et al., 2007)*.* Similarly, 9-day-old *aux1-7* seedlings only form half the number of LRPs formed in the wild type (Marchant et al., 2002)*.* When seeds of the *ProCYCB1:GUS* reporter line (a marker for cell division) were grown for 30 hours after germination and then subjected to a gravitropic bending (90° rotation) every 12 hours for 3.5 days, LRPs were observed at more than 90% of the bend sites (Lucas et al., 2008). The authors noted that Arabidopsis seedlings can reorient their PRs to the new gravity vector after gravistimulation as early as 4 hours after gravitropic bending (Lucas et al., 2008).

LRP induction by mechanical induction was less efficient in the mutants deficient in auxin transport, *ethylene insensitive 1(eir1-1 or pin2)*, and *aux1-7* mutant plants compared to the wildtype. However, emerged LRs were observed in >70% of roots (75% of *aux1-7* (n=40) and 83% for *eir1-1* (n-32)) when the tips of the PRs in these plants were amputated to test whether abnormal auxin transport from the root apical meristem prevents LRP formation (Ditengou et al., 2008). Bending intact *eir1–1DR5::GUS* roots resulted in the induction of LRPs in 100% of the roots studied (Ditengou et al., 2008).

Taken together, these data suggest that LRP initiation in response to mechanical induction is independent of PIN2 and that LRPs can be initiated in response to mechanical induction (via manual or gravitropic bending).

Gravity plays a major role in the directional growth of plants during morphogenesis; roots grow and develop in orientation to a preferred angle with respect to the gravity vector, called the gravity set point angle (GSA) (Ottenschläger et al., 2003; Digby and Firn,1995). When the roots sense a gravity-induced change in their growth orientation, they transduce this physical information into a physiological signal, this signal is transduced from the site of sensing (columella cells in the root apex (Ottenschläger et al., 2003; Sack. 1997)) to the site of response (the elongation zone), where differential elongation leads to the formation of a curvature (Strohm et al., 2013). Gravity-induced root growth is considered to be due to the differential distribution of auxin at the site of the curvature (Ottenschläger et al., 2003).

The Cholodny-Went model postulates that plant tropisms are caused by the lateral redistribution of a plant substance, which was later identified to be auxin (Went and Thimann, 1937; Firn and Myers, 1987; Firn, 1992; Firn et al., 2000;). Redistribution of auxin enables the initiation of LRPs (Benková et al, 2003). The redistribution of auxin during LRP formation in Arabidopsis is dependent on the repolarisation of the PIN-formed1(PIN1) auxin transporter from an anticlinal orientation during the early stages of LRP formation, and to the periclinal cell membranes at (Figure 1.9) (Benková et al., 2003), thereby redirecting auxin flux towards the tips of the growing LRPs at advanced stages (Figure 1.9) (Marhavý et al., 2014).

To understand the auxin dynamics behind the formation of LRPs at the sites of gravity-induced curvature (bend sites hereafter), Ditengou et al (2008) first observed *DR5::GUS* activity in LRPs induced at the bend sites under both manual and gravitropic bending conditions (Figures 1.8B & 1.8C) (Ditengou et al., 2008). However, the xylem cells impacted by manual bending did not express the PIN1 auxin efflux transporter in the elongation zone where bends are formed (Ditengou et al., 2008).

PIN1 is, however, expressed in the xylem cells in the elongation zone under gravitropic bending conditions, consequently, the authors used the more physiologically accurate gravitropic bending assay to study the PIN1-mediated auxin transport dynamics before LRP initiation at the bend site (Ditengou et al., 2008). PIN1 was found to be localised at the apical and basal regions on a single protoxylem located at the convex side of the elongation zone of the PR tip 3 hours after gravitropic bending (Figure 1.10A) (Ditengou et al., 2008). PIN1 localisation was only observed at the apical regions of a few basipetal protoxylem cells on the convex side of the elongation zone of the PR tip (Figure 1.10A) (Ditengou et al., 2008), this gravity-induced relocalization of the PIN1 serves to maintain local auxin maximum in developing xylem cells.

In pericycle cells, PIN1 relocalises to the concave sides of the gravitropically-induced bends; a diffuse cytosolic signal (of PIN1) close to the lateral wall adjacent to the vasculature was observed (Figure 1.10B) (Ditengou et al. 2008). This change in the localisation of the PIN1 signal was corroborated by a less intense *pDR5rev::3XVENUS-N7* signal in adjacent cells in the vasculature compared to the same cells on the convex side of the PR (Figure 1.10B) (Ditengou et al. 2008). Taken together, these observations suggest that gravitropic bending induces positional cues in the cell which determine the sites for LRP initiation, suggesting that the earliest event of LRP initiation after gravitropic bending is the relocalisation of PIN1 in protoxylem cells (Ditengou et al. 2008). The fact that LRPs can be induced due to gravitropic response in Arabidopsis under *in vitro* conditions, provides opportunities to synchronise LRP initiation in physiological and developmental studies (Xun et al., 2020; Qian et al., 2022) .

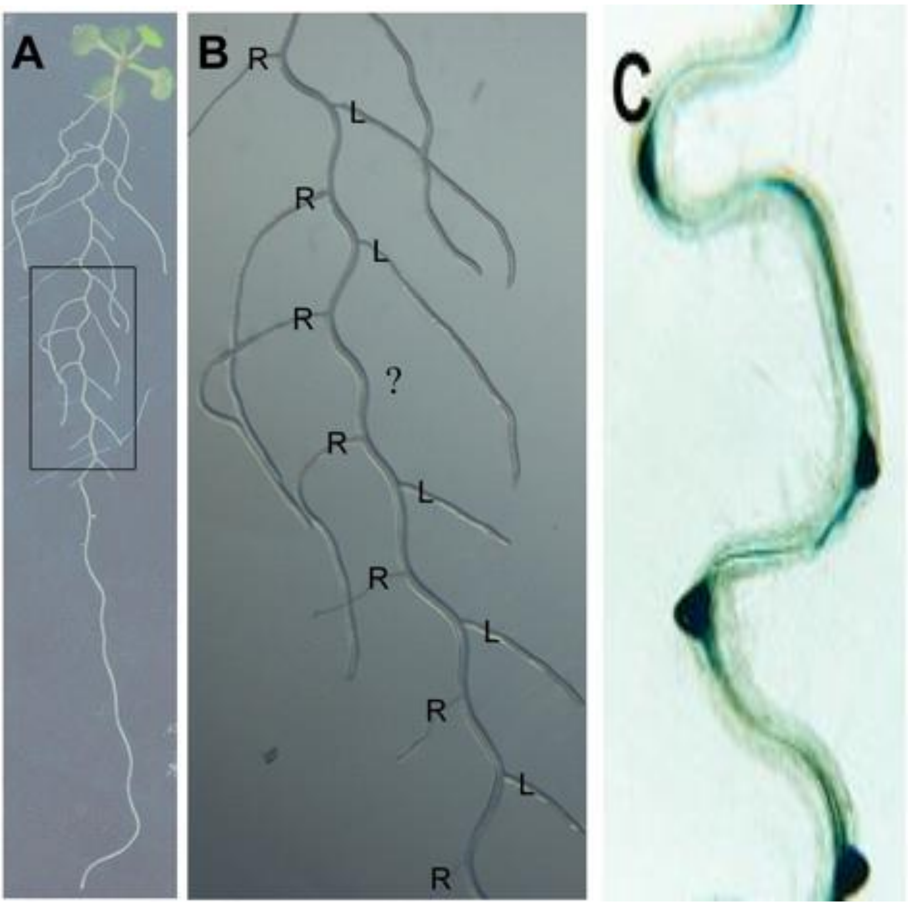


Figure 1.7 **Correlation between Arabidopsis root waving and lateral root positioning**. (A) Vertically grown Col-0 seedling with wavy growth pattern of the root. (B) Detail of boxed area in A showing the left (L)- right (R) alternating lateral roots.?, wave where lateral root is not apparent. (C) Root grown at 45° to enhance waving. Positions of lateral root primordia are marked by the *CYCB1;1::GUS* activity. Image was adapted from (De Smet et al., 2007)

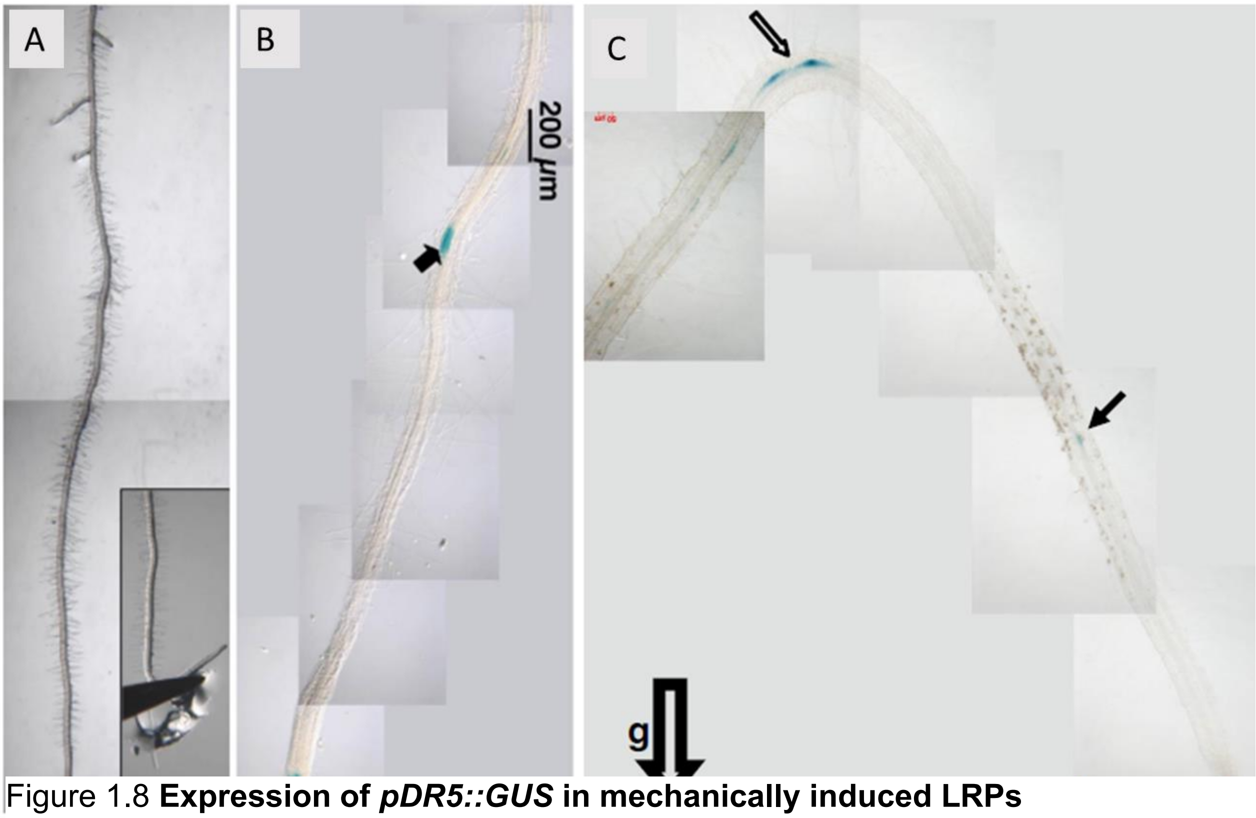


Figure 1.8 **Expression of *pDR5::GUS* in mechanically induced LRPs**

(A-C), LRP induction in mechanically bent 10-day-old *pDR5::GUS* transgenic plants.

(A) Before bending, inset indicates root tip manually bent with fine forceps.

(B) LRP as indicated by GUS signal (arrow).

(C) Enlargement of boxed area showing LRP initiation at the bend site, open arrow indicates gravity induced lateral root primordia, filled arrow shows a lateral root formed away from the bending region indicating that plant ability to form lateral roots is unaffected, gravity vector (g). Image was adapted from (Ditengou et al., 2008).

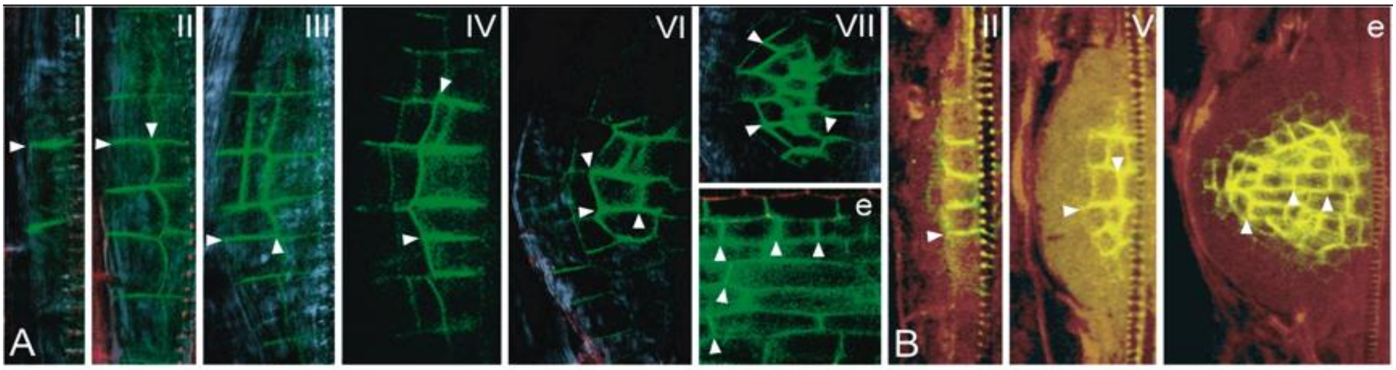


Figure 1.9 **Correlation of PIN1 Relocation and LRP formation**

(A and B) *PIN1: GFP* (A) and PIN1 (B) localization in developing LRP with gradual establishment of PIN1 at the lateral cell membranes.

Arrowheads indicate PIN1 polar localization (A, B). GFP signal in green (A), PIN1 signal in yellow (B). Image was adapted from (Benková et al., 2003).

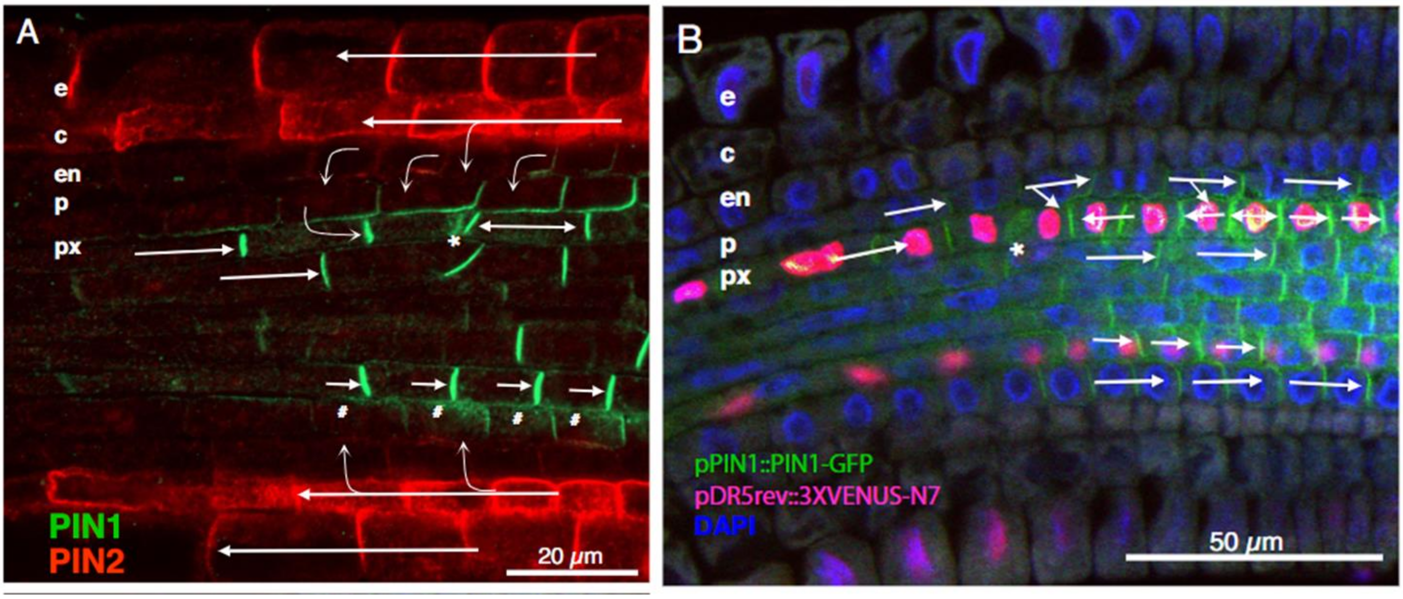


Figure 1.10 **PIN protein localization in gravistimulated roots of wild type and *pPIN1::PIN1- GFP*, *pDR5rev::3XVenus-N7* transgenic lines.**

(A) root elongation zone 3 h after gravistimulation. Immunolocalization with anti-PIN1(green) and anti-PIN2 (red). Epidermis (e), cortex (c), endodermis (en), pericycle (p), px(protoxylem), arrows indicate inferred auxin fluxes, asterisk (*) and hash (#) indicate diffuse cytosolic PIN1 localization. (B) PIN1-GFP (green) and DR5-Venus (pink), 5 h after gravistimulation, DAPI labelled nuclei (blue), asterisk (*) indicates protoxylem cell with diffuse *PIN1-GFP* signal. Image was taken from (Ditengou et al., 2008).

1.4 Scoring LRPs

Scoring of LRPs as used in this thesis is the quantification of the LRPs according to the various stages. LRP stages have been classically scored by root clearing, followed by a Normaski Differential Interference Contrast (DIC) microscopy according to a root clearing method developed by Malamy and Benfey (1997) for their pioneering work of characterising LRP stages (Malamy and Benfey, 1997). Root clearing is a histochemical method for making the root transparent to eliminate the need for sectioning of the root before microscopic analyses, thereby helping to understand the 3D cellular and tissue organisation of the root via deeper imaging of the root tissues (Attuluri et al., 2022; Richardson et al., 2021).

The classic root clearing method developed by Malamy and Benfey (1997) involves the treatment of whole mounts of PRs with 0.24 N HCl in 20% methanol, followed by a 15-minute incubation on a 57°C heat block. The roots are further treated with a solution containing 7% NaOH, 7% hydroxylamine-HCl, and 60% ethanol for 15 minutes at room temperature, Roots are then rehydrated for 5 minutes each in 40%, 20%, and 10% ethanol, and infiltrated for 15 minutes in 5% ethanol, 25% glycerol. Roots are then mounted in 50% glycerol on glass microscope slides and then subjected to a Normaski DIC microscopy where the various stages of LRPs are scored (Malamy and Benfey, 1997).

The classic method of root clearing has been improved by some authors to improve the speed, and efficiency and to preserve the cytological details of roots (Dubrovsky et al., 2009). For example, Dubrovsky et al (2006), added a few modifications to the clearing method described by Malamy and Benfey (1997), by increasing the incubation time and temperature of the 0.24 N HCl in 20% methanol treatment step (Dubrovsky et al., 2006; Malamy and Benfey, 1997).

Additionally, the infiltration in 5% ethanol and 25% glycerol was extended for 2h (per step) (Dubrovsky et al., 2006).Roots are further fixed in 30% (aq. v/v) glycerol containing 2% (v/v) Dimethyl sulfoxide (DMSO and left for at least 30 minutes at room temperature. Roots are then mounted in clearing solution and observed at least 1 h after the sample preparation. This clearing solution contains 4.2 M Sodium Iodide (NaI) and 8 mM Sodium thiosulfate ($Na_2S_2O_3$) prepared in 65% (aq. v/v) glycerol supplemented with 2% (v/v) DMSO. This alternative method ensures the storage of root samples before microscopic analyses and is less laborious (Dubrovsky et al., 2009).

These root-clearing-based methods for scoring LRP stages have not allowed for the nuclear migration stage to be scored. This stage can be considered as stage 0 since it is the preceding stage to the LR initiation stage which is often considered as stage 1 (De Rybel et al., 2010; Goh et al., 2012). The nuclear migration stage should be scored when quantifying LRP stages in physiological and developmental studies, as this stage will provide the starting point for tracking the developmental trajectory of LRPs, especially in response to a particular treatment under experimental conditions. In the original work by Malamy and Benfey, (1997), the first stage scored was stage 1 (Figure 1.3; (Malamy and Benfey, 1997). The same observation is true for the subsequent studies that took inspiration from the original authors (Dubrovsky et al., 2006; Dubrovsky et al., 2009). This may be that root fixation and clearing is a destructive process, and therefore may not have allowed live imaging of the nuclear migration process, which is required to accurately score the aforementioned process (De Rybel et al., 2010) or the authors did not aim to study the nuclear migration.

Fluorescent reporter lines, especially ones developed from genes involved in auxin response and signalling or transport (e.g., *PIN1;2;3;4;6, DR5, IAA14/SLR)* and other auxin-responsive genes involved in LRP formation (e.g., GATA23) have been used to visualise LRPs via microscopy (Fukaki et al. 2002; Benková et al., 2003; De Smet et al. 2007; Ditengou et al. 2008; De Rybel et al. 2010). In studies on LRP formation, these reporter lines have often been generated by fusing the promoters of the aforementioned genes to GUS, LRPs are then observed by histochemical staining followed by Normaski DIC microscopy (Benková et al., 2003; De Smet et al., 2007), although in some studies, the GFP reporters of these genes have been used, for example, *PIN1-GFP*, *pGATA23::NLS-GFP* (De Rybel et al., 2010), to mention a few. The use of GFP reporter lines offers a non-destructive alternative to the GUS-based reporter lines and allows for the live imaging of roots to score LRPs.

The best reporter line for studying the nuclear migration stage would be a GATA23-based reporter line, due to the role of GATA23 in the nuclear migration process. The best characterised reporter for studying nuclear migration is the pGATA23::NLS-GFP line developed by De Rybel et al (2010) in their study of the role GATA23 plays in nuclear migration (De Rybel et al., 2010) as already discussed earlier in this chapter. Root clearing methods have gone through diverse modifications, and one such method is the ClearSee method which allows roots of fluorescent reporter lines to be cleared without quenching the fluorescence (Kurihara et al., 2015). However, this method while useful in preserving the fluorescence of reporter assays is still very laborious and requires plant samples to be fixed as in the clearing methods discussed earlier.

GATA23-based reporter lines such as the *pGATA23::NLS-GFP* and *GATA23:H2B:3xmCherry/pUBI:3xGFP: PIP1,4,* (as described by (Barro et al. 2019)) (Figure 1.11) (De Rybel et al., 2010; Barro et al., 2019) score the nuclear migration stage, as well as subsequent LRP stages. These tools enables LRPs to be scored via live imaging by using epifluorescence microscopy and laser scanning confocal microscopy, without need for root clearing and fixation.

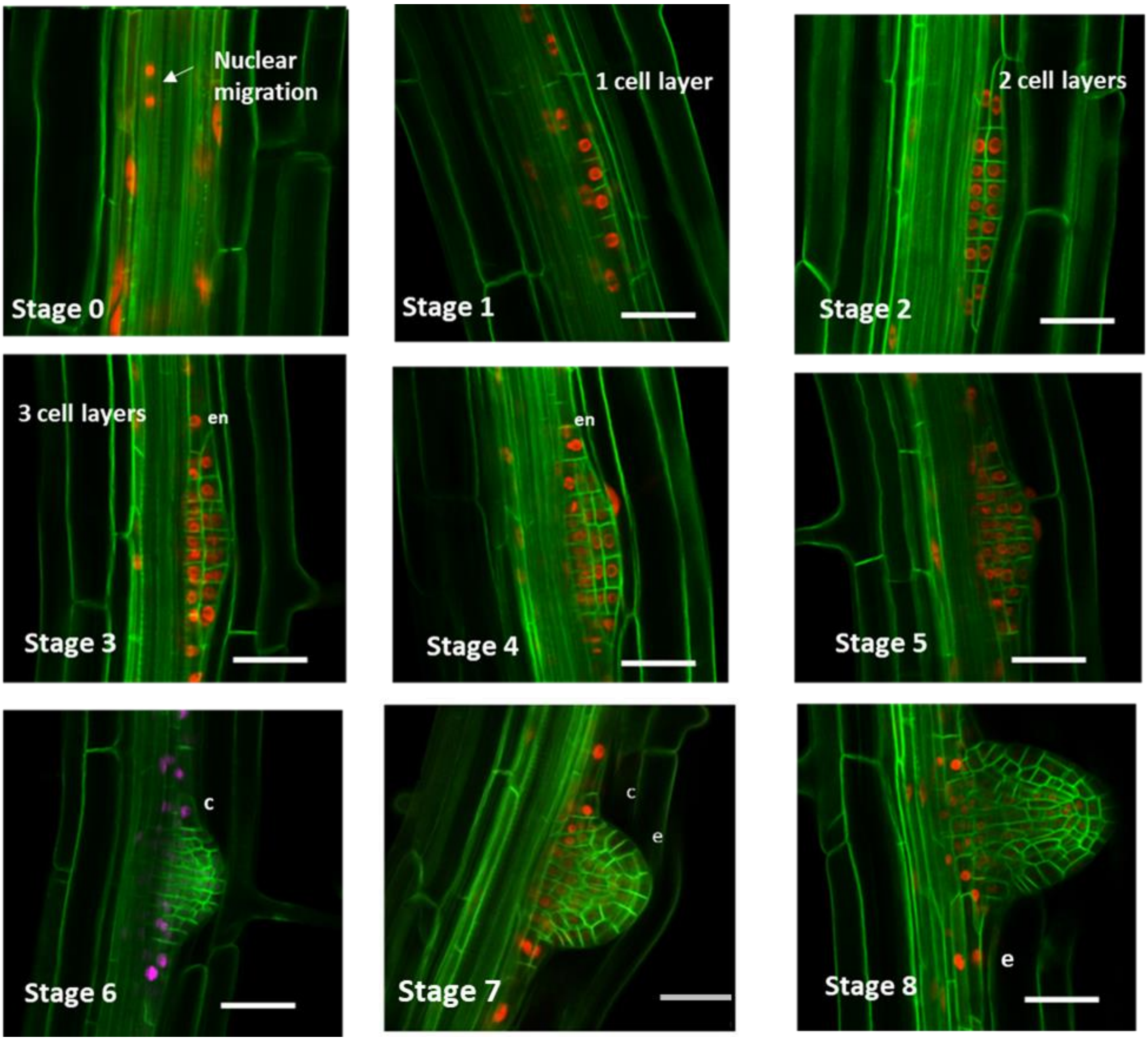


Figure 1.11 Images of LRP stages. Confocal images of xylem pole pericycle layer and LRP stages in roots of 7-day old seedlings of *GATA23:H2B:3xmCherry/pUBI: 3xmCherry:3xGFP:PIP1,*4 reporter line. GFP-based reporter lines with nuclear localisation signals allows the nuclear migration stage to be scored and eliminates the need for root clearing and fixation. en: endodermis, c: cortex, e: epidermis. Images taken by Frank Machin. Bar is 50 µm.

1.5 Regulation of LR formation by resources

LR formation is modulated by the physical environment, and external and internal resource availability (Malamy and Ryan, 2001; Malamy, 2005; Van Norman et al., 2013). Resources are materially required for plant growth and are generally limiting for growth, which, once assimilated by the plant, are transported from source (supply) to sink (demand) tissues and organs to be utilised for growth and developmental processes. Both externally acquired resources (e.g., water and mineral salts) and internally generated resources (e.g., fixed carbon, amino acids) are either re-invested into a growth or maintenance programme or stored for later utilisation (Bloom et al., 1985). The storage of plant resources is defined as the build-up of resources which is utilised for future physiological and developmental processes (Chapin et al., 1990).

Plant growth and developmental processes are underpinned by the ‘demand’ and ‘supply’ of resources from source to sink, respectively, therefore, when supply exceeds demand, or under changing environmental conditions, a given resource may be stored and remobilised later to support growth and development. For example, during the day when plants are photosynthesising, sucrose is utilised for growth and development, however, some of this sucrose is stored as starch which will be remobilised to support growth at night when the plant is no longer photosynthesising (Gibon et al., 2004). Similarly, the amino acid, proline accumulates in plants (in the shoot, stems, and roots during drought stress and phosphate starvation, but can serve as nitrogen reserves during cold periods of winter) (Verslues and Bray, 2006; Verslues, 2010).

Externally acquired resources serve as environmental signals that affect LR formation, this allows plants to thrive under varying soil conditions (Malamy , 2005). The supply of resources themselves has a profound impact on LR formation by altering the number, length, angle, and diameter of roots (Malamy and Ryan, 2001; Malamy, 2005). For example, moderate levels of exogenous nitrate stimulate LR emergence and outgrowth, as seen in the increase in LR numbers, while high nitrate has a systemic inhibitory effect on LR elongation (Zhang and Forde, 1998).

Phosphate (P) availability has a strong influence on LR formation, during phosphate starvation, primary root growth is inhibited, and LRP initiation and LR elongation is stimulated to allow for exploration of the different layers of the phosphate-rich topsoil (Williamson et al. 2001; Péret et al., 2011). P limitation upregulates the expression of the auxin receptor, *TIR1,* and increases the degradation of AUX/IAA. The increased degradation of AUX/IAA will allow *ARF19* and possibly other ARFs already bound to auxin response genes (AuxREs) to regulate their expression with a downstream effect on pericycle cell division (Nacry et al., 2005; Pérez-Torres et al., 2008).

Similarly, differential changes in nitrate levels have been reported to alter TIR1/AFB3 transcript levels (Pérez-Torres et al. 2008; Parry et al. 2009; Vidal et al. 2010) , and this might therefore affect all processes involved in LRP formation, as discussed earlier. It is, however, difficult to conclude the effect of these resources on TIR1/AFB3-mediated auxin perception and signalling in the absence of translation and protein stability data. Based in current data, mechanistic understanding of the effect of these nutrients on TIR1/AFB3 cannot be concluded, as their effect could be either direct as nutrient sources or indirect as signals via an unknown mechanism.

Under drought conditions, the lack of accessibility to water induces stress signals that repress LR elongation; however, patchy water availability serves as positional cues for LR patterning (Xiong et al. 2006; Robbins and Dinneny, 2015). Aside from the roles of externally acquired resources on LR formation with some discussed earlier, internally generated resources such as sucrose have also been suggested to promote LR formation. Subsequent sections in this chapter are focused on reviewing the current knowledge and gaps in establishing and understanding a causal relationship between sucrose availability and LR formation.

1.6 Sucrose synthesis

Sucrose is the main product of photosynthesis and the primary carbon transported in the phloem of plants (Lunn and ap Rees, 1990; Winter and Huber, 2000; Maloney et al., 2015; Flügge et al. 2016; Stein and Granot 2019). During photosynthesis, carbon dioxide (CO_2) is fixed in the chloroplasts via the Calvin cycle to yield triose phosphates (triose-P). Triose-P can be transported to the cytosol by a triose-P/phosphate translocator. In the cytosol, two triose-P molecules produce one fructose 1,6-bisphosphate (F1,6BP) molecule in a reaction catalysed by aldolase. F1,6BP is then metabolised to produce other hexose phosphates, such as fructose 6-phosphate (F6P) and glucose 6-phosphate (G6P). G6P can be used to form nucleotide sugars such as UDP-glucose (UDP-G), and UDP-G is combined with F6P to form sucrose 6-phosphate (sucrose-P) in a reaction catalysed by sucrose phosphate synthase (SPS).

Sucrose-P is dephosphorylated by sucrose phosphate phosphatase (SPP) to form sucrose (Ruan, 2014). The process of photosynthesis and sucrose synthesis is more complex and detailed than has been described in this section. However, this description is to provide a brief background to sucrose synthesis and to provide contextual background for discussing how sucrose is made available to root support growth and development (e.g., LR formation) in subsequent sections. A detailed description of sucrose synthesis is outside the context of this thesis.

1.7 Allocation of sucrose from the shoot to the root.

The allocation of sucrose from source tissues to sink provides the energy and carbon resources required for plant growth and ontogeny. Sucrose is hydrolysed in sink tissues into glucose and fructose to be utilised for growth and development. The availability of sucrose and its hexoses (fructose and glucose) has been reported to promote root growth and development (Hammond and White 2008; Booker et al. 2010; Mason et al., 2014; Wang and Ruan, 2016). Understanding sucrose transport is, therefore, crucial for understanding and establishing a causal relationship between sucrose availability and plant growth and development. The following section reviews our current understanding and gaps in knowledge of sucrose transport, hydrolysis, and how sucrose transport might promote LR formation.

1.7.1 Münch hypothesis and sucrose transport.

The vascular systems in plants allow for the transport of resources via bulk flow from source to sink. The xylem transports water and mineral nutrients from the soil to the shoot, while the phloem allocates photo-assimilates from source leaves to sinks such as young leaves and roots. The central and most widely accepted hypothesis for phloem transport is the Münch hypothesis which states that transport through the phloem results from the osmotically generated pressure differences between source and sink—transport occurs without energy input (Münch 1930; Knoblauch and Peters, 2017).

The Münch hypothesis suggests that sucrose is the main driver of phloem transport. The accumulation of sucrose in the phloem of source leaves attracts water from surrounding xylem vessels, increasing the turgor pressure of the phloem that drives the transport of phloem content toward sink tissues (Wang and Ruan, 2016).

In sink tissues, sucrose is hydrolysed by invertase and/or sucrose synthase (Ruan, 2014) which reduces the osmotic concentration in the phloem, thus the osmotic pressure reduces. This then creates a pressure differential between sink and source tissues, driving the bulk flow of sucrose. The transport of sucrose from source leaves to sink tissues involves the loading of the phloem (at the source) and the unloading of the phloem at the sink. The sieve element/companion cell (SE/CC) complex is a functional unit formed between the SE and the CC for long-distance phloem transport. In Arabidopsis, the SE/CC complex is symplastically isolated from the surrounding cells. This requires facilitated transport of sucrose to the cell wall (apoplast) before influx into the SE/CC complex (Braun, 2022). Sucrose first moves symplastically through the plasmodesmata of the mesophyll cells where it is synthesised, into the phloem parenchyma. The symplastic movement of sucrose is a passive process driven by difference in the concentration gradient between the mesophyll cells and the phloem (Li et al., 2022). The *Arabidopsis thaliana* Sucrose Will Eventually Be Exported Transporter 11 and 12 (AtSWEET11/12) then export sucrose from the phloem parenchyma cells into the apoplast (Figure 1.12) (Chen et al., 2012). SWEETs are facilitative diffusers; they transport sucrose across membranes along their concentration gradients (Chen et al., 2010; Chen et al., 2015).

AtSWEET11 and AtSWEET12 are localised to the plasma membrane and are expressed in the phloem parenchyma (PP) cells adjacent to the SE/CC complex of leaves (Chen et al., 2012). *atsweet11atsweet12* double mutant plants exhibit retarded growth (~35% smaller than wild type) and sugar and starch accumulation increased in leaves when grown initially under low light and transitioned to high light (Chen et al., 2012). Members of the SWEET family of membrane transporters are redundant in their function, as such it is possible that, SWEET members such as AtSWEET13 supplement sucrose transport in *atsweet11 atsweet12* double mutant line. This could explain why there was only a slight degree of growth defect in *atsweet11atsweet12* compared to the wild type. AtSWEET13 expression was upregulated approximately 16-fold in the double mutant, although normal expression levels in wild type leaves are low (Chen et al., 2012). The predicted redundancy of the AtSWEET11/12/13 in sucrose efflux from the phloem parenchyma to the apoplast can be tested and verified from physiological studies on *atsweet11/12/13* triple mutant lines, however, this mutant has not been characterised, and in the absence of phenotypic analyses of the *atsweet11/12/13* triple mutant it is impossible to test the aforementioned hypothesis.

Mature leaves of *atsweet11/12* double mutants contained higher levels of sucrose at the end of both the light and dark periods, suggesting a defect in the ability of *atsweet11/12* double mutants to transport sucrose from leaves (Chen et al., 2012). Defects in the ability of *atsweett11/12 double* mutants to export sucrose from leaves were further corroborated by direct 14CO_2-labeling experiments under low light conditions, where the *atsweet11/12* double mutants exported ~50% of fixed 14C compared to controls. Reduced or complete defects in the ability to export sucrose from leaves are likely to lead to reduced transport of sucrose to the root, thereby affecting root growth and development (Riesmeier et al., 1994; Gottwald et al., 2000). *atsweet11/12 double* mutants grown on sucrose-free media showed reduced PR growth as well. (Chen et al., 2012). Taken together, these data demonstrate that AtSWEET11/12 are involved in the transport of sucrose from the shoot to the root.

After sucrose export from the phloem parenchyma into the apoplast by AtSWEET11/12, sucrose is imported from the apoplast into the SE/CC complex by SUCROSE-PROTON SYMPORTER 2 (AtSUC2) also known as sucrose transporters (SUTs) (Figure 1.12) (Stadler and Sauer, 1996). AtSUC2 is expressed in CCs and the protein is localised to the plasma membrane (Gottwald et al., 2000; Srivastava et al., 2008; Stadler and Sauer, 2019). The *atsuc2* mutants fail to load sucrose into the phloem, accumulate anthocyanin in their leaves, show reduced primary root growth, and only produce a few viable seeds (Gottwald et al., 2000; Srivastava et al., 2008), suggesting unavailability of sucrose at the whole plant level to fuel the aforementioned developmental processes.

From the SE/CC, sucrose is transported along the transport phloem and unloaded at sink tissues. Three successive functional sections are defined along the phloem transport pathway; the collection phloem where the loading of sucrose into the SE/CC in the shoot occurs, the transport phloem where sucrose is transported from the shoot to the root, and the release phloem where unloading of sucrose occurs (De Schepper et al., 2013).

In the root, sucrose is unloaded from the SE into the phloem pole pericycle and diffuses from cell to cell throughout the root tip (Ross-Elliott et al., 2017). Ross-Elliot et al (2017) first investigated how and where small solutes such as sugars were unloaded in *Arabidopsis* roots (Ross-Elliott et al., 2017). They applied fluorescent phloem-mobile dyes on leaves and then followed their movement through the phloem. Using a gene expressed in immature protophloem sieve tube cells, they showed that dyes moved down to the first differentiating protophloem cell. This cell was called ‘PSE zero’. By measuring dye flow velocity in the protophloem sieve tube and assuming that flow velocity would decrease in the unloading zone, they predicted that the dye was being unloaded in a region between PSE zero to about 400 µm towards the mature part of the root.

To corroborate this result, the authors found numerous plasmodesmata in the walls of the protophloem sieve tube cells in this zone when they stained for callose which is present to a small extent in plasmodesmata. On the contrary, sieve tube walls were almost completely covered with callose in parts of the root more than 400 µm away from PSE zero, where flow velocities in the protophloem sieve tubes were constant. This amount of callose is likely to inhibit lateral solute movement in this zone. Taken together, these findings suggest that solutes such as sucrose flow towards the root tip in the mature metaphloem sieve elements until they reach the immature metaphloem.

In the immature metaphloem, they move into the differentiated protophloem sieve elements and are transported to the unloading zone, where they move laterally out of the protophloem sieve elements, then move symplastically into the phloem pole pericycle cell (Ross-Elliott et al., 2017). From the phloem pole pericycle, sucrose is transported through a combination of diffusion and bulk flow along a concentration gradient into surrounding cells (Ross-Elliott et al., 2017). It is unknown whether and how sucrose and/or its hexoses get to XPP cells.

It is, however, possible that sucrose and/or its hexoses diffuse apoplastically from the PPP cells into neighbouring XPP cells, where they potentially (i) provide the structural molecules for necessary for LR ontogeny, (ii) provide the energy necessary for LRP formation (iii) serve directly as signals that regulate aspects of LR-specific gene expression, and (iv) serve as stimuli that cue signalling networks that modulate LRP formation.

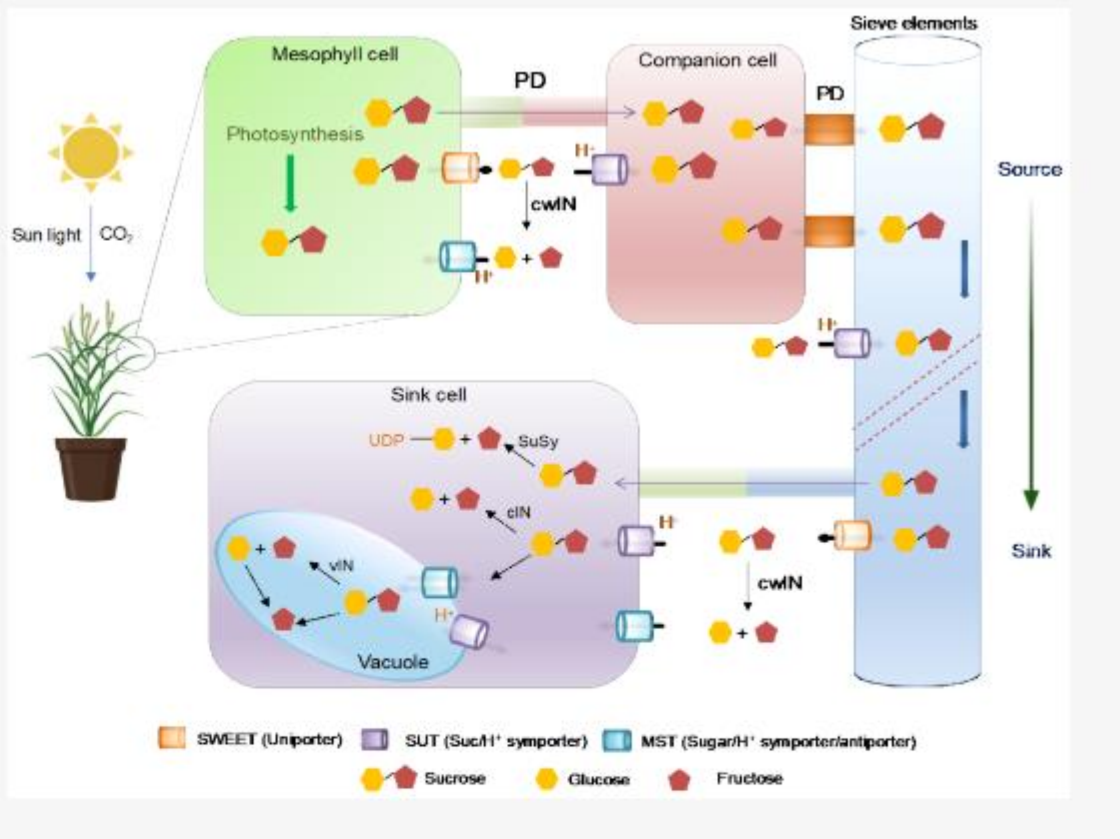


Figure 1.12 Schematic diagram showing the path of sucrose transportation from source to sink. The photosynthetically synthesized sucrose is transported out from mesophyll cells via SWEETs sugar transporters. Sucrose transporters (SUTs) accumulate sucrose in the sieve element/companion cell complex for long-distance distribution throughout the plant body. PD: plasmodesmata, cwIN: cell wall invertase, cIN: cytoplasmic invertase, SuSy: sucrose synthase, vIN: vacuole invertase, Glc: glucose, Fru: fructose. Image taken from Gautam et al., 2022.

1.8 Sucrose signalling

Sucrose, in addition to being an energy source for plant growth and development, has also been proposed to be a signal. Due to its high propensity to be hydrolysed into metabolites such as fructose, glucose, and trehalose 6-phosphate (T6P), which are themselves signals , it is challenging to unequivocally pinpoint sucrose-specific signalling processes (Yoon et al., 2021). However, in recent years, some evidence of physiological and developmental processes that are regulated by sucrose-specific signalling has come to light. For example, the regulation of the expression of ADP-glucose pyrophosphorylase (AGPase), the first limiting step in starch biosynthesis, catalyses the conversion of glucose 1-phosphate to ADP-glucose, which is a direct substrate for starch synthesis (Preiss, 1988).

In Arabidopsis, transcript levels of the AGPase genes, *ADP-glucose pyrophosphorylase large subunit 2* (*ApL2*), *ApL3*, and *ADP-glucose pyrophosphorylase small subunit* (ApS) increased several-fold when exogenous sucrose was fed to detached leaves in the dark. Glucose treatment was also administered, however, it was not as effective as sucrose (Sokolov et al.,1998). In this experiment, 6-7 weeks old Arabidopsis plants were kept in complete darkness for 6 hours to potentially carbon starve them or lower the levels of endogenous sugars before sugar-feeding experiments. Leaves of dark-grown plants were fed with 0.1 M and 0.3 M sucrose and glucose, and other osmotica (e.g., 0.1 M sorbitol and 6% PEG 8000) for 12 hours and total RNA fractions were extracted from leaves. Northern-blot analyses revealed increases in the transcripts levels of *ApS, ApL2,* and *ApL3* after the supply of sucrose or glucose to the detached leaves in the dark, whereas transcript contents of *ApL1* decreased under the same conditions. 0.1 M sucrose was slightly more effective after 12 h in inducing changes in transcript levels than 0.3 M sucrose. Transcript levels in *ApS, ApL2,* and *ApL3 u*nder glucose conditions were lower compared to those under sucrose treatment conditions (Sokolov et al.,1998). Increased transcript levels were also observed in *ApS, ApL2,* and *ApL3* genes in mocktreated leaves (water; no sucrose or glucose supply) under high light intensities (Sokolov et al.,1998).

Similar observations have also been made in sweet potato (*Ipomea batatas*), where the transcription of the *iAGPLI-1* gene (which encodes an isoform of the APL) was induced within 12 h after 3% exogenous sucrose supply to detached leaves based on a northern blot analysis. The same observation was made under light conditions; the supply of the same concentrations of glucose and fructose did not induce the expression of the *iAGPLI-1* gene at all, suggesting a sucrose-specific role in promoting the transcription of the *AGPase* gene (Harn et al., 2000). This sucrose-induced increases in the transcription of *AGPase* genes was, however, not transmitted to the translation of the protein as western blot analyses revealed that protein levels remained constant after exogenous sucrose supply, suggesting that the regulation of the *AGPase* expression by sucrose is post-transcriptional (Sokolov et al. 1998; Harn et al. 2000). Taken together, these observations suggest that starch biosynthesis, which is crucial for maintaining plant energy homeostasis is regulated by sucrose-specific signalling, and not metabolism.

Cell division, and cell expansion underpin all plant growth and development (Wang and Ruan, 2013). As discussed in section 1.3.1.3 of this chapter, LRP initiation and morphogenesis, and effectively RSA, is underpinned by cell division. Sucrose has been reported to regulate cell division in Arabidopsis, a correlation was found between the expression of CycD2 and CycD3 in response to sucrose supply (Riou-Khamlichi et al., 2000). In this study, seedlings of the Arabidopsis ecotype, *Landsberg erecta (*Ler-0*)* grown in liquid MS medium without sucrose for 8 days and transferred for 24 hours to a medium containing 3% exogenous sucrose were used to determine the effect of exogenous sucrose supply on CycD2 and CycD3 (Riou-Khamlichi et al., 2000).

Western blot analyses revealed that the expression of CycD2 and CycD3 was increased 5.8- and 3.6-fold, respectively, in response to exogenous sucrose supply, compared to control conditions where seedlings were left in a medium without exogenous sucrose (Riou-Khamlichi et al., 2000).

A follow-up experiment using 7-day-old Ler-0 seedlings which were transferred from a medium containing 3% exogenous sucrose to a medium without sucrose was conducted. At 48 hours after the transfer, the seedlings showed an 8-fold reduction in both CycD2 and CycD3 mRNA levels compared to control seedlings which were supplied with exogenous sucrose (Riou-Khamlichi et al., 2000). When control seedlings were transferred to a medium containing 0.5% or 1% exogenous sucrose, CycD2, and CycD3 mRNA levels both increased 15-fold within 6 hours after transfer. Similarly, when cells from Ler-0 were grown in suspension culture, where they were initially grown in a medium without sucrose, mRNA levels of both CycD2 and CycD3 were reduced approximately 2-fold. mRNA levels of CycD2 and CycD3 were increased 4-fold and 9-fold, respectively, within 4 hours after the addition of 3% sucrose. Additionally, CycD2 and CycD3 mRNA levels were observed to be increased by the addition of glucose to carbon-starved cells (Riou-Khamlichi et al., 2000). Taken together, these observations suggest that sucrose plays a key role in the cell cycle by regulating the transcription of CycD2 and CycD3 genes which are key regulators of the G1 phase of the cell cycle in plants.

In tobacco BY-2 cells, glucose was found to regulate the transcription of cycD2;1, D3;2, A3;2, and B1;2 (Hartig and Beck, 2005). A3 and B1 cyclins are required to drive G1/S and G2/M transitions, while D cyclins as previously discussed are involved in the G1 phase of the cell cycle, by serving as sensors to external stimuli to regulate the cell cycle (Menges et al., 2005; Meijer and Murray, 2000; Wang and Ruan, 2013), this observation, combined with the observations by Riou-Khamlichi et al (2000), discussed earlier suggest that glucose serves as a signal to regulate the cell division in plants.

Other studies have also shown the role of sucrose in regulating the expression of cell cycle genes, notably, the effect of glucose in initiating the G2/M transition by repressing transcription of the negative regulator TPR (Tetratricopeptide repeat)-DOMAIN SUPPRESSOR OF STIMPY (TSS) in shoot meristematic tissues, thereby activating the expression of key cell cycle components required for G2/M transition, such as CYCB1;1 and CDKB1;1 (Skylar et al., 2011). In this study, the understanding that the cell cycle is arrested at the G2 phase in XPP cells in the PR (Beeckman et al., 2001), made the authors reason that the same could be expected for XPP cells in the hypocotyl. The authors, therefore, asked whether the supply of exogenous sucrose to excised hypocotyls would induce the re-entry of XPP cells into the cell cycle and whether this potential re-entry into the cell cycle is auxin-dependent or independent (Skylar et al., 2011).

CYCB1;1::GUS activities were detected around the vascular bundle, where the XPP cells are located after incubation of excised hypocotyls on a medium containing 1.5% exogenous sucrose for 6 days, compared to no *CYCB1;1::GUS* in hypocotyls grown on media containing 0% exogenous sucrose. In the presence of 10 µM NPA, sucrose was still able to induce *CYCB1;1::GUS* activity in the XPP cells, but at a reduced level (Skylar et al., 2011). CDKA1 and CDKB1;1 GUS activities were also induced in response to sucrose supply in the presence of NPA, similarly as the aforementioned. The XPP cells in the sucrose-treated hypocotyls could not divide after re-entry into the cell cycle after prolonged incubation on sucrose. These observations, therefore, led to the conclusion that sucrose is sufficient to activate the cell cycle in G2-arrested cells but insufficient to induce mitosis (Skylar et al., 2011).

With the addition of 2,4-D to the media, there was a differential response of excised hypocotyl XPP cells based on the presence or absence of exogenous sucrose in the media. In the presence of sucrose, XPP cells divided rapidly and formed large cell mass around the vasculature, with high levels of *CYCB1;1::GUS* activity. However, in the absence of exogenous sucrose, few divisions were observed and divisions ceased ever after prolonged incubation, as evidenced by no *CYCB1;1::GUS* activity. The authors, therefore, predicted that exogenous 2,4-D was only able to induce divisions in XPP cells when there is an availability of cellular sugar, however, in the absence of sugars, 2,4-D alone is unable to sustain either proliferation or cell cycle gene expression (Skylar et al., 2011). Other experiments in the same study showed that sucrose activates the re-entry of G2 into the cell cycle by suppressing the activity of TSS, a detailed discussion of this study is outlined in the paper by Skylar et al (2011) (Skylar et al., 2011). Taken together, these observations show that sucrose plays a prominent role in cell division in Arabidopsis and that sucrose requirement for G2 to M transition may be a prerequisite for the auxin-mediated cell division in plants.

Sucrose has been reported to mediate other physiological and developmental processes in plants such as floral induction (Lejeune et al., 1993; Sun et al., 2017), photosynthesis, expression of key genes involved in metabolic processes (Nagata et al., 2012), anthocyanin biosynthesis (Teng et al., 2005). Details of all the signalling role of sucrose in mediating the aforementioned physiological and developmental processes, together with other roles of sucrose as a signal in plants are reviewed elsewhere (Horacio and Martinez-Noel, 2013; Yoon et al., 2021; Choudhary et al., 2022).

1.9 Role of sucrose in LR formation.

In *in vitro* studies in plant biology, there is a standard practice of growing Arabidopsis in a culture medium containing exogenous sucrose. This routine of adding exogenous sucrose into the culture medium may have hindered the full recognition of sucrose's specific morphogenic role in root growth (Kircher and Schopfer, 2012). However, some studies have made efforts to attempt to establish a causal relationship between the availability of sucrose and/or its hexoses and root growth and development (e.g., PR growth, adventitious root formation, and LR formation) (Reed et al. 1998; Freixes et al., 2002; Takahashi et al., 2003; Karthikeyan et al. 2007; Lee-Ho et al. 2007; Macgregor et al. 2008; Kircher and Schopfer 2012).

In physiological experiments aimed at establishing a causal relationship between sucrose availability and LR formation, various methods of ensuring sucrose availability to plants have been used: (i) exogenous sucrose feeding to plants, and/or, (ii) manipulation of the conditions that stimulate sucrose synthesis (e.g., increased light intensities and high CO_2 levels). In a study by Lee Ho et al (2007), the growth of 14-day-old Col-0 plants under varying CO_2 levels: ambient (360 µmol/mol) and elevated (900 µmol/mol) under 0% exogenous sucrose conditions, higher LR numbers were observed under elevated CO_2 levels, compared to ambient CO_2 levels (Lee-Ho et al., 2007).

These observations suggest increased endogenous sucrose levels under elevated CO_2 levels, and these increased sucrose levels had a stimulatory effect on some aspect, or probably all aspects of LR formation However, a conclusion of increased endogenous sucrose levels on specific processes involved in LR formation cannot be made based on the data presented by the authors as the authors did not study the aforementioned. The authors aimed to study the effect of elevated CO_2 levels and sucrose concentrations on the overall RSA, as such, they observed increased total root system numbers and lengths when the same plants as mentioned earlier in this section were grown under a combination of varying exogenous sucrose concentrations (0%, 1%, 2%, 2.5%, 4.5%, and 5%) and elevated CO_2 levels (Lee-Ho et al., 2007). Taken together, their study shows that sucrose, both exogenous and endogenous stimulates the development of RSA, and for the context of this thesis, LR formation, although as already mentioned, the details of the aspect of LR formation sucrose promotes cannot be concluded from that study.

Increased light intensities are expected to promote endogenous sucrose levels, and low light intensities affect Calvin cycle genes and enzymes and carbon assimilation in plants (Freixes et al., 2002; Sharkey et al., 2007). In a study by Freixes et al (2002), increased rates of LR elongation were observed under increasing light intensities (120, 290, and 460 µmol m−2s−1), under 0% exogenous sucrose conditions with the highest elongation rate at the highest light intensity (460 µmol m−2s−1) in Col-0 plants of developmental age up to 13-day-old (11-13days after sowing) (Freixes et al., 2002). LR elongation rates were increased under conditions where increasing light intensities were combined with various exogenous sucrose concentrations (0.5% and 2%). The addition of exogenous sucrose of concentration as low as 0.5% was able to restore the reduced LR elongation phenotype under low light intensity conditions (120 µmol m−2s−1). There were no statistically significant differences in LR elongation between plants grown under intermediate and high light intensity (290 and 460 µmol m−2s−1) and 0.5% exogenous sucrose, and those grown under intermediate and high light intensities (290 and 460 µmol m−2s−1) and 2% exogenous sucrose (Freixes et al., 2002), this, therefore, suggests a threshold effect of sucrose on LR elongation.

The authors also report increases in LR and LRP density (number of LR/ LRPs/ length of PR [cm]), at high sucrose concentrations and light intensities. LR densities ranged from 2·2 cm−1 (120 µmol m−2s−1, 0% exogenous sucrose) to 4·2 root cm−1(460 µmol m−2s−1; 2% sucrose), LRP densities were similarly increased under combinatory conditions of intermediate light intensity and 0.5% exogenous sucrose, suggesting a stimulatory effect of sucrose on LRP initiation (Freixes et al., 2002).

The authors also measured hexose (fructose and glucose) and sucrose levels in 15-day-old Col-0 plants the in the apical (elongation zone:2·5 mm) region of the PR and LRs, as well as in the regions of the PR spanning from 5 mm from the apex to the first emerged LR (labelled as sub-apical region: 5 to 20–25 mm from the PR apex), where LRPs are expected to develop, as LRPs development is known to be restricted to this zone—the differentiation zone (the 2-6 mm region from the PR apex is the known designated region for LRP initiation (Dubrovsky et al., 2000). The delimitation of this region was probably based on the observation of a stage LRP at a region of the PR 6-10 mm from the apex and the emergence of the first observed 20-25 mm from the apex (Freixes et al., 2002).

Higher concentrations of both hexose and sucrose were observed in the differentiation zone first 2 mm region from the root tip than in distal regions (Freixes et al., 2002). The authors observed increasing hexose and sucrose concentrations with increasing light intensities at the differentiation zone this, therefore, could explain why LRs and LRP densities were increased with increasing light intensities. The authors later reported no significant relationship between either sucrose and glucose levels, or sucrose and fructose, furthermore, a strong relationship was observed between LR elongation rate and hexose concentration ($r \leq = 0.64$) (Freixes et al., 2002).

Taken together, this study suggests that sucrose availability at the PR tip, and its concomitant hydrolysis into hexoses, and their further transport to the XPP cells in the differentiation zone stimulates LRP initiation and morphogenesis. The authors of this study did not go further to investigate how the availability of sucrose and/or its hexoses to XPP leads to the initiation and morphogenesis of LRPs. And precisely what aspect of LRP development the availability of sucrose and its hexoses potentially regulate, as they did not study or report data on the distribution of LRPs among the stages as described (Malamy and Benfey,1997).

The distribution of LRPs among stages are the developmental stages of LRP morphogenesis, with stage 1 being the LRP initiation stage, emergence of LRPs is also known to begin after stage 4 (Goh et al., 2016). Therefore, LRP distribution among developmental stages in response to exogenous sucrose supply or varying light intensity or elevated CO_2 levels would provide insights into potential checkpoints of regulation by sucrose. Additionally, sucrose and its hexoses, as already discussed in earlier sections of this chapter are both resources and signals, so the question from such a study becomes, whether sucrose simply provides the energy and the carbon skeletons required for LRP development or sucrose as a signal regulates components of the LR formation apparatus.

The aforementioned gap in knowledge can be said for other subsequent developmental and physiological studies aimed at establishing a causal relationship between sucrose availability and LR formation. For example, in a study by MacGregor et al (2008), the supply of 1% exogenous sucrose was reported to increase LR lengths in 5-day-old Col-0 plants, more profoundly than equimolar amounts of glucose, fructose, and G6P and the authors went further to show that the supply of exogenous sucrose, and specifically the contact of the shoot with sucrose in the agar media is sufficient to increase LR lengths, suggesting an effect of sucrose on LR elongation and outgrowth (Macgregor et al., 2008).

The authors went on to show reduced LR length under conditions where Col-0 plants were supplied the non-metabolised phosphorylated glucose analogue, 3-*O*-methyl glucose, which led them to conclude that it is the metabolism of sucrose, and signalling that regulates LR emergence, they seem to strengthen their conclusion by showing increased LR lengths in the hexokinase mutant, *gin2-1* which is incapable of glucose signalling, but is capable of glucose metabolism. These conclusions are inaccurate as discussions of sucrose signalling in section 1.8 of this chapter showed that there could be sucrose-specific signalling. Additionally, in the absence of data on the effect of exogenous sucrose on the LRP distribution among stages as mentioned for the studies discussed earlier, it is impossible to make substantiative conclusions on the effect of sucrose and any treatment on LR emergence and LR formation in general.

There is other scattered evidence in the literature about the role of sucrose of RSA in Arabidopsis, but these studies take the form of those discussed herein determining the effect of light intensities on LR lengths (Reed et al., 1998), the effect of exogenous sucrose supply on LR lengths (Takahashi et al., 2003; Karthikeyan et al., 2007). All these studies are plagued with the same gaps in knowledge discussed earlier; a lack of a mechanistic understanding of how sucrose stimulates LR formation, and which aspect of LR formation sucrose promotes. This observation led to the need for the studies that will be presented in the ensuing chapters of this thesis, which attempted to investigate the role of sucrose in promoting LRP initiation and morphogenesis.

As discussed earlier, sucrose is both a resource and a signal. As a resource, sucrose is required for LR formation, just like any other growth and developmental process in plants. However, the dual role of sucrose makes it an interesting molecule to study in relation to LR formation, as, not only could it provide the energy and the building blocks for LR development and ontogeny, but it could also be a signal that controls several aspects of LR formation.

Sucrose could have both direct and indirect regulatory roles in LR formation. Sucrose could indirectly control LRP initiation and morphogenesis and LR emergence by controlling aspects of auxin homeostasis. Sucrose has been suggested to promote auxin biosynthesis, transport, and signalling in Arabidopsis by increasing the transcript levels of the genes that encode the proteins required for these processes (Lilley et al., 2012; Sairanen et al., 2012; Zhang et al., 2020). In these studies, the supply of exogenous sucrose upregulated expression of some early auxin response genes, including *INDOLE-3-ACETIC ACID17* (*IAA17*), *IAA19*, *IAA29*, and *SMALL AUXIN UP-REGULATED15* (*SAUR15*) in isolated shoots (Lilley et al., 2012). The non-metabolisable sucrose analogue, turanose was shown to induce elevated auxin levels in the basal and apical regions of the cotyledon, the hypocotyl, the PR apex, and the elongation zone, as demonstrated by increased DR5 activity in 5-day-old *DR5::GUS* transgenic plants (Gonzali et al., 2005).

This turanose-induced auxin maximum was found to precede adventitious root formation, additionally, the supply of turanose was also found to lead to increased IAA levels in the Arabidopsis ecotype, Columbia glabra (gl1-1) (Gonzali et al., 2005). Taken together, these observations suggest a role of sucrose signalling in auxin response, and potentially biosynthesis and transport. The supply of exogenous sucrose increased the expression levels of several genes that encode the enzymes that catalyse auxin biosynthesis, for example, the expression levels of *YUCCA flavin monooxygenase 2*(*YUC2*) and *YUC6* were elevated in response to the supply of 5% exogenous sucrose compared to water and an equimolar amount of mannitol in peach (Zhang et al., 2020). There were, in addition, sucrose-induced increases in IAA levels in the roots of peach, which resulted in increased LR numbers and total root system length; the supply of exogenous sucrose increased IAA levels in the root by 34.04%, compared to the supply of water, and a 2-fold increase compared to mannitol (Zhang et al., 2020). Additionally, in the same study, the supply of exogenous sucrose upregulated the transcription of auxin transport genes, *PIN1*, *PIN3,* and *PIN3,* suggesting a role of sucrose in auxin transport.

Although these data are only transcript data and there was no data on the effect of exogenous sucrose on translation and protein stability, thereby providing insights on whether there is a translational and post-translation regulation of these genes and those aforementioned by sucrose, this data provides a viable and falsifiable hypothesis that could be tested. It was also, later shown in the study that Sucrose non-fermentation-1-protein kinase-1 (SnRK1) which is an energy sensor and can sense intracellular sugar levels (Li and Sheen, 2016; Rolland et al., 2006) partakes in the sucrose-mediated auxin response and biosynthesis. This was shown by elevated expression of the Arabidopsis Tryptophan Aminotransferase1 (*AtTAA1)* which is involved in (Zhao, 2012), as well as other auxin biosynthesis, *AtYUC2*, and *AtYUC6* and auxin transport-related genes *AtPIN1*, *AtPIN2*, and *AtPIN3* (Zhang et al., 2020).

Taken together, these data suggest that sucrose and component of sucrose signalling regulate auxin biosynthesis, transport, and response or signalling. This, therefore, supports the possibility that sucrose could indirectly regulate LR formation by being upstream of the auxin biosynthesis, transport, and signalling which are crucial for all aspects of LR formation (Santos Teixeira and Ten Tusscher, 2019). Possibly and more crucially via indirect regulation of the asymmetric cell division that underpins LRP initiation and morphogenesis which is the basis of RSA as already discussed elsewhere in this chapter.

Sucrose could also possibly directly regulate LRP initiation and morphogenesis of the asymmetric division. As discussed in section 1.8 of this chapter, sucrose controls the transcription of various components of the cell cycle in plants, especially the cyclins which are vital for cell cycle progression. Therefore, it could be that sucrose would not only provide the energy required for the division of XPP cells, but also, as a signal, would regulate their re-entry into the cell cycle. As already discussed earlier, XPP cells in the root are arrested at the G2/M transition and that not only is their re-entry into the cell cycle activated by an auxin maximum (Beeckman et al., 2001), but sucrose could either be at the upstream of this activation or it could directly regulate this activation.

1.10 Light availability as a tool for studying the effect of endogenous sucrose on LR formation.

Light captured by the shoot provides the energy needed for photosynthesis, thereby influencing sucrose biosynthesis, transport, and availability to promote lateral root development (Kircher and Schopfer, 2012). Light intensity is expected to increase endogenous sucrose levels as discussed in section 1.9 of this chapter, and also discussed in section 1.9 affects LR elongation and outgrowth (Freixes et al., 2002). The energy-providing effect of light for sucrose synthesis means that light availability and intensity can be manipulated under experimental systems to study the effect of endogenous sucrose availability and levels on LR formation. Such studies have been done in Arabidopsis, with an example of the Freixes et al (2002) study discussed in section 1.9 (Freixes et al., 2002).

Another example of such studies is one by Miotto et al (2021). In this study, the effect of exogenous sucrose in fully illuminated seedlings, illuminated shoots only, and dark roots (condition was created by covering 3/4 of the Petri dish length with black paper surrounding), and seedlings (both shoot and roots) grown in total darkness was studied (Miotto et al., 2021). Analyses of the roots of 7 days after germination (DAG) seedlings grown in the absence of exogenous sucrose under fully illuminated seedlings or illuminated shoots and dark roots revealed similar PR lengths. However, seedlings grown in total darkness D seedlings showed shorter PR lengths. The supply of exogenous sucrose increased PR growth in plants under all conditions, however, to less extent under total darkness conditions (Miotto et al. 2021). Additionally, the growth of plants under low light intensity (40 μmol m−2s−1) under all illumination conditions, the absence of exogenous sucrose in media inhibited LR emergence. However, the supply of 1% exogenous sucrose could restore LR formation (Miotto et al., 2021).

Analyses of the effect of the NPA (1 μM) on PR growth under varying light intensities (40, 60, 80, and 105 μmol m−2s−1) under illuminated shoots and dark roots conditions, revealed that reduction in PR growth by both NPA and IAA (1 μM) compared to the control (1/2 MS medium (MS), 0 μM IAA and NPA). PR growth was strongly inhibited by IAA regardless of the light intensity compared NPA which led to increasing PR growth with increasing light. Consequently, the authors concluded that the illumination of the shoot promotes polar auxin transport to promote root growth, and not necessarily increasing root IAA concentration (Miotto et al., 2021).

Similar to sucrose, light availability has been proposed to promote auxin transport from shoot to root via regulating (PIN1 levels in a CONSTITUTIVE PHOTOMORPHOGENESIS1(COP1)-dependent manner. This results in differential modulation of root PIN1 and PIN2 levels (Sassi et al., 2012). Light has also been proposed to promote cell divisions in the RAM. In the same study by Miotto et al (2021), decreased *CYCB1;1:GUS* activity was observed in plants grown in total darkness, compared to plants grown under partial illumination (roots covered and shoot illuminated), suggesting a reduced cell division under total darkness conditions. This reduced cell division in the RAM is evidenced by the reduction in the size of the RAM, which was restored upon return of plants to light (Laxmi et al., 2008). Under complete darkness, PIN2 abundance is reduced due to localisation in the vacuole, compared to the expected subcellular localisation in the plasma membrane (Laxmi et al., 2008). This defect in the intracellular distribution of PIN2 is expected to cause a defect in polar auxin transport within the RAM, thereby impacting cell division in the RAM.

The observation of the effect of light on the root system, and specifically LR formation inspired a study on the effect of light on energy homeostasis by Muralidhara et al (2020). In this study, the authors studied the effect of low light and extended darkness conditions on LRP initiation. They did this by first growing 8 DAG 0 seedlings under low-light conditions (15 μmol m^{-2} s^{-1}) for 1 to 5 days; Increased LR densities were observed under conditions with up to 3 days of low-light treatment (Muralidhara et al., 2021). Plants grown under low light conditions for longer periods showed reduced LR densities compared to control conditions; this reduced LR density phenotype was independent of the length of PR (Muralidhara et al., 2021). An extension of the night cycle by 6 hours (under 16hour/light, 8hour/dark regime) led to a decrease in PR growth, creating an artifact of an increased LR density, as the LR numbers were not different from the control light conditions. However, a 4-hour extended darkness treatment (termed short-term unexpected darkness) led to significantly increased LR numbers and densities, independent of the length of the PR.

The authors also reported higher *pGATA23::GFP* activity in response to 4 hours of extended darkness, suggesting increased LRFC founder cell specification, and higher LRP numbers were quantified under the aforementioned condition, compared to control light conditions (Muralidhara et al., 2021). This short-term unexpected darkness-induced increased LR density phenotype was further reported to be associated with reduced levels of hexoses, sucrose, and T6P, as well as the activation of SnRK1 (a low-energy stress marker).

The aforementioned study does not study the effect of extended darkness over longer periods (similar to the initial 6 hours and longer), to understand how the LRP initiation and morphogenesis phenotype would be. And although the media used for the study contained 1% exogenous sucrose, the question remains, would LRP initiation be increased under slightly more intense carbon starvation and what would the distribution of the LRPs at stage 1 which is the LRP initiation stage be like? How would the distribution of LRPs among the various developmental stages be, if their experiments were executed under conditions with absolutely no sucrose availability? To answer these questions, this thesis set out to understand the involvement of light as an energy source for sucrose synthesis on LRP initiation and morphogenesis.

Light, just like sucrose is both an energy source and a signal, light as a signal is known to be stem-piped to the root where it activates phytochrome receptors (Lee et al., 2016). Phytochrome receptors in the root induce the expression of the light-dependent transcription factor, *ELONGATED HYPOCOTYL (HY5)*. HY5 has been shown to inhibit LR emergence, by decreasing the plasma membrane abundance of the auxin transporters, LAX3 and PIN3 (van Gelderen et al., 2018). This prevents auxin transport from the PR tip to growing LRPs, hence inhibiting their emergence. LAX3/PIN3-mediated accumulation of auxin in overlaying tissues of the parental root is required for nascent LRPs to emerge (Lucas et al., 2013; Péret et al., 2013).

It is worth noting that the aforementioned study was conducted under low red: far-red (R:FR) conditions, which is associated with reduced auxin synthesis (van Gelderen et al., 2018). This thesis is aimed at studying the effect of sucrose, both endogenous and exogenous on LRP development and while it would be interesting and it is crucial to separate the effect of the energy-giving and signalling roles of light as pertaining LRP development, such a study is outside of the context of this thesis.

1.11 Thesis rationale

Several hormones, signals, and transcription factors are crucial for lateral root formation; however, signals and hormones are not materially required for growth. Carbon (sucrose) provides the energy and structural molecules required for cell growth and division. There is evidence about the stimulatory effect of sucrose, both endogenous and exogenous on LR numbers and densities, LR elongation, as well as LRP numbers, as explained earlier. However, these studies have not determined the effect of sucrose on the distribution of LRPs among the various developmental stages to determine the potential effect of sucrose on LRP development. And to also determine whether or not there is a checkpoint at which sucrose controls LRP development. Therefore, the study presented in this thesis set out to understand whether and how sucrose, both endogenous and exogenous controls LRP development using a combination of plant physiology and cell biological approaches.

1.12 Thesis aim and questions.

1.12.1 Thesis aim.

The overall aim of the thesis was to understand whether and how sucrose promotes LRP development (initiation and morphogenesis).

1.12.2 Thesis questions.

The specific questions asked in this thesis are as follows:

1. What is the effect of sucrose on LRP development?
2. What is the effect of longer periods of extended darkness-induced carbon starvation on LRP development?

Chapter 2 Materials and Methods

2.1 Introduction and rationale for chapter

The study described in this thesis was executed using physiology and cell biology approaches. These physiology and cell biology methods were crucial to answer the questions asked in this thesis, such as the effect of sucrose availability on the distribution of LRP stages. Results generated from using these methods would provide the foundation for formulating hypotheses that would be tested using molecular genetics approaches.

2.2 Plant Materials

2.2.1 Wild type

Col-0: The Arabidopsis Col-0 accession was used as a wild type where indicated.

pGATA23: NLS-GFP: A reporter line containing a GATA23 promoter fused to NLS and GFP (De Rybel et al., 2010) was used as the wild type where indicated. The reporter line was a gift from Tom Beeckman, Ghent University. This line was used in the studies in chapters 3 and 4.

GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4: This reporter line was used where indicated. The reporter line was a gift from Alexis Maizel (Barro et al., 2019). This line was used in the study in chapter 5.

2.3 Plant Growth and conditions

2.3.1 Soil-grown plants

2.3.1.1 SEED GERMINATION AND GROWTH ROOM CONDITIONS

Seeds were aliquoted to 1.5 mL Eppendorf tubes, and 1 mL of 0.1% micro agar solution was added. Seeds were cold stratified at 4°C for 2 days before sowing. The temperature in growth rooms was maintained at 21°C for short-day conditions, photoperiod was 12h/light, 12h/dark for long-day conditions, the temperature was maintained at 22°C, at a photoperiod of 16h/light, 8h/dark. Light intensity in long day room was 200 µmol/m^2/s for long day room and. Humidity was maintained at 65% for day conditions and 50% for night conditions.

2.3.2 Plate-grown plants

2.3.2.1 MEDIA PREPARATION AND SEED STERILISATION

All media used consisted of 0.5 x MS salts (Murashige and Skoog 1962) and MES at a final concentration of 25mM and pH of 5.8. Media contained 0%, 0.3%, 0.6%, or 1% sucrose and 1% tissue culture grade AGAR PHYTO (P1003.1000; Melford Laboratories Limited).

All seeds were sterilised by aliquoting at most 50 seeds into 1.5 mL Eppendorf tubes. 1 ml of 70% ethanol with 0.05% Tween (500 µl/L) was added to tube. Tubes were shaken for 15 minutes. 70% ethanol and 0.05% Tween solution were discarded; seeds were washed with 95% ethanol for 5 minutes and allowed to dry for 5 to 10 minutes.

2.3.2.2 SEED IMBIBITION

500 µl of 0.1 agar was added to ethanol-sterilised seeds and cold stratified at 4°C for 2 days.

2.3.2.3 PREPARATION OF PLATES FOR PLANT GROWTH

All plants for plate-based experiments were pre-grown for 10 days and transferred onto desired treatment plates. Plates were prepared by microwaving solidified agar media with 0.5x MS/MES, 0.6%, 0.3% or 0% sucrose, and 1% agar. Molten agar media were cooled down to 60°C; 75 ml of molten media was pipetted into square plates and allowed to set. Plants used in the study in chapter 3 were pre-grown on 0.3% sucrose, while the plants used in the studies in chapters 4 and 5 were pre-grown on 0.6%, unless otherwise stated in the respective chapters.

2.4 Preparation of materials for filter paper/Parafilm strip experiments

2.4.1 Preparation and sterilisation of Parafilm strips

Parafilm strips were either 13 x 2 cm or 11 x 2 cm in dimensions based on the direction of their placement on the media plate as would be discussed in chapter 4 of this thesis. Parafilm strips were sterilised by soaking them in 95% ethanol for 60 minutes. Ethanol-sterilised Parafilm strips were dried on autoclave-sterilised paper towels under a Laminar flow hood for 60 minutes. Parafilm strips of dimensions, 11 x 2 cm were used in the study in chapter 3, and Parafilm strips of dimensions 13 x 2 cm were used in the studies described in chapters 4 and 5.

2.4.2 Preparation and sterilisation filter paper strips

Filter paper strips were either 12 x 1.5 cm or 10 x 1 cm in dimensions. Filter paper strips were prepared for autoclaving by wrapping 20 pieces of 12 x 1.5 cm strips or 30 10 x 1 cm strips in two layers of aluminium foil. Filter paper strips were dry autoclaved at 120°C for 1 hour.

2.4.3 Preparation of filter paper strips for experiments

Filter paper strips were either soaked in 1 ml (for 10 x 1 cm strips) or 1.5 ml (for 12 x 1.5 cm) of either 1% or 0% sucrose unless otherwise stated in the results section.

2.4.4 Placing Parafilm and filter paper strips on media.

Parafilm strips were placed onto media plates with flat-edged forceps sterilised in 95% ethanol and air-dried under a flow hood for 5 minutes. 13 x 2 cm Parafilm strips were either placed horizontally and 11 x 2 cm Parafilm strips diagonally at 45^{o} onto media plates. Filter paper strips soaked in either 1% or 0% sucrose were laid in the middle of their designated Parafilm strips. 12 x 1.5 cm filter strips were placed onto 13 x 2 cm Parafilm strips, whereas 10 x 1 cm filter paper strips were placed onto 11 x 2 cm Parafilm strips.

2.4.5 Pre-growth.

Seedlings for Parafilm/filter paper experiments and non-Parafilm/filter paper experiments were pre-grown for 10 days on 0.5x MS/MES media containing either 0.3% or 0.6% sucrose and 1% agar.

2.4.6 Transferring pre-grown seedlings onto sucrose treatment plates.

Pre-grown seedlings were transferred onto media containing either 0% sucrose or various concentrations of sucrose (0.3%, 0.6%, and 1%). Shoots of seedlings were in contact with filter paper soaked in either (0%, 0.3%, 0.6%, and 1%) or 1% and 0% sucrose, while the roots were either in contact with the media without sucrose or in contact with media with various concentrations of sucrose (0%, 0.3%, 0.6%, and 1%). Seedlings were transferred onto sucrose treatment plates by first washing them in distilled water and placed onto filter paper strips with

95% ethanol-sterilised forceps. The tip of the PR was marked to differentiate between pre-transfer PR growth and post-transfer growth during root growth analyses.

2.4.7 Transferring pre-grown seedlings for non-Parafilm/filter paper strip experiments.

Pre-grown seedlings were washed in sterile water and transferred onto media with different concentrations of sucrose. Both shoots and roots of seedlings were in contact with media.

2.5 Post-transfer growth and conditions.

Plants were grown for up to 6 days in a Percival plant growth cabinet. All plate-grown plants were maintained at long-day conditions, with a photoperiod of 16 hours/8 hours day/night with a temperature of 21°C/16°C day/night; light intensity in the growth cabinet was maintained at 150 μmol/m^2/s.

2.6 Root growth and development analyses.

2.6.1 Image acquisition

Plates were imaged at the end of the designated growth period. Plates were imaged from the back using a Nikon D60 digital camera. Before images of plates were taken, a calibration image was taken from a calibration plate which was used to set the scale for post-image analyses. Plates were illuminated by using a light source connecting to a homemade imaging station.

2.6.2 Root measurements

2.6.2.1 FIJI IMAGEJ ANALYSES

The Fiji Image J software (https://imagej.nih.gov/ij/) was used to analyse root growth. The scale of measurement was set using the calibration image described in 2.5.1. The scale of measurements was set as pixels/mm.

2.6.2.2 MEASUREMENT OF PRIMARY ROOT GROWTH

Primary root growth was determined by measuring the length of the primary root from the previously marked tip of the PR before transfer to the tip of the PR at the end of the post-transfer growth period. Primary root lengths were measured by using the segmented line on Fiji Image J. Primary root length was measured in mm.

2.6.2.3 QUANTIFICATION OF EMERGED LATERAL ROOTS

Lateral roots were scored by counting the number of visible lateral roots per post-transfer primary root. Visible lateral roots are those lateral roots that have emerged out of the PR and look just like the PR (Malamy and Benfey 1997). In chapter 3, LRs were scored as both stage 8 and stage (Figure 2.1).

2.7 Quantification of LRPs

LRPs were quantified using epifluorescence microscopy and imaged using confocal microscopy.

2.7.1 Epifluorescence microscopy

Epifluorescence microscopy was used to score LRP stages. A Nikon E600 microscope was used. A mercury lamp was used to generate UV light, and root samples were viewed using different fluorescence filters. To score LRP in the *pGATA23: NLS-GFP* background, the GFP filter was used, and to score LRP in the *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4,* the mCherry filter was used. Microscopy was done using 10x, 20x, and 40x objective lenses.

2.7.2 Mounting samples for epifluorescence microscopy

Only post-transfer roots (as mentioned in section 2.5.2.2) were used for microscopy. Root samples were mounted in distilled water on a slide and meticulously covered with either a 22 x 22mm or 24 x 50 mm coverslip.

2.7.3 Confocal microscopy

An Olympus IX70 inverted confocal microscope with Argon and Helium-Neon lasers was used for confocal imaging. Images were taken using 20x, 40x, or 60x water immersion lenses. GFP and propidium iodide fluorescence was visualised using both 488nm and 543nm excitation wavelengths, a 510 long-pass filter (BA510IF), a 530 short-pass filter (BA530RIF), and a 585/640 band-pass filter (BA585-640). Confocal images were captured using a Kalman scan with 4 individual scans of the same image, to minimise noise-to-signal ratio.

2.7.4 Mounting samples for confocal microscopy

GATA23:H2B:3xmCherry/pUBI: 3xmCherry:3xGFP: PIP1,4 root samples were mounted in distilled water, instead of propidium iodide due to the presence of a membrane reporter (GFP: PIP1,4) while *pGATA23: NLS-GFP* root samples were mounted in propidium iodide at a concentration of 5mg/ml. Slide preparation was as described in section 2.6.2.

2.7.5 Image conversion and analyses

Confocal images were saved as Fluoview Tag Image File Format (TIFF) images. FIJI Image J was used to convert the images into RGB format, to allow for false colours to be added to the channels. Z stack images were resliced into digital cross sections using the reslice function in FIJI Image J. Image stacks were converted to RGB format and saved as multiple TIFF image files. New images from the stack were created using the resliced function, incorporating all layers. Images were enlarged and all sides were made equal using the resize function in FIJI Image J.

2.8 Scoring LRP

LRPs were scored according to 8 stages (stages 1-8) as classified by (Malamy and Benfey 1997), the nuclear migration stage as stage 0, and outgrown lateral roots as stage 9. Additionally, to accurately identify and score the different stages of LRP, the ground state xylem pole pericycle cell layer was characterised.

Ground state xylem pole pericycle cell layer: The pericycle cells are long and thin when LRPs are not yet initiated. The nuclei are lens-shaped (Figure 2.1A)

Stage 0: An LRP was scored as stage 0 when it had two adjacent cells with their nuclei aligned or migrating toward their common cell wall. The nuclei of the stage 0 LRPs are round (Figure 2.1B).

Stage 1: A stage 1 LRP is characterised by one cell layer (Figure 2.1C)

Stage 2: A stage 2 LRP is characterised by two cell layers (Figure 2.1D).

Stage 3: A stage 3 LRP is characterised by three cell layers (Figure 2.1E).

Stage 4: A stage 4 LRP is characterised by four cell layers. The LRP penetrates the endodermis of the parent root (Figure 2.1F).

Stage 5: An LRP was scored as stage 5 if it emerges out of the endodermis of the parent root and penetrates the cortex (Figure 2.1G).

Stage 6: An LRP was scored as stage 6 when it emerges out of the parental cortical layer and penetrates the epidermis (Figure 2.1H).

Stage 7: An LRP was scored as stage 7 when it is just penetrated the epidermis of the parent root but has not emerged out of it (Figure 2.1I)

Stage 8: A lateral root organ was scored as stage 8 when it emerges out of the epidermis of the PR, and it sits right on top of the epidermis (Figure 2.1J).

Outgrown lateral root: An outgrown and elongated LR. An LR was scored as outgrown when it was no longer sitting atop the epidermis and had elongated (Figure 2.1K).

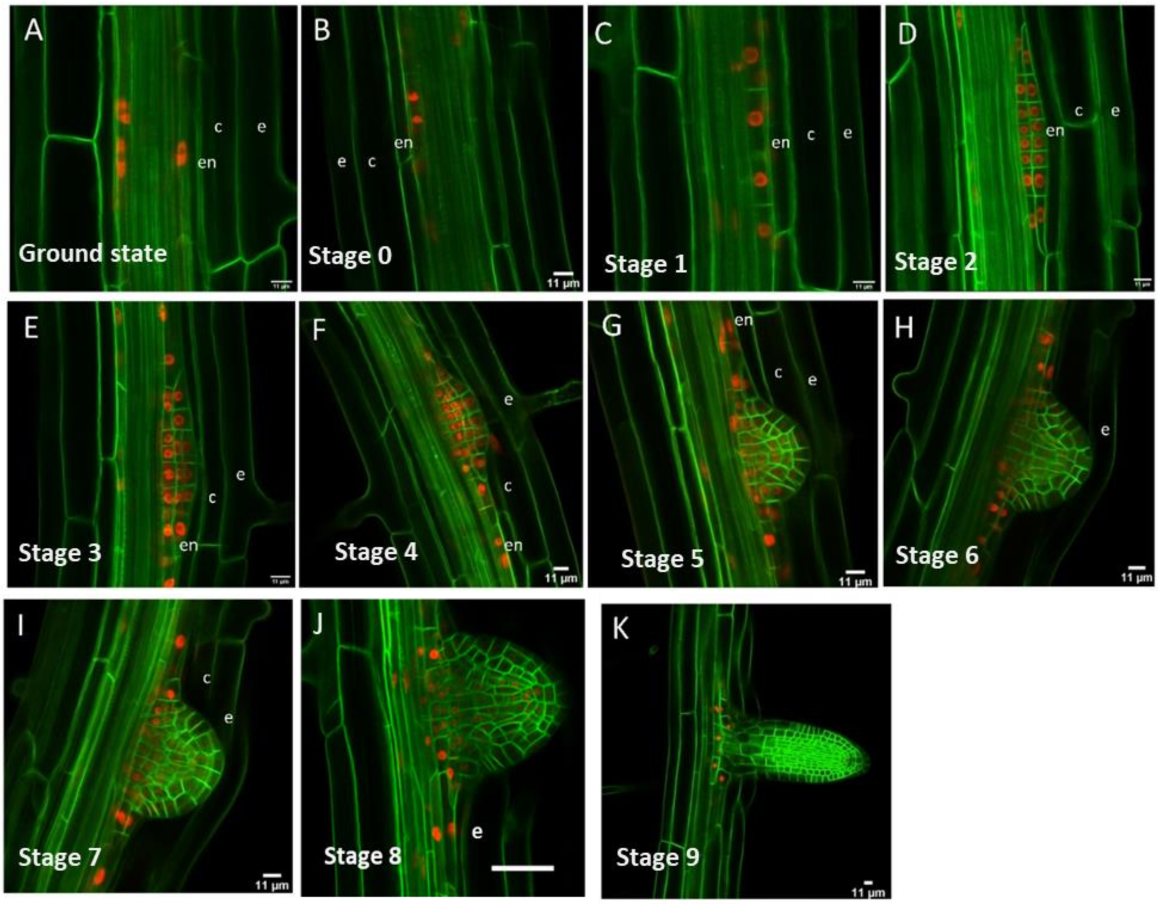


Figure 2.1 **Confocal images of xylem pole pericycle layer and LRP stages in roots of 7-day old seedlings of GATA23:H2B:3xmCherry/pUBI: 3xmCherry:3xGFP:PIP1,4 reporter line.**

(A) Ground state xylem pole pericycle cell layer. (B) Stage 0 LRP characterised migration of two adjacent nuclei. (C) Stage 1 LRP characterised by one cell layer. (D) Stage 2 LRP characterised by two cell layers. (E) Stage 3 LRP characterised by three cell layers. (F) Stage 4 LRP characterised by four cell layers and the penetration of the parental endodermal layer. (G) Stage 5 LRP characterised by the emergence out of the parental endodermal layer and penetration into the cortex. (H) Stage 6 LRP characterised by emergence out of the cortex and penetration into the epidermis. (I) Stage 7 LRP characterised by near-emergence out of the epidermis. (J) Stage 8 LRP characterised by emergence out of the epidermis, and the LRP sitting atop the epidermis. (K) Outgrown lateral root, characterised by elongation of the emerged lateral. en: endodermis, c: cortex, e: epidermis. Bar for figure J is 50 μm. Images taken by Frank Machin.

2.9 Calculation of lateral root organ densities.

2.9.1 Normalisation of lateral root organs to primary root length

Lateral root, LRP, and total lateral root organ (lateral root + LRP numbers) densities were calculated by dividing the total numbers of lateral roots or LRP or both by the length of the post-transfer primary.

LRP/LR ratios were calculated using the formula below:

LRP/LR ratios = $\frac{Total\ LRP\ numbers}{Total\ LR\ numbers}$

2.9.2 Calculation of stage-specific LRP density

The densities of lateral roots were also calculated on a per-stage basis as described section in 2.8. This was important to accurately interpret the effect of sucrose treatment at individual LRP stages.

2.10 Preparation of box for dark treatments

Boxes of dimensions 25.4 x 15 x 13 cm (height x length x width) were covered with three layers of aluminium foil to make them light-tight. Wooden racks of smaller dimensions than the light box were placed in the box to ensure plant growth plates could be placed vertically. Plates were placed in light-tight boxes and covered with opaque tape with adhesive and then with brown Sellotape.

2.11 Statistics and data visualisation

All quantitative data were analysed using Excel 2016 or PAleontological STatistics (PAST) software (version 4.12b) (Hammer-Muntz et al., 2001). Datasets were subjected to normality tests datasets were considered normal if the p-value was > 0.05 (Ghasemi and Zahediasl, 2012). Welch's t-test was performed on all datasets that were normally distributed but had unequal sample sizes. A level of statistical significance was set at $p < 0.05$. Significant differences in LRP distribution among stages at various time points in response to exogenous sucrose supply were determined by chi-square tests in the PAleontological STatistics (PAST) software (version 4.12b). Chi-square tests were conducted because the data collected (LRP stages) are categorical variables. Statistically significant differences in the progression of LRPs in response to exogenous sucrose supply at various time points were determined by the non-parametric two-sample test, the Mann-Whitney U test (Mann and Whitney, 1947). All data were visualised using Excel 2016. Violin plots were generated using boxplotR: (http://shiny.chemgrid.org/boxplotr/), and all column graphs were generated using Excel 2016.

Chapter 3 Supplying exogenous sucrose to shoot promotes primary root growth and the developmental progression of lateral root primordia.

3.1 Introduction

Exogenous sucrose promotes primary root (PR) growth and lateral root (LR) formation (Freixes et al., 2002; Takahashi et al., 2003; Lee-Ho et al. 2007; Macgregor et al. 2008; Kircher and Schopfer 2012). In a physiological experiment, when 4daig wild type Columbia (Col-0) seedlings were subjected to treatments that inhibited various aspects of photosynthesis ((i) the removal of CO_2 from the atmosphere in the light, and (ii) preventing greening with the carotenoid synthesis inhibitor (norflurazone), short PRs were formed (Kircher and Schopfer, 2012). The addition of 30 mM sucrose restored the short root phenotypes to long roots similar to PR lengths under control conditions (Kircher and Schopfer, 2012).

Growth of wild-type Col-0 plants grown under high light intensities and sucrose concentrations developed long PRs and high LR numbers (Freixes et al., 2002). High LR numbers and long LR lengths have also been observed under elevated CO_2 conditions (which promotes photosynthesis) (Lee-Ho et al., 2007). Taken together, these observations suggest the role of sucrose (whether endogenously supplied or exogenously supplied) in promoting PR growth and LR formation. Efforts to establish a causal relationship between sucrose and LR formation in most of the studies discussed earlier have been mostly based on determining LR numbers and LR lengths in response to either exogenous sucrose supply or conditions that potentially stimulate endogenous sucrose synthesis (Freixes et al., 2002; Takahashi et al., 2003; Macgregor et al., 2008). In a study by Macgregor et al (2008) (Macgregor et al., 2008), although they quantified LRPs, these LRPs were not categorised into the various stages as established by Malamy and Benfey, 1997 (Malamy and Benfey,1997).

Quantifying and measuring LR numbers and lengths in response to sucrose treatment only gives insights into the emergence and outgrowth of LRs, and not the LR initiation and LRP morphogenesis processes that precede LR emergence and outgrowth. This approach of establishing a quantitative relationship between sucrose and LR formation does not account for its likely role in promoting LR initiation and LRP morphogenesis. LR initiation and LRP morphogenesis are the basis for root system development in plants (Dubrovsky et al., 2006). As such, determining the response of LR initiation and LRP morphogenesis to exogenous sucrose treatment as demonstrated by the distribution of LRPs among the various stages will provide an opportunity to identify potential checkpoints of regulation by sucrose. Identification of these checkpoints will enable a mechanistic understanding of how sucrose promotes LR initiation and LRP morphogenesis to be determined.

The conventional method for growing Arabidopsis on Petri dishes involves contact of both shoot and root with sucrose via agar media. This suggests that sucrose is taken up by both the shoot and the root which does not happen under soil conditions. Roots do not take up sucrose under soil conditions—sucrose is synthesised in the shoot and supplied to the root and other carbon sink organs via long-distance transport (via bulk flow) through the phloem (Durand et al., 2018). Phloem transport of sucrose from shoot to root, carries alongside it other metabolites such as auxin and amino acids, which could have roles in regulating LRP formation (Swarup et al., 2001;.Vanneste et al., 2009). Auxin especially is a master regulator of LRP formation (De Rybel et al., 2007; De Smet et al. 2007; Vermeer et al. 2014). This suggests that the conventional method of supplying exogenous sucrose to plate-grown Arabidopsis is flawed. This is a problem because biological insights from physiological experiments conducted this way may not recapitulate the relationship between sucrose and LR formation under soil conditions.

Under *in vitro* conditions, when both the shoot and the root are in contact with sucrose via the media, the root is considered to take up sucrose, instead of the shoot, this was evidenced by higher expressions of AtSWEET11 and AtSWEET12 in the root compared to the shoot (Papaioannou et al., 2018).

Under *ex vitro* conditions, although expression levels of AtSWEET12 were low in both the shoot and root, the expression of levels was significantly more profound in the shoot than in the root (Papaioannou et al., 2018). Taken together, these observations suggest that under *in vitro* conditions, sucrose is taken by the root and transported to the shoot. Taking this prediction into consideration, the transport of any shoot-derived molecule or metabolite that may be crucial for LRP formation may not be observed under *in vitro* conditions when both shoot and root are in contact with sucrose via the media. Consequently, the conventional method for the growth of Arabidopsis plants *in vitro* is unsuitable for physiological studies aimed at establishing a causal relationship between sucrose availability and root growth and development.

Physiological studies may be conducted under soil conditions, where endogenous sucrose levels could be controlled by growing Arabidopsis plants in growth environments (e.g., plant growth rooms) where light intensities are varied (e.g., high light intensity: e.g., 150 $\mu mol/m^2/s$ vs low light intensity: e.g., 70 $\mu mol/m^2/s$). Or where different CO_2 concentrations are varied. However, scoring LRP stages will require the collection of root samples from the soil and subsequent washing of root samples before scoring LRPs. This whole process may lead to the loss of root samples, and potentially useful data.

‘Shovelomics’, which is a process whereby roots are excavated from the soil with a concomitant visual scoring is widely used to study root system architecture under soil conditions (Burridge et al., 2016; Arifuzzaman et al., 2019). The aforementioned method could lead to the loss of fine root samples, especially LRs (Takahashi and Pradal, 2021). Rhizoboxes with an attached imaging station which allows the growth patterns of RSA, such as the total root area, length, etc., to be measured have been reported (Bontpart et al., 2020).

However, even if such root phenotyping systems are used in studies aimed at establishing a causal relationship between sucrose availability on LRP formation in Arabidopsis under soil conditions, only the lengths of numbers, lengths, and densities of LRs can be quantified under such systems.

To score LRP stages, root samples would still need to be harvested and washed, which is not only labour-intensive, but the fine nature of the Arabidopsis root will lead to a massive loss of root samples and potentially useful data, compared to other plants species with thicker roots such as the crown roots of monocots. Therefore, agar media plates remain arguably the best experimental system to study LRP formation in Arabidopsis, and in the context of this study, to establish a relationship between sucrose availability and LRP formation.

A method that allows the supply of exogenous sucrose directly to the shoot and not to the root is needed to study the relationship between sucrose availability and LRP formation in Arabidopsis. An experimental method that allows the shoot to be isolated from the media using ethanol-sterilised Parafilm strips has been described by Macgregor et al (2008) (Macgregor et al., 2008). Macgregor et al (2008) showed that contact of the shoot with media containing sucrose was essential for the stimulatory effect of sucrose on LR emergence and outgrowth as demonstrated by increased LR lengths in response to sucrose.

They showed this by growing seeds of *lateral root development2* (l*rd2*) mutants close to the edge of a sterile Parafilm strip such that, the seeds would grow onto the Parafilm strip (Macgregor et al. 2008). The *lrd2* mutant exhibits increased LR emergence and outgrowth (Deak and Malamy 2005), therefore, it served as a great assay for studying the regulation of LR formation under the conditions of their study (Macgregor et al., 2008). Their assay showed decreased LR lengths and numbers, and similar observations were made in other mutants with reduced permeability in the leaves due to defects in cutin biosynthesis (Macgregor et al., 2008). There were no visible LRs observed when the shoots of wild-type Col-0 plants were prevented from contacting the media. Analyses of the percentage of LRPs that emerged to become LRs showed that LRPs were formed under the conditions where the shoot was isolated from the sucrose-containing media, but they were unable to emerge (Macgregor et al., 2008).

This LR emergence and outgrowth phenotype in the wild-type Col-0 plants was restored by adding 100 µl drops of agar containing 1% exogenous sucrose to the shoot of individual seedlings (Macgregor et al,. 2008). Drops of 1% exogenous sucrose solution was also able to restore the LR emergence and outgrowth phenotype compared to equimolar amount of mannitol and drops of water (Macgregor et al., 2008). The authors, therefore, concluded that contact of the shoot with sucrose is sufficient to promote LR emergence and outgrowth. Although, the study conducted by Macgregor et al (2008) does not dissect the role sucrose might be playing in promoting LRP initiation (stage 1) and morphogenesis, their experimental method inspires the development of an experimental method that allows sucrose to be supplied directly to the shoot without contact of the root with sucrose via media due to the reasons discussed earlier. The study presented in this chapter developed such a method as will be described in sections 3.3 and 3.4 of this chapter.

LRPs have been scored by root clearing and differential interference contrast microscopy (Malamy & Benfey, 1997; Dubrovsky et al., 2006; Dubrovsky et al., 2009). However, this method of scoring does not allow stage 0 LRPs to be visualised and scored. As discussed in chapter 1 of this thesis, stage 0 is the nuclear migration stage and serves as the starting point from which the developmental trajectory of LRPs can be tracked, and most importantly, it is the ‘marker stage’ for LR initiation (De Rybel et al., 2010). Scoring stage 0 LRPs is crucial in determining whether a particular treatment has stimulated or promoted LR initiation by determining the differences between the numbers of stages 0 and 1 in the presence and absence of the treatment. Consequently, LRP markers such as the *pGATA23::NLS-GFP* reporter lines (De Rybel et al., 2010) that allow for stage 0 LRPs to be scored via live imaging using epifluorescence microscopy can be used in physiological experiments that require various stages of LRPs to be quantified.

There are other well-characterised and well-known LRP markers such as the enhancer trap reporter line, J0121, which is specifically expressed in xylem pole pericycle (XPP) cells that could be used to score LRP stages (Laplaze et al., 2005; Dubrovsky et al., 2006). However, the main difference between the J0121 enhancer trap line and the *pGATA23::NLS-GFP* reporter is that stage 0 LRPs cannot be observed in the J0121 line because it uses endoplasmic reticulum-targeted GFP (ER-GFP) which does not allow the nucleus to be observed (Laplaze et al., 2005). Other LRP markers such as PIN-based or DR5-based fluorescent reporter lines may be used, however, a GATA23 fluorescent reporter line remains the best-characterised line for scoring the nuclear migration stage due to the role of GATA23 in the nuclear migration process (De Rybel et al., 2010). Therefore, the *pGATA23::NLS-GFP* reporter line which was developed by De Rybel et al (2010) to study nuclear migration (De Rybel et al., 2010), and which still marks subsequent LRP developmental stages was used to score LRPs in the study presented in this chapter.

Sucrose is also an osmotic agent in addition to being a major carbon source for plant growth *in vitro*, consequently, higher concentrations may cause osmotic stress (Cui et al., 2010). *In vitro* drought stress studies typically use osmotic stress as the drought stress equivalent under soil conditions (Verslues and Bray 2006). The addition of osmotica such as mannitol or sorbitol to plant growth media reduces the osmotic potential or the water potential of the growth media, thereby causing osmotic stress (Deak and Malamy 2005; Roycewicz and Malamy 2012). Lower water potential in the growth media reduces the ability of the root to absorb water from the media, thereby hampering growth. ≥ 60 mM mannitol causes a reduction in the developmental progression of LRPs and a reduction in the lengths of LRs (Deak and Malamy, 2005; Xiong et al. 2006; Macgregor et al., 2008). The addition of 60 mM mannitol has been hypothesised to reduce sucrose uptake by the shoot under *in vitro* conditions which leads to a reduction in LR lengths (Macgregor et al., 2008). In physiological studies mannitol and sorbitol have been used interchangeably (Claeys et al., 2014). Therefore, the osmotic effect of exogenous sucrose was controlled for using sorbitol due to its readily availability in the research laboratory where the study presented in this chapter was conducted.

3.2 Objective(s) and questions

3.2.1 Objectives

The main objective of the study presented in this chapter was to determine the effect of exogenous sucrose on the LRP formation; but first to develop a method that allows the supply of exogenous directly to the shoot due to the reasons discussed in section 3.1 of this chapter and to determine whether there is a difference between the response of LR formation (as determined by LRP numbers and densities; LR numbers and densities) to the supply of exogenous sucrose supplied directly to shoot and the supply of exogenous sucrose to both shoot and root.

3.2.2 Question

Is there a difference between the response of LRP formation to the supply of exogenous directly to the shoot and the supply of exogenous sucrose to both the shoot and the root?

3.3 Methods

3.3.1 Generic experimental design for chapter

- **Seeds sterilisation**: pGATA23::NLS-GFP or Col-0 seeds, where indicated were ethanol sterilised and cold stratified for 2 days (Figure 3.1).

- **Pe-growth**: Seeds were pre-grown on 0.3% sucrose for 10 days, and 7 days where indicated (Figure 3.1).

- **Transfer**: Seedlings were transferred onto various sucrose plates as described in section 3.3.2 (Figure 3.1).

- **Root analyses**: PR lengths were measured using Fiji ImageJ and LR and LRP numbers were scored using Fiji ImageJ and epifluorescence microscopy, respectively (Figure 3.1).

3.3.2 Preparation of plates for experiments

Plates for validation experiments were prepared by adding 20% solutions to 500 mL MS agar media (0.5x MS/MES and 1% agar) to achieve desired final concentrations (0%, 0.3%, 0.6%, and 1%) (Table 3.1).

Plates for experiments aimed at dissecting the effect of the supply of sucrose to shoot alone on LRP progression were prepared by adding no sucrose (0% sucrose) to 500 mL MS agar media (0.5x MS/MES and 1% agar). Plates for experiments aimed at dissecting the effects of the supply of exogenous sucrose to root alone on LRP progression were prepared by adding various concentrations of sucrose (0%, 0.3%, 0.6%, and 1%) to 500 mL of MS agar media (0.5x MS/MES and 1% agar).

Plates for experiments aimed at dissecting the effects of the supply of exogenous sucrose to both the shoot and root on LRP progression were prepared by adding different volumes of sucrose (Table 3.1) to 500 mL of MS agar media (0.5x MS/MES and 1% agar) to achieve various final sucrose concentrations (0%, 0.3%, 0.6%, and 1%).

Plates for experiments aimed at dissecting the effects of increased osmolarity associated with 1% on LRP progression were prepared by adding various adding 20% sucrose solution to MS agar media to achieve desired concentrations (0%, 0.3%, 0.6%, and 1%). Various volumes of sorbitol (Table 3.2) were also added to the same MS agar media to achieve the equivalent of 1% sucrose sorbitol needed to have equivalent osmolarity of 1% sucrose and a final media volume of 500 mL. 75 mL of all media were pipetted into Greiner BIO-ONE square Petri dishes.

Table 3.1 Volumes of 20% sucrose added to media to achieve desired sucrose concentrations and a final media volume of 500 mL.

DESIRED SUCROSE CONCENTRATION	VOLUME OF 20 % SUCROSE TO ALIQUOT FROM 20% SUCROSE STOCK SOLUTION TO BE ADDED TO MEDIA (FINAL VOLUME: 500 ML)	VOLUME OF MEDIA (FINAL VOLUME:500 ML)
1%	25 mL	475 mL
0.6%	15 mL	485mL
0.3%	7.5 mL	492.5 mL
0%	0	500 mL

3.3.3 Preparation of filter paper strips

Filter paper strips for experiments aimed at dissecting the effect of sucrose supplied to shoot alone on PR growth, LR, LR density, and LRP numbers were soaked in 1 mL of various concentrations of sucrose (0%, 0.3%, 0.6%, and 1%). Filter paper strips for experiments aimed at dissecting the effect of sucrose supplied to root alone on PR growth, LR, LR density, and LRP numbers were soaked in 1 mL 0% sucrose (sterile water). Filter paper strips for experiments aimed at dissecting the effect of sucrose supplied to both the shoot and root on PR growth, LR, LR density, and LRP numbers were soaked in1 mL of various concentrations of sucrose (0%, 0.3%, 0.6%, and 1%). Filter paper strips for experiments aimed at dissecting the effect of increased osmolarity associated with 1% sucrose on PR growth, LR, LR density, and LRP numbers were soaked in 1 mL 0% sucrose solution (sterile water) (Tables 3.1& 3.2), 1% sorbitol and 1% sucrose (Table 3.3).

3.3.4 Molar equivalents of sucrose and sorbitol used in experiments.

Osmolarity = molarity x the number of particles that dissociate in solution.

1% w/v sucrose solution = $1g/100ml$ =0.029 M =29 mM

Osmolarity (1% sucrose) = 29 mosmol/L because sucrose doesn't dissociate in solution.

Molecular weight (sorbitol) = 182.17 g/mol

Molecular weight (sucrose) = 342.2965 g/mol

Sorbitol Mol equivalent mass of sucrose:

1M sorbitol = 182.17 g

1M sucrose = 342.3 g

182.17/342.3 = 0.532

Stock solutions:

20% Sucrose = 200 g/l = 200 $g/342.3g/mol$ =0.58 mols

20% Sorbitol = 200 g/l = 200 $g/182.17g/mol$ = 1.1 mols

Table 3.2 The volumes of sorbitol added to media to obtain equimolar equivalents of 1% sucrose.

SUCROSE CONCENTRATION	VOLUME OF 20 % SUCROSE TO ADD TO MEDIA TO ACHIEVE A FINAL VOLUME OF 500 ML	VOLUME OF 20% SORBITOL ADDED TO MEDIA TO ACHIEVE A FINAL VOLUME OF 500 ML
1%	25 mL = [29 mM]	0
0.6%	15 mL = [17.4 mM]	5.25 mL
0.3%	7.5 mL = [8.9 mM]	9.15 mL
0% (osmolarity control)	0	13.2 mL
0% (negative control)	0	0

Table 3.3 The volume of sorbitol added to sterile water to obtain an equimolar equivalent of 1% sucrose.

SUCROSE CONCENTRATION	VOLUME OF 20% SUCROSE ADDED TO STERILE WATER TO ACHIEVE A FINAL VOLUME OF 1 mL	VOLUME OF 20% SORBITOL ADDED TO STERILE WATER TO ACHIEVE A FINAL VOLUME OF 1 mL
1% sucrose	50 µl= [29 mM]	0
0% (osmolarity control)	0	26.4 µl = [29 mM]
0% (negative control)	0	0

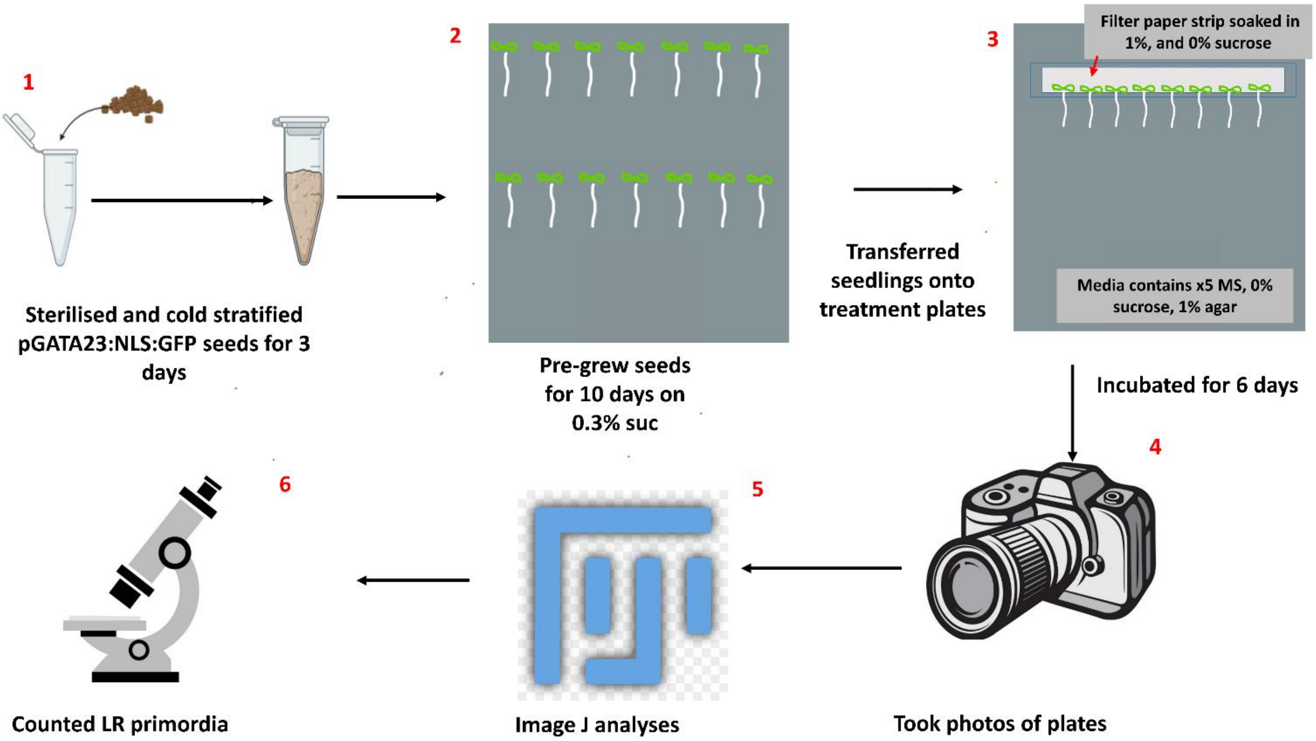


Figure 3.1 **Generic experimental design for the filter paper/Parafilm strip experiments.**

(1) pGATA23::NLS-GFP seedlings were sterilised as described in section 3.3 and cold stratified for 2 days. (2) Seeds were pre-grown on 0.3% sucrose for 10 days, (3) transferred onto various treatments, and incubated for 6 days. (4) Images of plates were taken using a Nikon D60 digital camera. (5) PR lengths were measured using segmented lines tools in Fiji ImageJ. (6) LRs and LRPs were scored using epifluorescence microscopy.

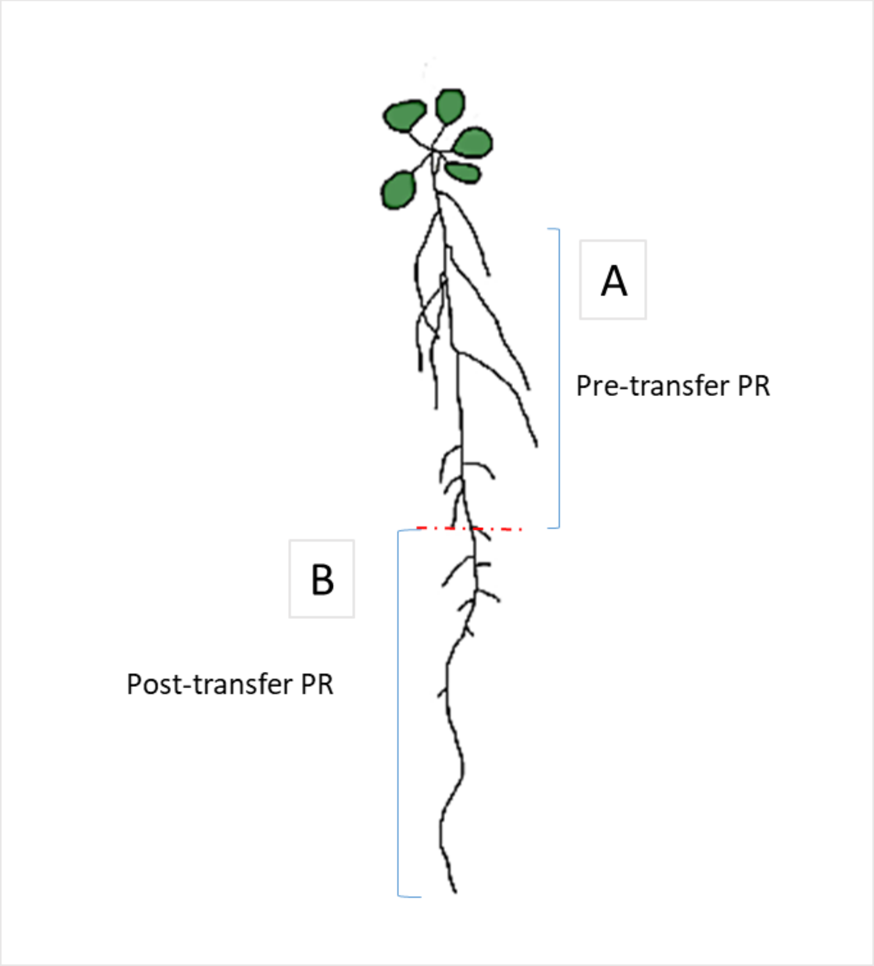


Figure 3.2 **Schematic of section of primary root analysed.**

(A) PR growth after 10 days of pre-growth on 0.3% sucrose—designated as pre-transfer. (B) PR growth after 6 days incubation after transfer—designated as post-transfer PR. This is the section of the root analysed. The post-transfer PR was analysed to determine PR growth and LR numbers; it was also cut (red dashes) from the pre-transfer PR and subjected to epifluorescence microscope to LRPs as described in section 2.7 of chapter 2. Image was adapted from (Xiao et al. 2020).

3.4 Results

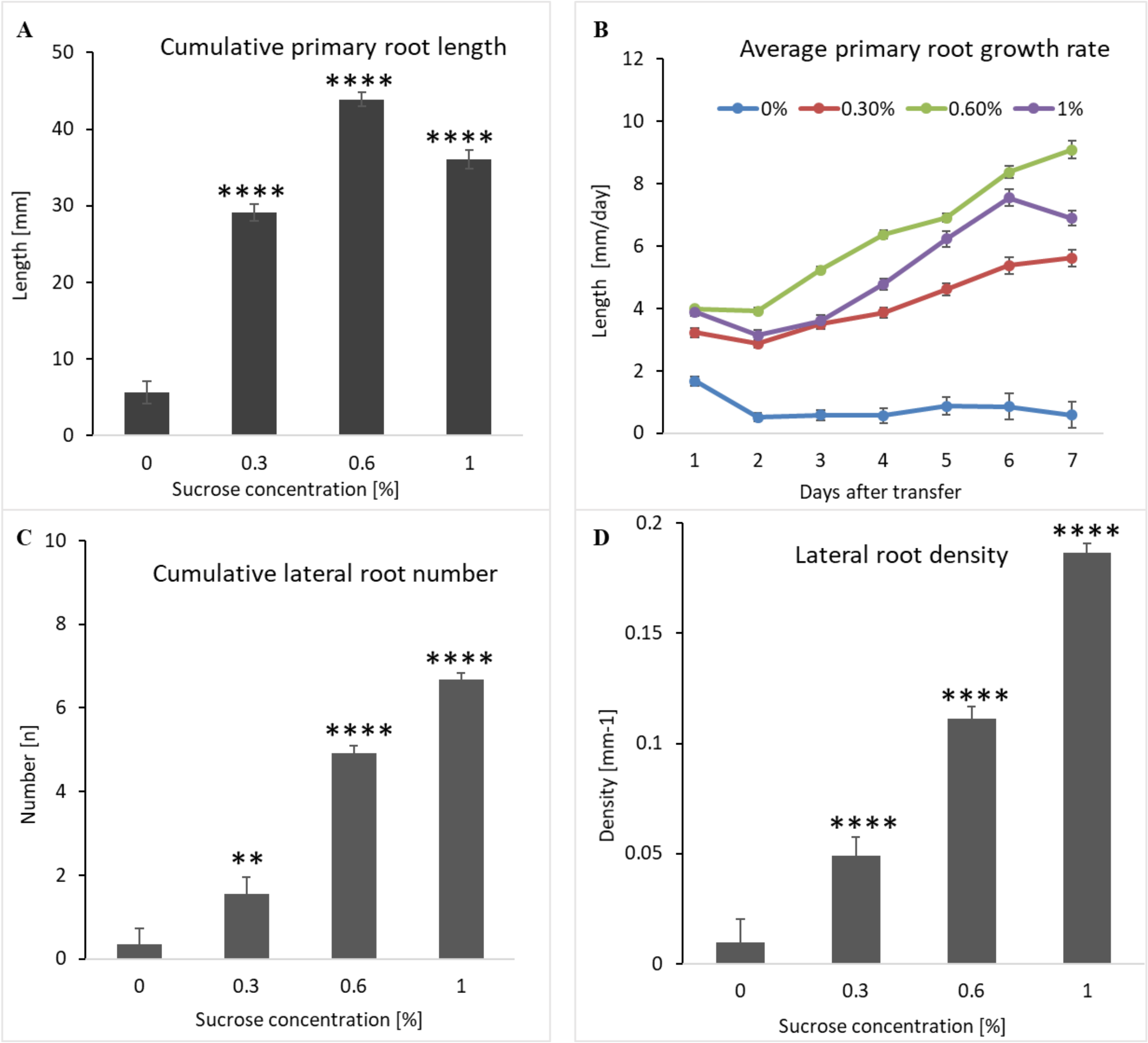
3.4.1 Validation of the effects of exogenous sucrose PR growth and LR outgrowth

To begin to understand how sucrose promotes LR formation, the stimulatory effects of sucrose on LR outgrowth as reported in various studies (Freixes et al., 2002; Takahashi et al., 2003; Macgregor et al. 2008) was validated. To do this, the effects of different sucrose concentrations on PR growth and parameters of LR formation (e.g., LR numbers and densities) as has been reported by some of the studies discussed earlier was studied. 7-day-old Col-0 seedlings were transferred directly onto MS media containing different concentrations of exogenous sucrose (0%, 0.3%, 0.6%, and 1%) and grown for additional 7 days. The various concentrations of exogenous sucrose were chosen to determine whether there was a dose-response relationship between exogenous sucrose and root growth. Plates were oriented vertically to enable visualisation of the roots. The tip of the PR was marked each day and root growth was measured at the end of the experiment.

The results showed that there were significantly higher PR growth rates and PR lengths in seedlings grown on 0.6% sucrose compared with those grown on 1% (Figures 3.3A & 3.3B). Seedlings grown at lower sucrose concentrations (0% and 0.3%) showed lower primary root growth (Figures 3.3A & 3.3B). The effects of exogenous sucrose on LR outgrowth were determined by evaluating the effects of the different concentrations of exogenous sucrose on LR numbers and LR density (LRD). The data shows a significant increase in LR numbers and LRD, with increasing exogenous sucrose concentrations (Figures 3.3C &3.3D). Seedlings grown on 1% sucrose produced higher LR numbers and showed higher LRD than those on 0.6% sucrose and lower sucrose concentrations (0.3% and 0%) had the least effects on LR parameters.

The data shows a dose-responsive relationship between exogenous sucrose and LR outgrowth, a non-linear relationship between exogenous sucrose availability. Taken together, this data validates the stimulatory effect of exogenous sucrose on LR outgrowth (Freixes et al., 2002; Takahashi et al., 2003; Macgregor et al., 2008).

In the experiments presented herein, seedlings were selected for transfer based on PR length (10 –12 mm). Typically, there was no root growth after transfer to 0% sucrose (Figure. 3.3E), however, PR growth was observed in some seedlings on 0% sucrose plates (Figure. 3.3E). The seedlings that had PR growth were observed to have a more developed shoot than those that did not. Suggesting that the seedlings that did not experience PR growth under 0% exogenous sucrose conditions could not synthesise endogenous sucrose during the duration of the experiment. The seedlings that had PR growth under 0% exogenous sucrose conditions were fewer across plates (Figure. 3.3E), suggesting a non-uniform development of the shoot system among seedlings on a plate. Consequently, to ensure uniform development of the shoot among seedlings, and similar response to exogenous sucrose after transfer, seedlings were pre-grown for 10 days for the experiments discussed in subsequent sections. A 10-day pre-growth period was chosen because the most uniform development of shoots under 0% exogenous conditions was found in 10-day-old seedlings.


A
Cumulative primary root length
Length [mm]
50
40
30
20
10
0
****
****
****
0
0.3
0.6
1
Sucrose concentration [%]
B
Average primary root growth rate
0%
0.30%
0.60%
1%
Length [mm/day]
12
10
8
6
4
2
0
1
2
3
4
5
6
7
Days after transfer
C
Cumulative lateral root number
Number [n]
10
8
6
4
2
0
**
****
****
0
0.3
0.6
1
Sucrose concentration [%]
D
Lateral root density
Density [mm-1]
0.2
0.15
0.1
0.05
0
****
****
****
0
0.3
0.6
1
Sucrose concentration [%]

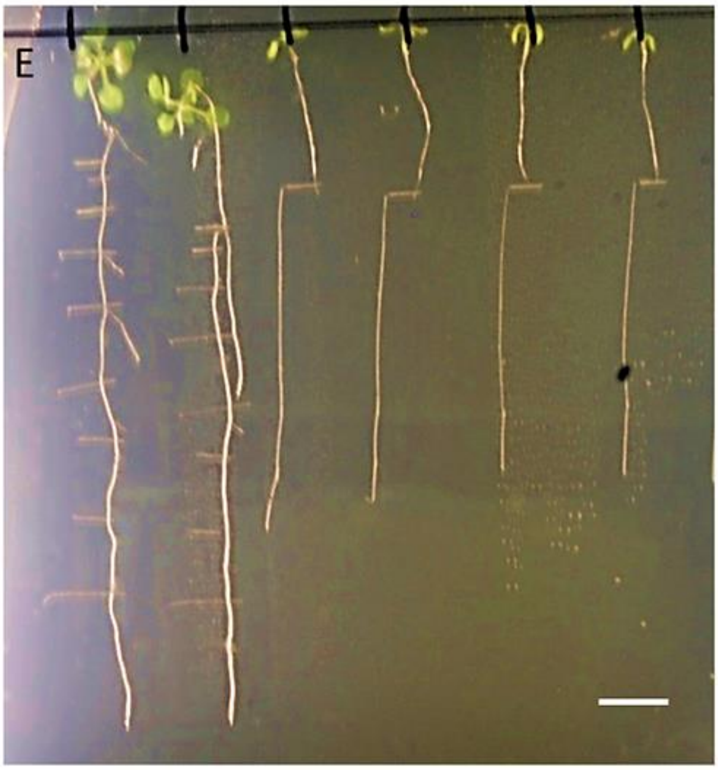


Figure 3.3 **Validation of the effect of exogenous sucrose on PR growth and LR outgrowth**

(A) Increased cumulative PR growth in response to different concentrations of exogenous sucrose. (B) Increased PR growth rate in response to different concentrations of exogenous sucrose. (C) Increased cumulative LR numbers in response to different concentrations of exogenous sucrose. (D). Increased LR densities in response to different concentrations of exogenous sucrose. (E) Variation in root growth on 0% sucrose based on primary leaf primordia. ** $p< 0.01$, ****$p<0.00001$, from 0% sucrose by a two-sided Welch's t-test; $n = 90$. Data were pooled from three independent experiments. Bar is 229 μm.

3.4.2 Exogenous sucrose supplied via shoot promotes LRP progression.

A filter paper /Parafilm strip experimental system that allows exogenous sucrose to be supplied to the shoot by soaking the filter papers in desired concentrations of sucrose was developed (Figure 3.1). This was important to begin to determine the difference between the effect of exogenous sucrose supplied directly to the shoot and the effect of exogenous sucrose supplied to both the shoot and root on LRP formation. This experimental method requires autoclave sterilised filter paper strips to be placed onto ethanol-sterilised strips. Sucrose-soaked filter paper strips are placed on Parafilm strips to prevent sucrose from leaking into the media, thereby defeating the purpose of preventing contact of the root with sucrose via the media. Using this system, various approaches for supplying sucrose to plants were used, sucrose was supplied: (i) directly to shoot with the contact of root with sucrose via the media, (ii) directly to root via media, without contact of the shoot with sucrose (filter paper strips were soaked in 0% sucrose solution), which is a control, and (iii) to both shoot and root, with both shoot and root in contact with sucrose, via the filter paper and media, respectively.

To study the effect of the supply of exogenous sucrose supplied directly to the shoot on LRP formation, various concentrations of exogenous sucrose (0%, 0.3%, 0.6%, and 1%) were supplied directly to the shoots of 10-day-old *pGATA23::NLS-GFP* seedlings. The 0% sucrose or sucrose control was supplied as distilled water, and not as sorbitol or MS salts in this experiment because the effect of osmotic stress on the distribution of LRP stages is a separate question to be answered in subsequent sections of this chapter. Plants were grown for 6 days after transfer and PR growth, LRP numbers and densities, as well as LR numbers and densities, were quantified. LRPs were categorised into early stages (≤ stage 3) and later stages (> stage 3). This categorisation of LRPs into early stages and later stages was to allow for an understanding of whether sucrose might have distinct roles in promoting LRP formation in the early and later stages. This was due to the known biphasic nature of LRP formation that categorises LRPs into early and later stages (Goh et al. 2016; Laskowski et al. 1995). The biphasic concept of LRP formation categorises the process into the early morphogenesis phase and the late meristem formation phase (Goh et al., 2016), however, this study categorised LRPs into the early LRP initiation stages (stages 0 to 3: ≤ stage 3) and later emergence stages (stages 4 to 7: > stage 3). This categorisation was intended to ensure ease of understanding based on data collected on whether sucrose might have separate roles at the initiation and emergence stages. The categorisation of LRPs into early stages has often included stage 4 (Goh et al. 2016), however, in this study, stage 4 LRPs were classified as later stages due to the reasons discussed earlier.

Quantification of LRPs in the post-transfer root (Figure 3.2) revealed that the supply of exogenous sucrose directly to the shoot promotes LRP progression from ≤ stage 3 to > stage 3 (Figures 3.4A & 3.4B). There was a statistically significant decrease in the numbers of ≤ stage 3 LRPs under 1% sucrose conditions, compared to the increase in ≤ stage 3 LRP numbers in the absence of exogenous sucrose (Figure 3.4A). In contrast, there was a statistically significant reduction in the numbers of > stage 3 LRPs under 0% sucrose conditions and a statistically significant increase in the numbers of > stage 3 LRPs in the presence of exogenous sucrose (Figure 3.4B). The distribution of LRPs among the aforementioned stages in response to various concentrations of exogenous sucrose was independent of the length of the PR (Figures 3.4E & 3.4F), indicating a real effect of sucrose treatment on LRP morphogenesis. Taken together, this data suggests that the supply of exogenous sucrose via the shoot promotes the progression of LRPs from ≤ stage 3 to > stage 3.

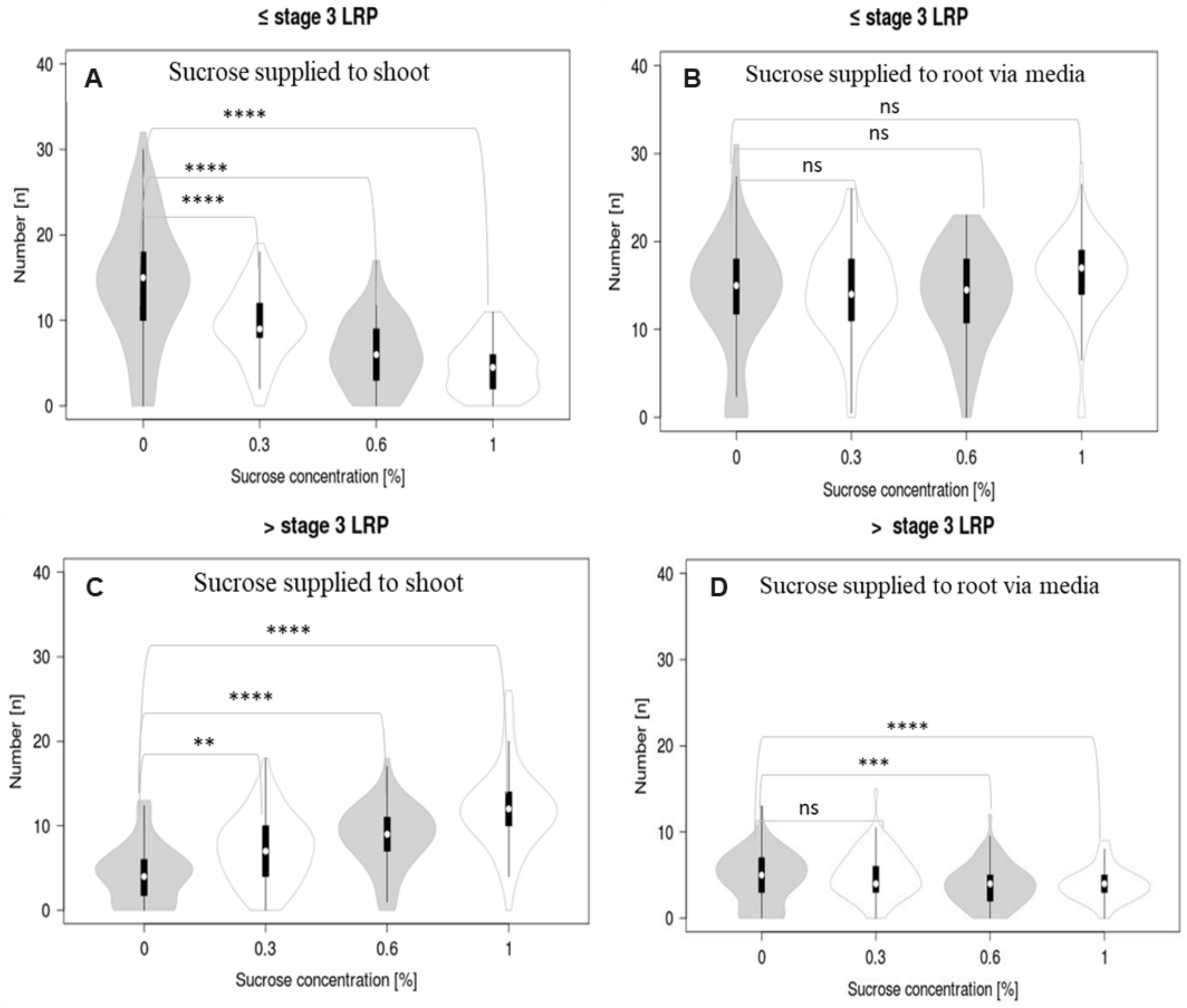
As a control, the same concentrations of exogenous sucrose as previously described (0%, 0.3%, 0.6%, and 1%) were supplied to the root via the MS agar media, with the shoot in contact with filter paper soaked in distilled water. This was done to ensure the shoot was treated similarly to experimental conditions where exogenous sucrose was supplied directly to the shoot. The data revealed that there were higher numbers of ≤ stage 3 LRPs in all exogenous sucrose treatments (0%, 0.3%, 0.6%, and 1%) with no statistically significant differences among treatments (Figure 3.4C). Additionally, there were lower numbers of > stage 3 LRPs in all exogenous sucrose treatments (Figure 3.4D). Again, the distribution of LRPs among the aforementioned stages was independent of the length of the PR (Figures 3.4G & 3.4H). Taken together, these data suggest a reduction in LRP progression.

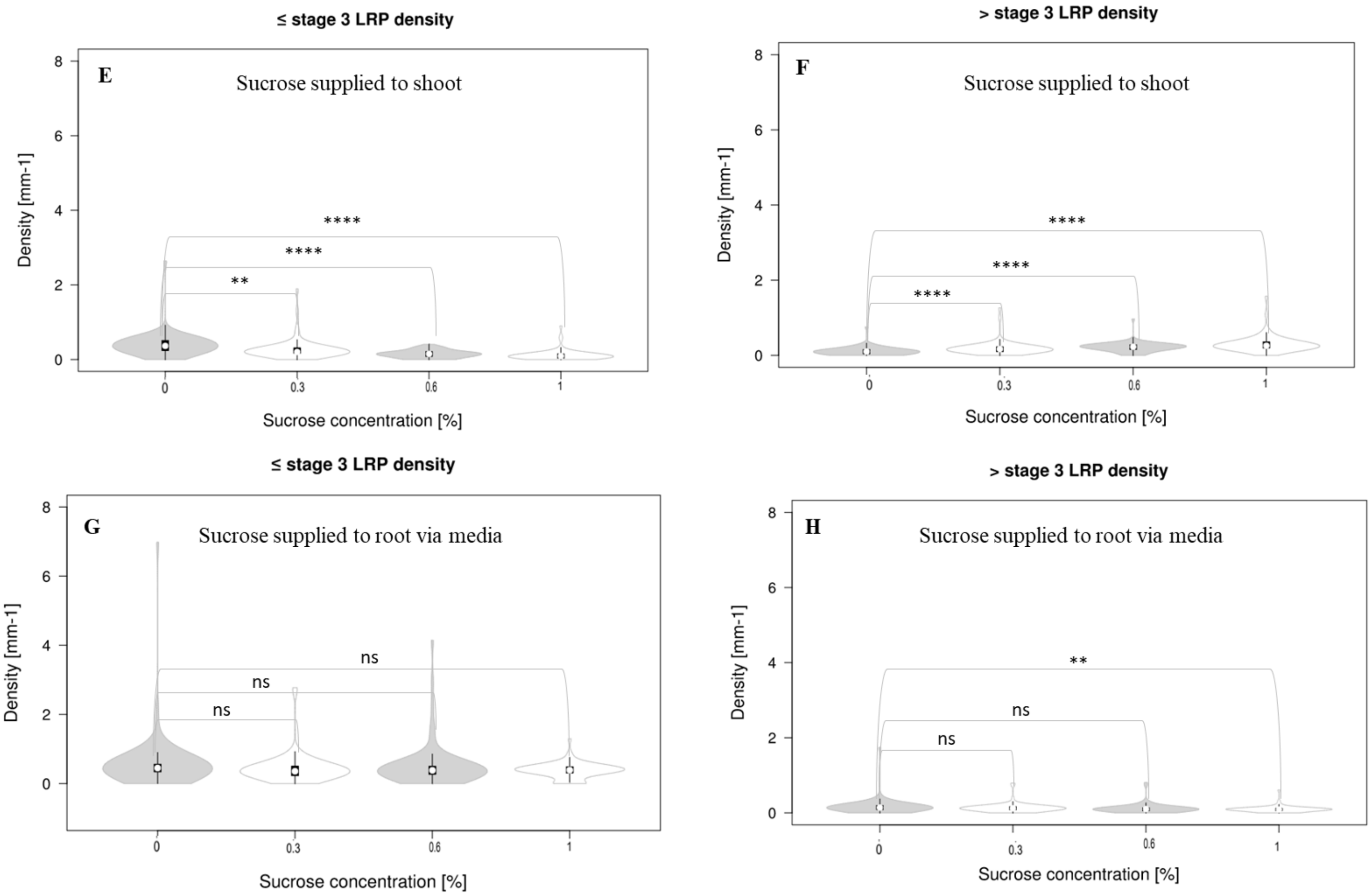
To supply exogenous sucrose to both, shoot and root, similar concentrations of exogenous sucrose as mentioned earlier were used. The experimental setup was as described earlier in this section. Plants were grown for 6 days, and the effect of exogenous sucrose was determined.

The data revealed a high accumulation of LRPs in ≤ stage 3 under all exogenous sucrose conditions (Figure 3.4I), although the statistical analyses show a statistically significant difference in LRP distribution among ≤ stage 3 under 0% and 1% exogenous sucrose conditions, an almost 3-fold reduction in > stage 3 LRPs compared to numbers of ≤ stage 3 (Figures 3.4I & 3.4J) under all exogenous sucrose conditions suggest a reduction in LRP progression from early stages to later stages. This is similar to the LRP progression phenotype under conditions where exogenous sucrose was supplied to the root alone (Figures 3.4C & 3.4D). Again, the observed reduction in LRP progression from early stages to later stages was independent of PR length (Figures 3.4K & 3.4L). Taken together, this data suggests that exogenous sucrose must be supplied directly to the shoot under *in vitro* conditions to observe a statistically significant promotion of LRP progression from early stages to later stages and that the uptake of sucrose, and potentially its transport to the root promotes the developmental progression of LRPs. This could be due to the transport of a shoot-derived factor that promotes the developmental progression of LRPs.

In addition, the representation of lateral root organs (LRs and LRPs) in stacked column graphs showed their distribution under the 3 different approaches for supplying sucrose (Figures 3.5A, 3.5B & 3.5C). The distribution of the lateral root organ numbers showed that percentages of > stage 3 LRPs were almost two-fold lower for 0% and 0.3% treatments under conditions when exogenous sucrose was supplied to both shoot and root than when exogenous sucrose was supplied to only the root (Figure 3.5C vs 3.5B), this observation coupled with higher numbers of ≤ stage 3 LRPs under the former conditions, compared to the latter shows that reduction in LRP progression was more pronounced under conditions where sucrose was supplied to both shoot and root than under condition where sucrose was supplied to the root only.

This could be due to some stress to the plants due to sucrose in the filter paper, as well as in the media, thus reducing the developmental progression of LRPs or just some other contributing factor that is not immediately evident from the data presented herein. That notwithstanding, the need for exogenous sucrose to be supplied directly to the shoot, without a supply to the root in *in vitro* studies of LRP development is glaring to study from the data presented herein, especially due to the reproducibility of results presented herein (datasets were pooled from 3 independent experiments).


≤ stage 3 LRP
A
Sucrose supplied to shoot
****
****
****
Number [n]
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
≤ stage 3 LRP
B
Sucrose supplied to root via media
ns
ns
ns
Number [n]
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
> stage 3 LRP
C
Sucrose supplied to shoot
****
****
**
Number [n]
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
> stage 3 LRP
D
Sucrose supplied to root via media
****
***
ns
Number [n]
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]


≤ stage 3 LRP density
E
Sucrose supplied to shoot
Density [mm-1]
Sucrose concentration [%]
0
0.3
0.6
1
0
2
4
6
8
****
****
**
> stage 3 LRP density
F
Sucrose supplied to shoot
Density [mm-1]
Sucrose concentration [%]
****
****
****
≤ stage 3 LRP density
G
Sucrose supplied to root via media
Density [mm-1]
Sucrose concentration [%]
ns
ns
ns
> stage 3 LRP density
H
Sucrose supplied to root via media
Density [mm-1]
Sucrose concentration [%]
**
ns
ns

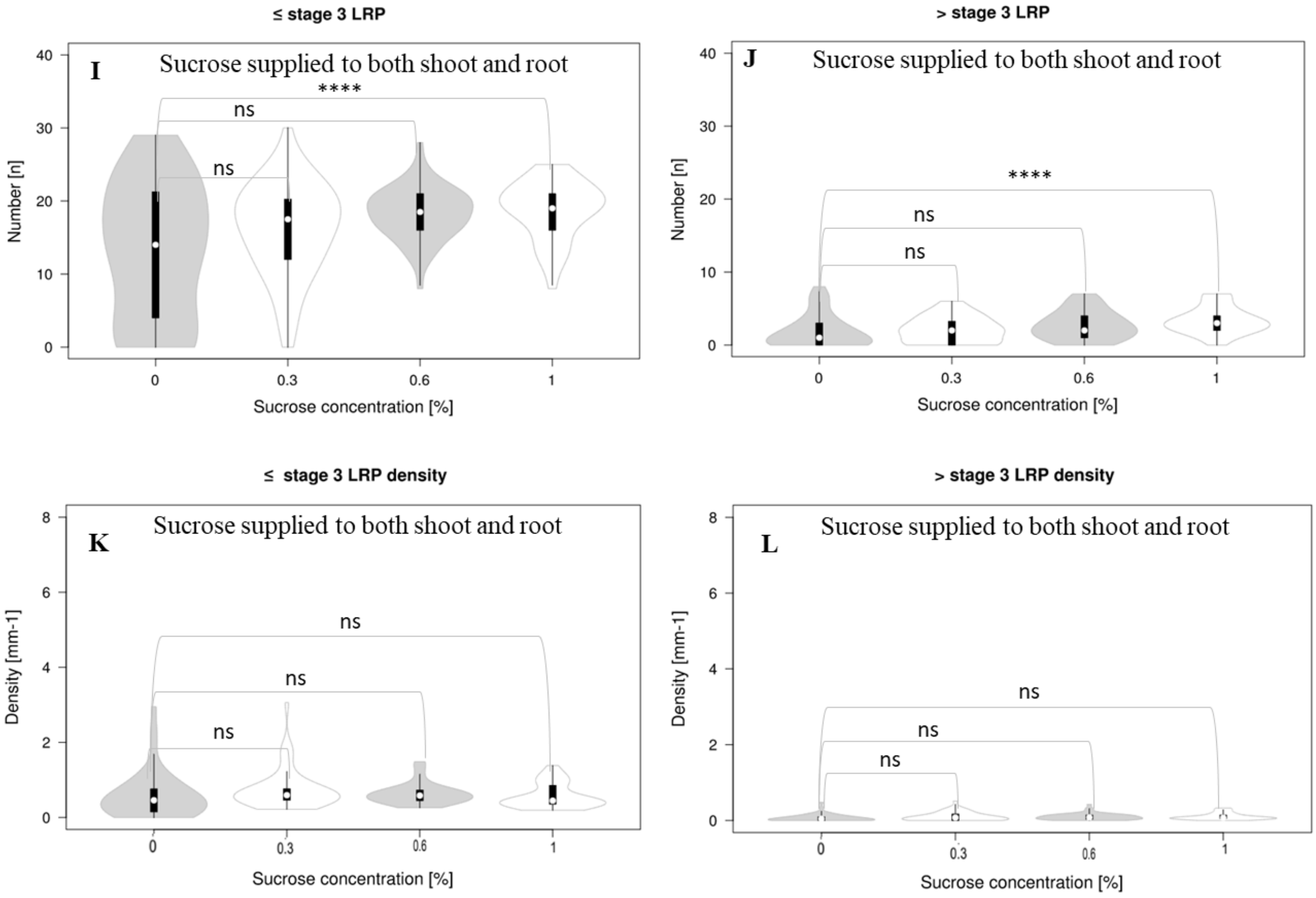
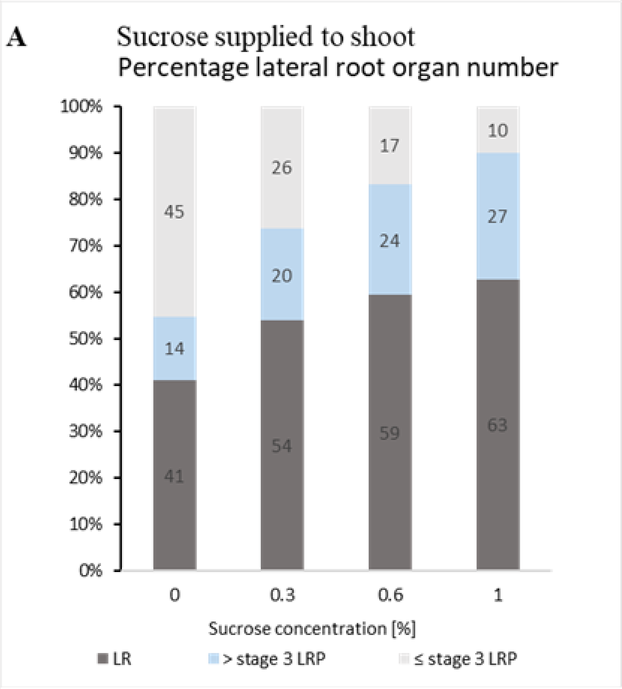
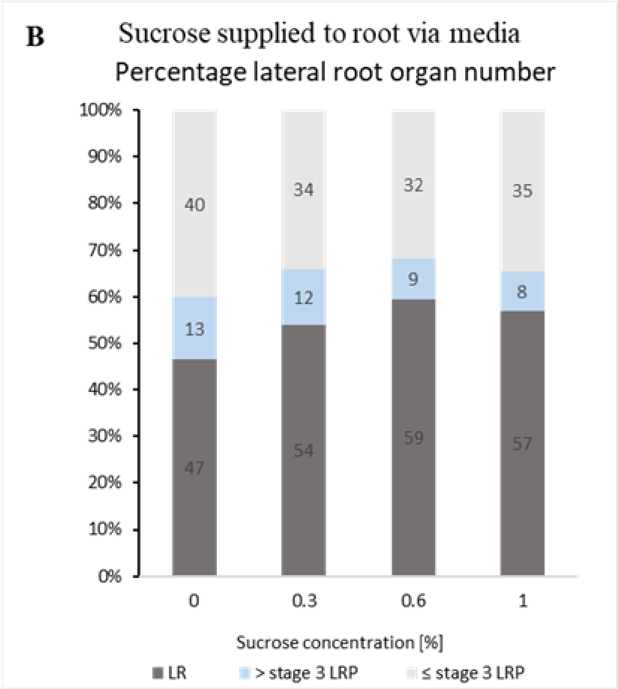


Figure 3.4 **Progression of LRPs is dependent on how sucrose is supplied.**

(A-D) Supply of exogenous sucrose to root only promotes progression of LRPs from early stage (≤ stage 3) to later stage (≥ stage 3). (B) supply of sucrose only to the root leads to the concentration of LRPs in early stages in both the absence and presence of sucrose compared to (A) supply of sucrose directly to the shoot (C) supply of exogenous sucrose directly to the shoot leads to the concentration of LRPs in later stages compared to (D) to the supply pf exogenous sucrose to the root only. (E-F) Supply of exogenous sucrose to shoot increases stage indicated LRP densities at the indicated stages. (G-H) The effect of exogenous supplied to root via media on LRP progression from ≤ stage 3 to > stage 3 is independent on the length of the PR. (I-J) supply of exogenous sucrose to both shoot and root reduces the progression of LRPs from early stage (≤ stage 3) to later stage (≥ stage 3). (K-L) The effect of exogenous supplied to both shoot and root via media on LRP progression from ≤ stage 3 to > stage 3 is independent on the length of the PR. ** $p< 0.01$, ****$p<0.00001$, ns >0.05 from 0% sucrose by a two-sided Welch's t-test; n =72 for (Figures A-D). Data were pooled from three independent experiments. n = 24 for each independent experiment. n = 48 for (Figures E-F); datasets were pooled from 2 independent experiments; n=24 for each independent experiments. Violin represents the distribution of data points from all roots analysed.


A
Sucrose supplied to shoot
Percentage lateral root organ number
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
45
14
41
26
20
54
17
24
59
10
27
63
0
0.3
0.6
1
Sucrose concentration [%]
■ LR
■ > stage 3 LRP
■ ≤ stage 3 LRP


B
Sucrose supplied to root via media
Percentage lateral root organ number
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
40
13
47
34
12
54
32
9
59
35
8
57
0
0.3
0.6
1
Sucrose concentration [%]
■ LR
■ > stage 3 LRP
■ ≤ stage 3 LRP

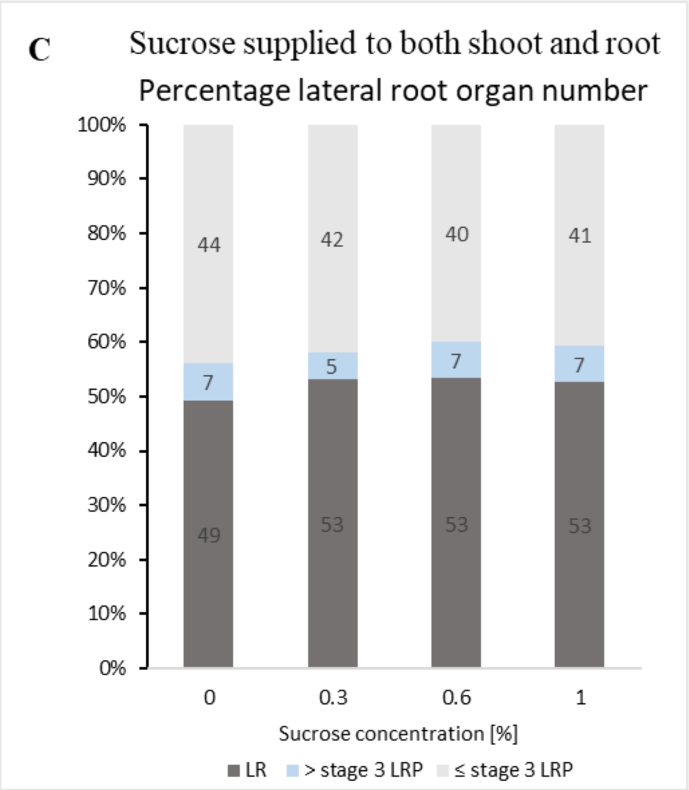
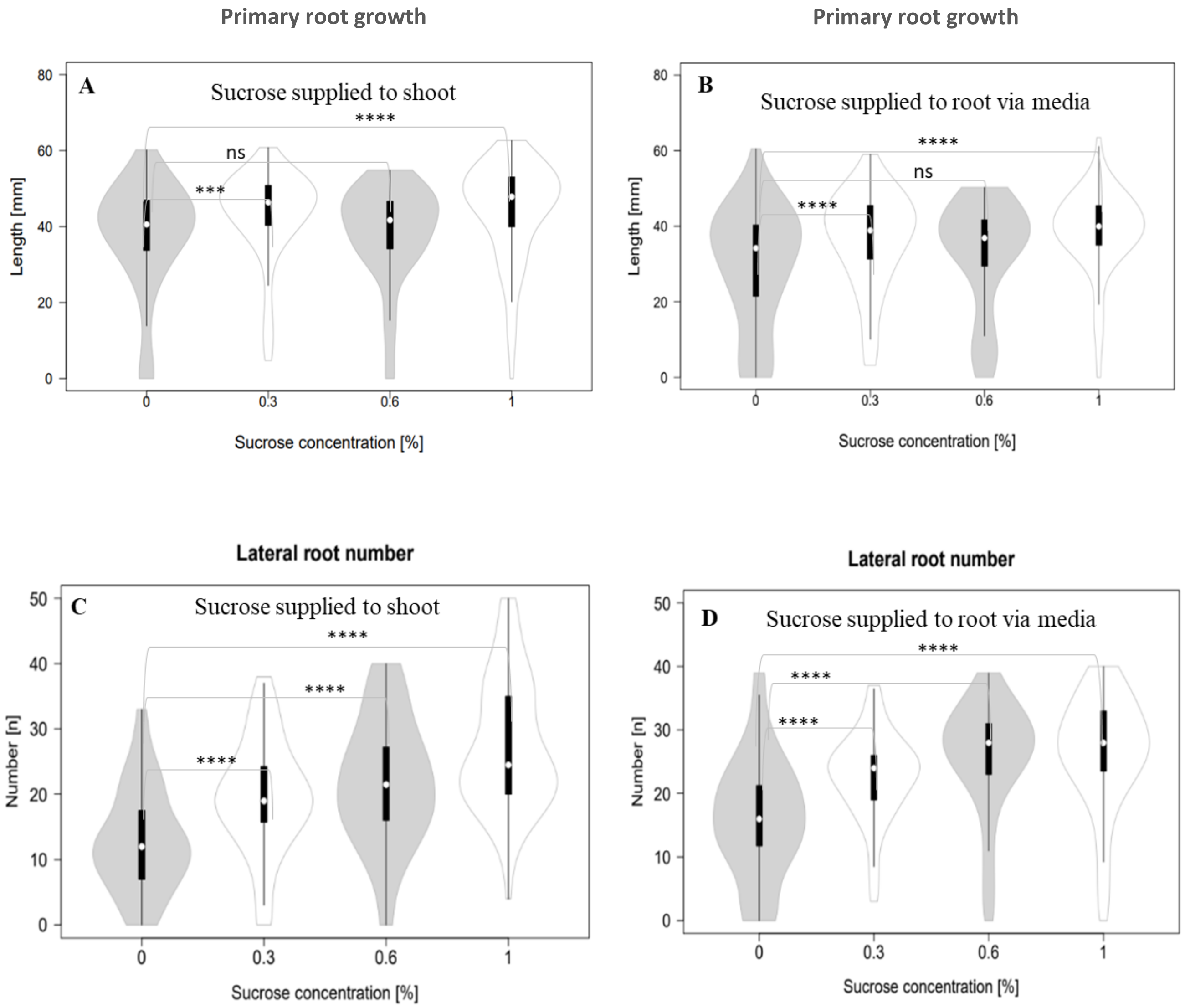

C
Sucrose supplied to both shoot and root
Percentage lateral root organ number
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
44
7
49
42
5
53
40
7
53
41
7
53
0
0.3
0.6
1
Sucrose concentration [%]
■ LR
■ > stage 3 LRP
■ ≤ stage 3 LRP

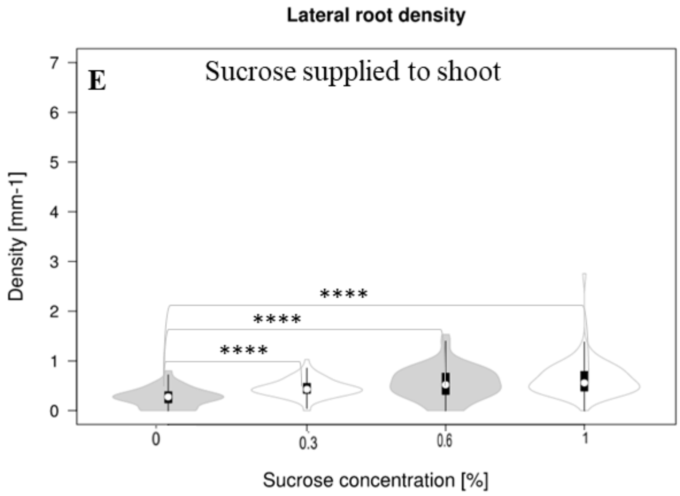
Figure 3.5 **Distribution of lateral root organs under indicated conditions.**

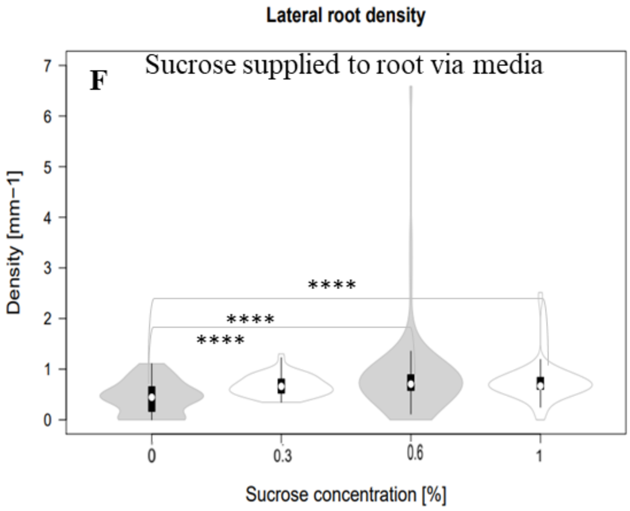
(A) Distribution of lateral root organs under conditions where exogenous sucrose was supplied to shoot (B-C) Numbers of > stage 3 LRPs were almost two-folds lower for 0% and 0.3% treatments under conditions when exogenous sucrose was supplied to both shoot and root (C) than when exogenous sucrose was supplied to only the root. Datasets were pooled from three independent experiments for (Figures 5A & 5B); n = 24 for each independent experiment. Datasets were pooled from 2 independent experiments for (Figure 5C); n=24 for each independent experiments.

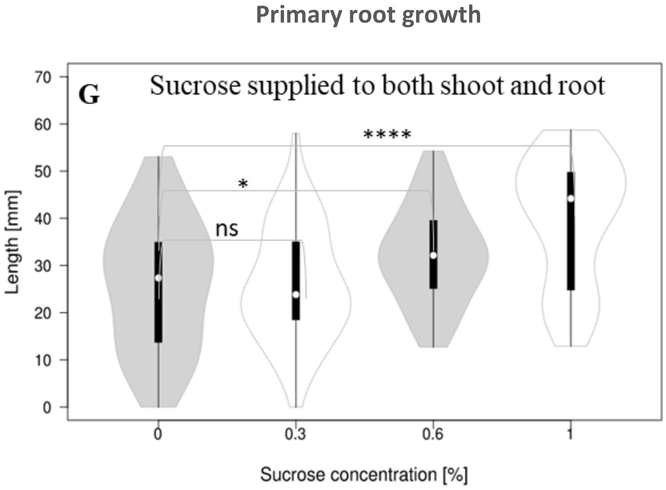
3.4.3 PR growth and LR outgrowth are independent of how sucrose is supplied to plants under *in vitro* conditions.

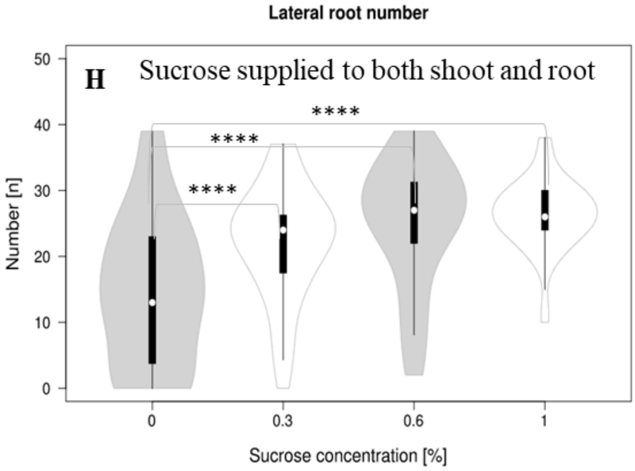
There were in addition surprising increases in PR lengths, LR number, and density from the same experiment as described earlier in this section 3.4.2 in the presence of different concentrations of exogenous sucrose compared to the absence of exogenous sucrose supplied directly to shoot (Figures 3.6A, 3.6C & 3.6E) and directly to root via media (Figures 3.6B, 3.6D & 3.6F). Although there were increases in PR lengths and LR numbers in the presence of exogenous sucrose supplied to both shoot and root (Figures 3.6G & 3.6H), there were no statistically significant differences in LR density among sucrose treatment (Figure 3.6I). Although uptake of sucrose by roots does not occur under soil conditions, this data suggests that PR growth and LR outgrowth are independent of how exogenous sucrose is supplied under *in vitro* conditions, indicating a potentially different pathways for the regulation of LR outgrowth by sucrose. Such a mechanism of regulation cannot be predicted based on the data predicted in this section.


Primary root growth
A
Sucrose supplied to shoot
Length [mm]
0
20
40
60
80
****
ns
***
0
0.3
0.6
1
Sucrose concentration [%]
Primary root growth
B
Sucrose supplied to root via media
Length [mm]
****
ns
****
Sucrose concentration [%]
Lateral root number
C
Sucrose supplied to shoot
Number [n]
50
40
30
20
10
0
****
****
****
Sucrose concentration [%]
Lateral root number
D
Sucrose supplied to root via media
Number [n]
****
****
****
Sucrose concentration [%]


Lateral root density
E
Sucrose supplied to shoot
Density [mm-1]
7
6
5
4
3
2
1
0
****
****
****
0
0.3
0.6
1
Sucrose concentration [%]


Lateral root density
F
Sucrose supplied to root via media
Density [mm−1]
7
6
5
4
3
2
1
0
****
****
****
0
0.3
0.6
1
Sucrose concentration [%]


Primary root growth
G
Sucrose supplied to both shoot and root
Length [mm]
70
60
50
40
30
20
10
0
****
*
ns
0
0.3
0.6
1
Sucrose concentration [%]


Lateral root number
H
Sucrose supplied to both shoot and root
Number [n]
50
40
30
20
10
0
****
****
****
0
0.3
0.6
1
Sucrose concentration [%]

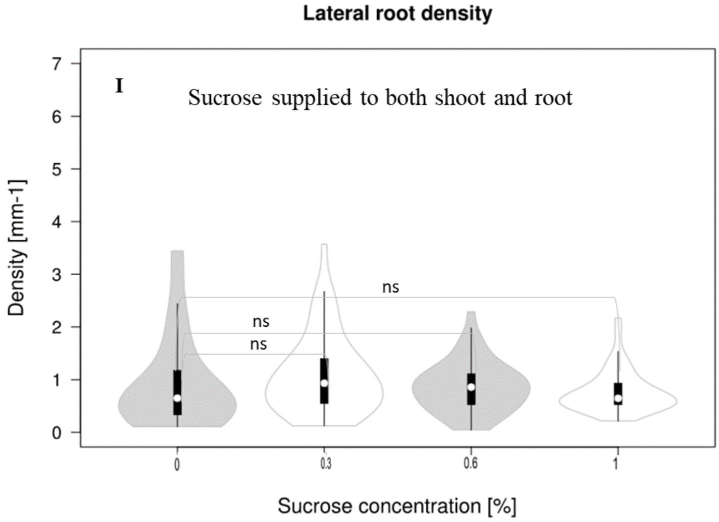

Lateral root density
I
Sucrose supplied to both shoot and root
Density [mm-1]
7
6
5
4
3
2
1
0
ns
ns
ns
0
0.3
0.6
1
Sucrose concentration [%]

Figure 3.6 **Primary root growth and lateral root outgrowth are independent of how sucrose is supplied.**

(A) Primary root growth in 16-day old p*GATA23:NLS-GFP* plants. Exogenous sucrose was supplied to shoot via filter paper.

(B) Primary root growth in in 16-day old *pGATA23: NLS-GFP plants*. Exogenous sucrose was supplied to root via MS agar media.

(C) Lateral root numbers in 16-day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to shoot via filter paper.

(D) Lateral root numbers in 16-day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to root via MS agar media.

(E) Lateral root density in 16-day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to shoot via filter paper.

(F) Lateral root density in 16-day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to root via MS agar media.

(G) Primary root growth in -day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to both shoot (via filter paper) and root (via media).

(H) Lateral root numbers in 16-day old *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to both shoot (via filter paper) and root (via media).

(I) Lateral root density in *pGATA23::NLS-GFP* plants. Exogenous sucrose was supplied to both shoot (via filter paper) and root (via media).

*** $p < 0.001$, ****$p<0.00001$, ns >0.05 from 0% sucrose by a two-sided Welch's t-test; n =72 for (Figures A-F). Data were pooled from three independent experiments. n = 24 for each independent experiment. n=48 for (Figures G-I); datasets were pooled from 2 independent experiments; n=24 for each independent experiments. Violin represents the distribution of data points from all roots analysed.

3.4.4 LRP:LR ratios corroborate the effect of exogenous sucrose supply directly to shoot the developmental progression of LRPs.

Further analyses of the data generated in section 3.4.2 revealed significantly higher LRP: LR ratios in the absence of sucrose under both conditions where exogenous sucrose was supplied to only shoot, and to only root via media (Figures 3.7D & 3.7E). High total LRP numbers were observed in the absence of exogenous sucrose under both conditions, and rightly so as shown in (Figures 3.7A & 3.7B). The total LRP numbers under all exogenous sucrose conditions in the conditions where exogenous sucrose was supplied directly to the root were statistically similar, corroborating the previous conclusion of a reduction in the developmental progression of LRPs under this condition.

This results is quite surprising because LR numbers were significantly different among all sucrose treatments under this condition, with the highest LR numbers observed at the highest exogenous sucrose concentration (Figures 3.5B & 3.6D). With a reduction in LRP progression under this condition, it is expected that LR numbers will be reduced, because LRPs become LRs, and if these LRPs are somewhat arrested at the early stages, then reduced LR numbers should be observed. This very confusing results should have warranted further experimentation, however, the purpose of the developing the filter paper/Parafilm strip experimental system has been achieved by showing that there is a difference in response of LRP formation to the supply exogenous sucrose directly to the shoot, without contact of the root with sucrose and directly to the root via the media without contact of the shoot with sucrose. Therefore, further investigation was not done, as the method of supplying exogenous to the root is unnatural as discussed in section 3.1 of this chapter.

Comparatively, and as expected, there were significantly higher total LRP numbers in the absence of exogenous compared to all other exogenous sucrose conditions in the condition where exogenous sucrose was supplied directly to the shoot without a contact of the root with sucrose via the media (Figure 3.7A). This indicates the progression of LRPs to eventually become LRs in response to exogenous sucrose supply under this condition, and the reduction in the progression of LRPs in the absence of exogenous sucrose as additionally evidenced by lower LR numbers (Figure 3.5A).

Under conditions where exogenous sucrose was supplied to both shoot and root, the highest total LRP numbers were observed in the absence of exogenous sucrose conditions, whereas the lowest total LRP numbers were observed in the presence of exogenous sucrose (Figure 3.7C). This suggests increases in LRP morphogenesis in response to exogenous under this condition, compared to the control to the condition (where exogenous sucrose was supplied directly to the root, without a contact of the shoot with sucrose). This increase in LRP numbers did not reflect in their developmental progression from early stages. Again, and confusingly, high LR numbers were observed under this condition in response to exogenous sucrose supply (Figures 3.5C & 3.6H). This approach of supplying exogenous sucrose was not used for subsequent experiments, due to similar reasons as that for the condition where exogenous sucrose was supplied to the root only via the media.

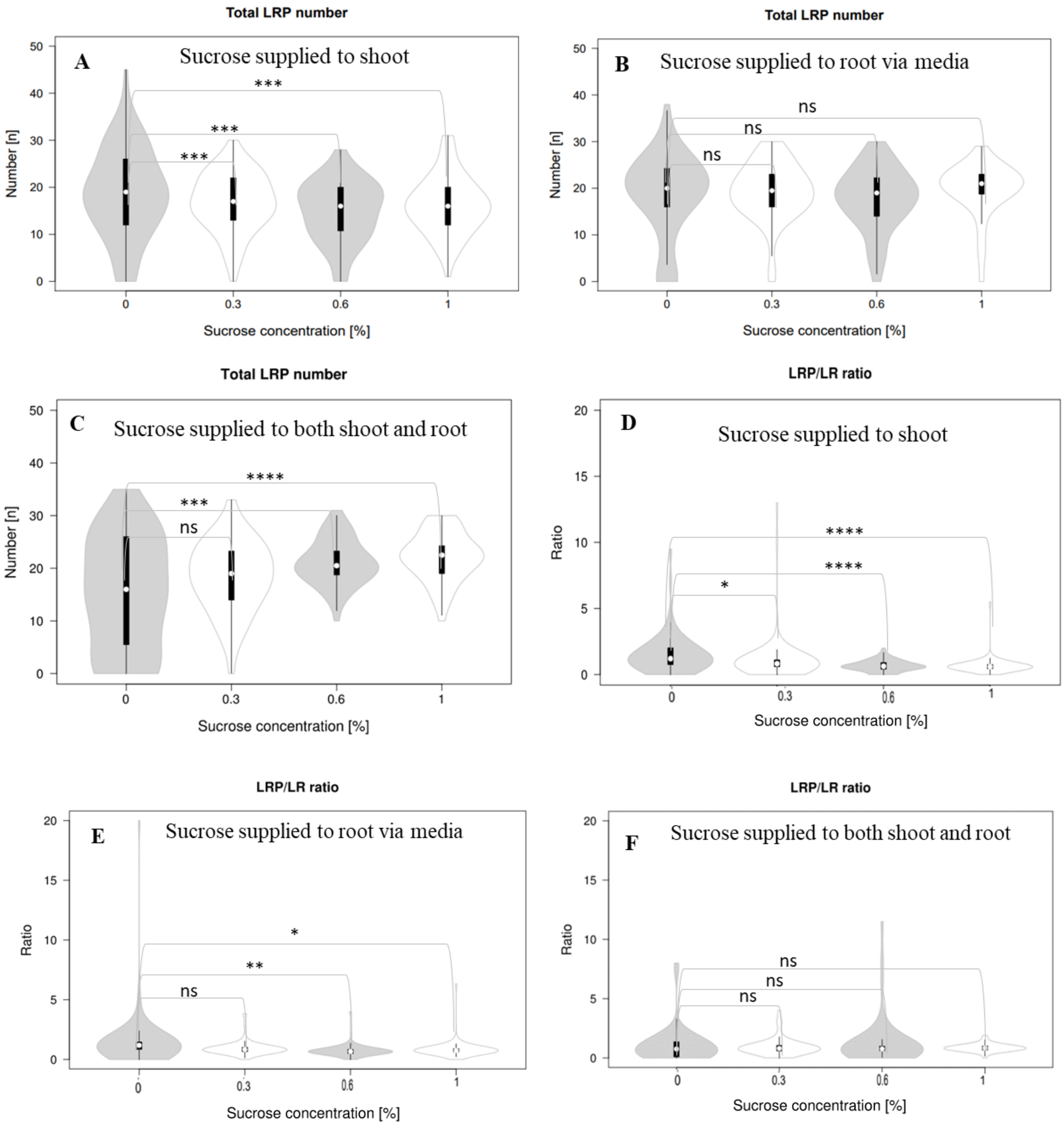

Total LRP number
A
Sucrose supplied to shoot
***
***
***
Number [n]
50
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
Total LRP number
B
Sucrose supplied to root via media
ns
ns
ns
Number [n]
50
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
Total LRP number
C
Sucrose supplied to both shoot and root
****
***
ns
Number [n]
50
40
30
20
10
0
0
0.3
0.6
1
Sucrose concentration [%]
LRP/LR ratio
D
Sucrose supplied to shoot
****
****
*
Ratio
20
15
10
5
0
0
0.3
0.6
1
Sucrose concentration [%]
LRP/LR ratio
E
Sucrose supplied to root via media
*
**
ns
Ratio
20
15
10
5
0
0
0.3
0.6
1
Sucrose concentration [%]
LRP/LR ratio
F
Sucrose supplied to both shoot and root
ns
ns
ns
Ratio
20
15
10
5
0
0
0.3
0.6
1
Sucrose concentration [%]

Figure 3.7 **LRP: LR ratios are increased in the absence of exogenous sucrose.**

(A) Total LRP numbers in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to shoot via filter paper. (B) Total LRP numbers in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to root via media. (C) Total LRP numbers in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to both shoot (via filter paper) and root (via media). (D) LRP: LR ratios in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to shoot via filter paper. (E) LRP: LR ratios in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to root via media. (F) LRP: LR ratios in 16-day old pGATA23::NLS-GFP plants. Exogenous sucrose was supplied to both shoot (via filter paper) and root (via media).*p<0.05, **p<0.01, *** p< 0.001, ****p<0.00001, ns >0.05 from 0% sucrose by a two-sided Welch's t-test; n =72 for (Figures A-F). Data were pooled from three independent experiments. n = 24 for each independent experiment. n = 48 for (Figures G-I); datasets were pooled from 2 independent experiments; n = 24 for each independent experiments. Violin represents the distribution of data points from all roots analysed.

3.4.5 Control for the osmotic effect of exogenous sucrose using sorbitol.

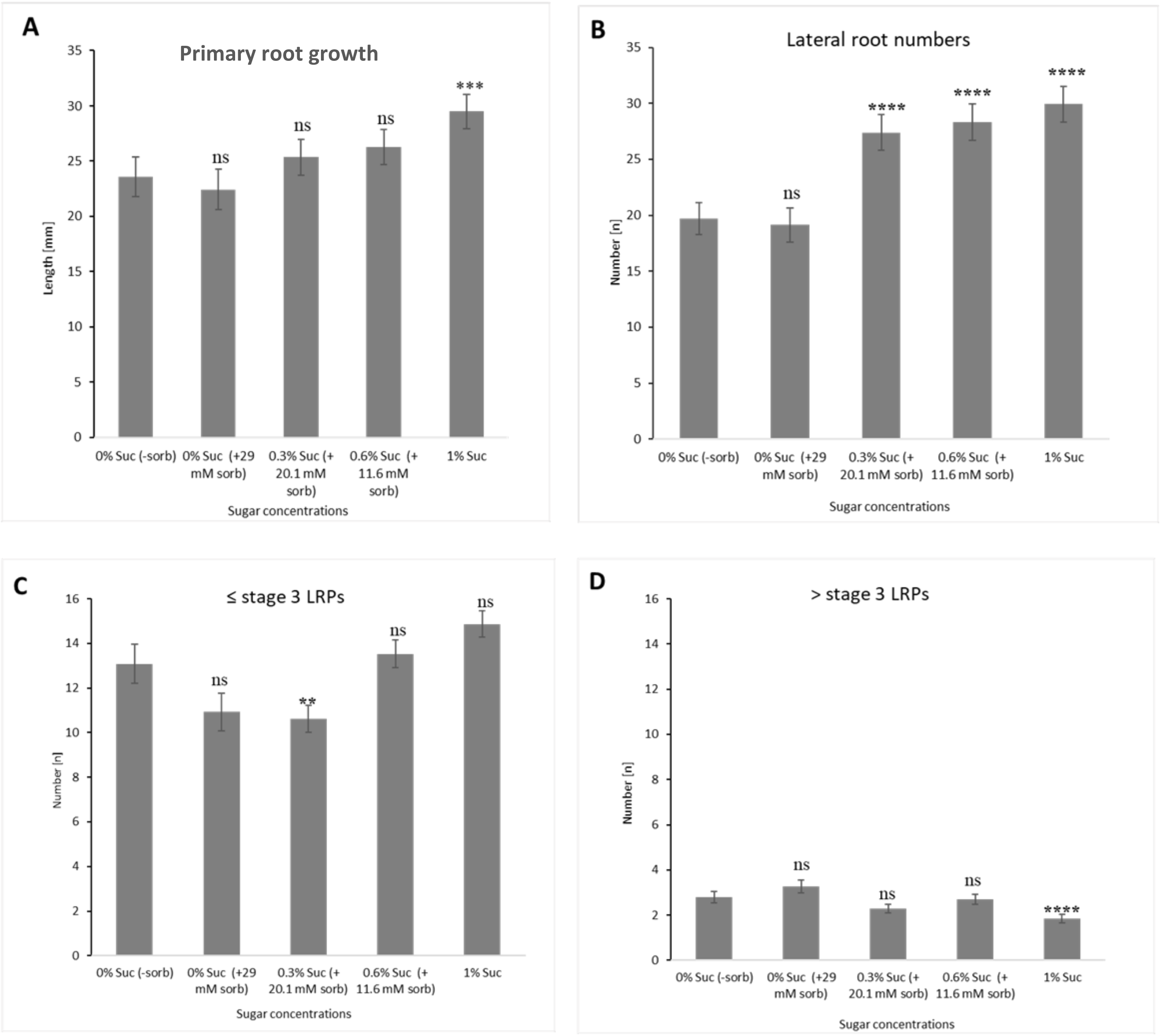
Increasing sucrose concentrations increases osmotic pressure potentially causing osmotic stress (Cui et al., 2010). Osmotic stress in roots has been hypothesised to prevent the uptake of sucrose by the shoot (Macgregor et al., 2008). The inability of the shoot to take up exogenous sucrose from the filter paper or the media would lead to a decreased progression of LRPs from ≤ stage 3 to > stage 3 based on the data in (Figures 3.4A & 3.4B). It was, therefore, the aim of this study to separate the potential osmotic stress effect of sucrose from the known effect on LR formation based on the hypothesis that: supplying an equimolar amount of sorbitol to have the osmolarity of 1% sucrose to root will decrease: (1) LR, and (2) LRP numbers. Sorbitol is a sugar alcohol that can mimic osmotic stress in plants when added to plant growth media by increasing the osmotic pressure in the media, and has been used by various authors to mimic drought stress under *in vitro* conditions (Verslues and Bray 2006). Therefore, adding sorbitol to the plant growth media is expected to stress the plant and limit developmental processes such as the developmental progression of LRPs.

The aforementioned hypothesis was tested under both the experimental methods where exogenous sucrose was supplied to only the shoot via the filter paper and only the root via media. To test this hypothesis, equimolar amounts of sorbitol were added to ½ strength MS agar media containing various concentrations of sucrose (0%, 0.3%, and 0.6%) to achieve the osmolarity of 1% sucrose and a final volume of 500 mL for the experimental method where exogenous sucrose was supplied to the root, while an equimolar amount of sorbitol was added to sterile water to achieve the osmolarity of 1% sucrose and a final volume of 1 mL under the experimental condition where sucrose was to only the shoot. The data revealed that exogenous sucrose still increases LR numbers in the presence of sorbitol (Figures 3.8B & 3.9B) similar to (Figures 3.6C & 3.6D). There were still higher numbers of ≤ stage 3 LRPs compared to > stage 3 LRPs under both 0% and 1% exogenous sucrose conditions in the presence of sorbitol under conditions where exogenous sucrose was supplied only to the root via media (Figures 3.8C & 3.8D) similar to (Figures 3.4C, 3.4D & 3.4H). This observation is independent of the length of the PRs (Figures 3.8E & 3.8F).

There were, however, a more increased PR growth under stress conditions compared to the control (Figures 3.9A), this was surprising because that was not the case under the conditions where sugars were supplied to only the root (Figure 3.8A). Under that condition, reduced PR growth and LR numbers were observed under stress conditions. It is noteworthy that in both approaches of supplying sugars to the plants, no statistically significant differences in PR growth were found (Figure 3.8A & 3.9A). That notwithstanding, normalisation of LR numbers to the PR length under conditions where sugars were supplied to only the shoot revealed that the increased PR growth did not affect LR numbers (3.9G), this was also evidenced by the similar LR numbers under both control and stress condition (3.9B).

There were also higher numbers of > stage 3 LRPs compared to ≤ stage 3 LRPs under 1% sucrose conditions in the presence of sorbitol under conditions where exogenous sucrose was supplied to the shoot (Figures 3.9C & 3.9D) similar to (Figures 3.4A & 3.4B). There was a reduction in the number > stage 3 LRPs and an increase in ≤ stage 3 LRPs in under 0% exogenous sucrose conditions in the presence of sorbitol (Figures 3.9C & 3.9D) similar to (Figures 3.4A & 3.4B). This observation was once again independent of the lengths of the PRs (Figures 3.9E & 3.9F) as was observed under the conditions where exogenous sucrose was supplied to only the root via media.

The distributions of lateral root organs under the conditions where exogenous sucrose was supplied to only the root via media (Figure 3.8H) were also similar to those in (Figure 3.5B). Additionally, the distribution of lateral root organs (Figure 3.9H) under conditions where exogenous sucrose was supplied to the shoot was similar to those in (Figure 3.5A). Taken together, these data suggest that the concentration of sucrose supplied to the root in experiments aimed at dissecting the effect of supplying sucrose to the root alone did not impact the observed effect on LRP progression. Also, the data suggest that the highest exogenous sucrose concentration (1%) used in the study presented in this chapter may not have caused osmotic stress in the plants studied.


A
Primary root growth
Length [mm]
35
30
25
20
15
10
5
0
***
ns
ns
ns
0% Suc (-sorb)
0% Suc (+29 mM sorb)
0.3% Suc (+ 20.1 mM sorb)
0.6% Suc (+ 11.6 mM sorb)
1% Suc
Sugar concentrations
B
Lateral root numbers
Number [n]
35
30
25
20
15
10
5
0
ns
****
****
****
0% Suc (-sorb)
0% Suc (+29 mM sorb)
0.3% Suc (+ 20.1 mM sorb)
0.6% Suc (+ 11.6 mM sorb)
1% Suc
Sugar concentrations
C
≤ stage 3 LRPs
Number [n]
16
14
12
10
8
6
4
2
0
ns
**
ns
ns
0% Suc (-sorb)
0% Suc (+29 mM sorb)
0.3% Suc (+ 20.1 mM sorb)
0.6% Suc (+ 11.6 mM sorb)
1% Suc
Sugar concentrations
D
> stage 3 LRPs
Number [n]
16
14
12
10
8
6
4
2
0
ns
ns
ns
****
0% Suc (-sorb)
0% Suc (+29 mM sorb)
0.3% Suc (+ 20.1 mM sorb)
0.6% Suc (+ 11.6 mM sorb)
1% Suc
Sugar concentrations

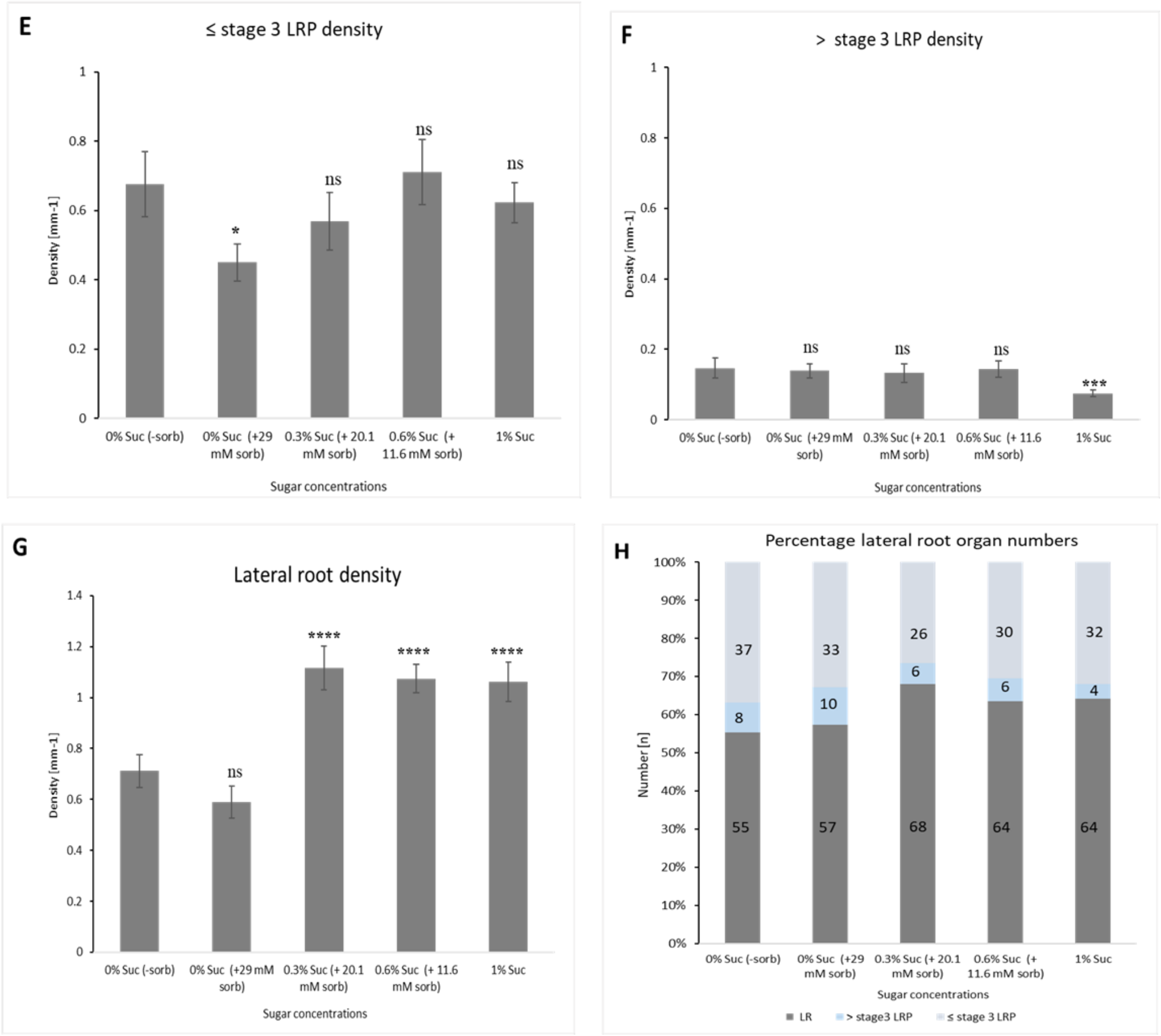
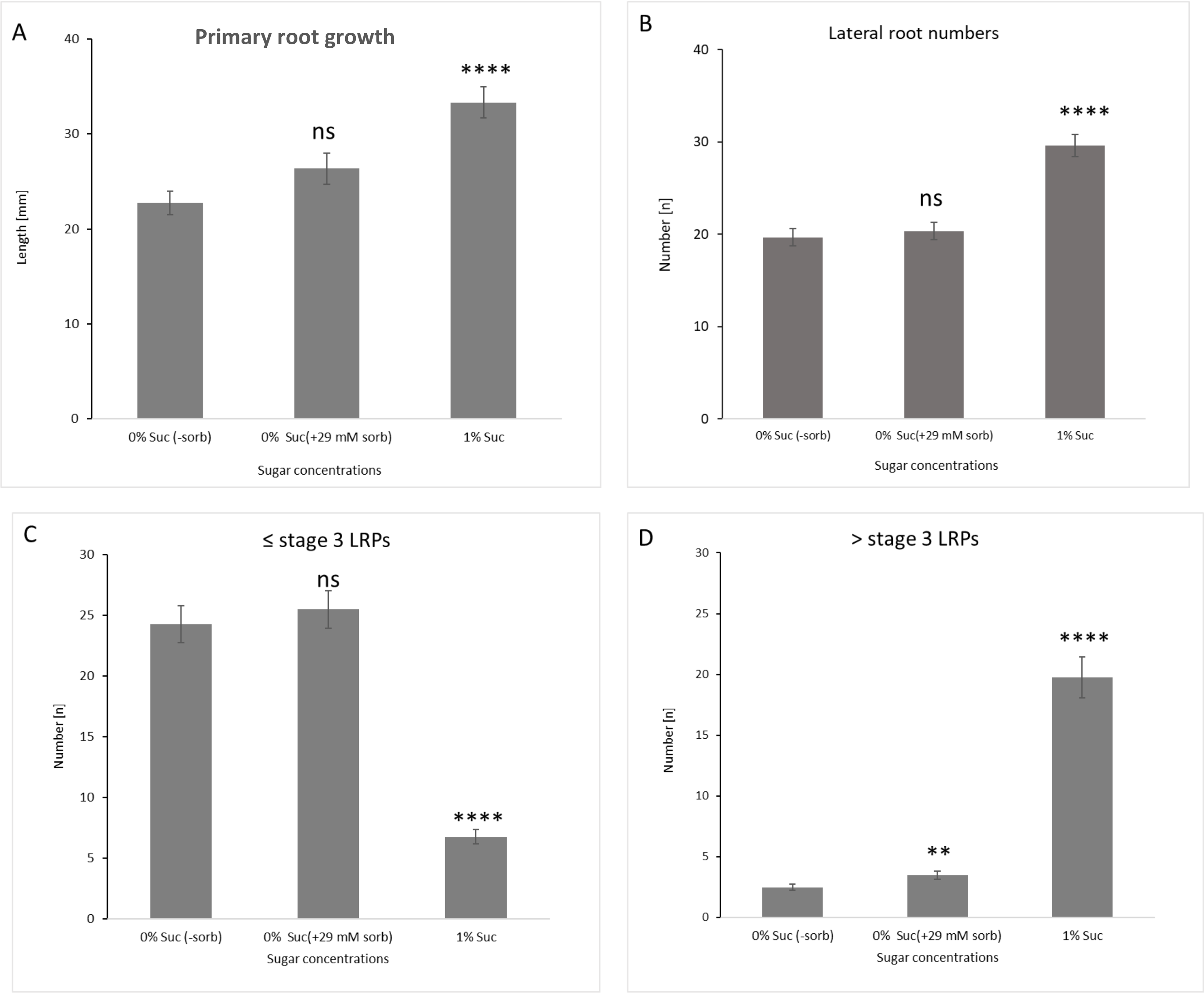


Figure 3.8 **Control for the osmotic effect of sucrose using sorbitol under experimental conditions where exogenous sucrose was supplied to root via media**.

(A) Increased PR growth in the response to sucrose supplied to root via media in the presence of osmotic stress. (B) Increased LR numbers in the response to sucrose supplied to root via media in the presence of osmotic stress. (C) Increased ≤ stage 3 LRP in the response to sucrose supplied to root via media in the presence of osmotic stress. (D) Reduced ≥ stage 3 LRP in the response to sucrose supplied to root via media in the presence of osmotic stress. (E) ≤ stage 3 LRP densities in the response to sucrose supplied to root via media in the presence of osmotic stress. (F) > stage 3 LRP densities in response to sucrose supplied to root via media in the presence of osmotic stress. (G) Increased LR densities in the response to sucrose supplied to root via media in the presence of osmotic stress. (H) Progression of ≤ stage 3 LRP to ≥ stage 3 LRPs is reduced in the presence of osmotic similar to (Figure 5B). * $p < 0.05$,** $p< 0.01$, ****$p<0.00001$, ns >0.05 from 1% sucrose by a two-sided Welch's t-test; n =72. Data were pooled from three independent experiments. n = 24 for each independent experiment.


A
Primary root growth
Length [mm]
0
10
20
30
40
ns
****
0% Suc (-sorb)
0% Suc(+29 mM sorb)
1% Suc
Sugar concentrations
B
Lateral root numbers
Number [n]
0
10
20
30
40
ns
****
0% Suc (-sorb)
0% Suc(+29 mM sorb)
1% Suc
Sugar concentrations
C
≤ stage 3 LRPs
Number [n]
0
5
10
15
20
25
30
ns
****
0% Suc (-sorb)
0% Suc(+29 mM sorb)
1% Suc
Sugar concentrations
D
> stage 3 LRPs
Number [n]
0
5
10
15
20
25
30
**
****
0% Suc (-sorb)
0% Suc(+29 mM sorb)
1% Suc
Sugar concentrations

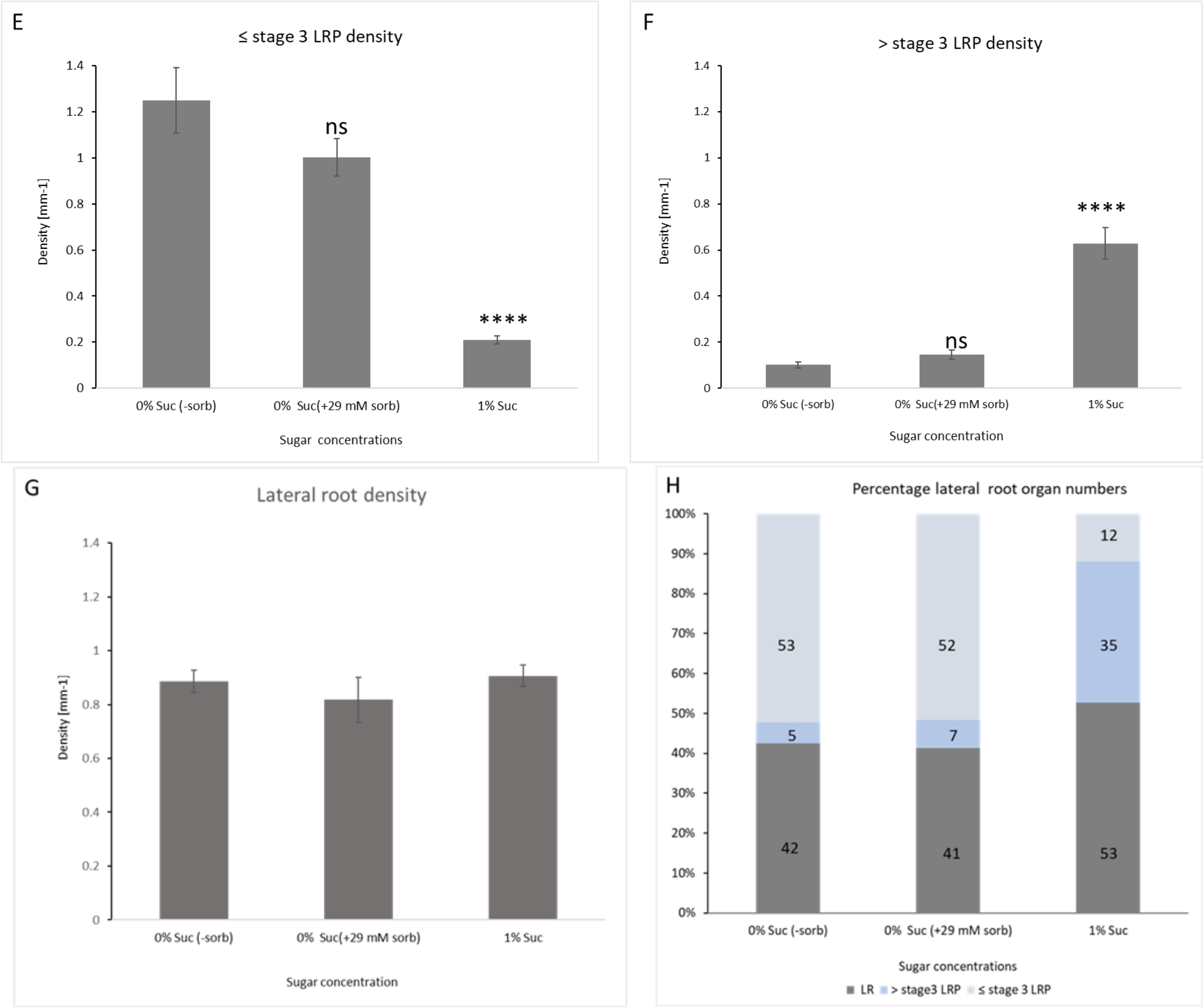


Figure 3.9 **Control for the osmotic effect of exogenous sucrose using sorbitol under experimental conditions where exogenous sucrose was supplied to the shoot only.**

A) Increased PR growth in the response to sucrose supplied to shoot in the presence of osmotic stress. (B) Increased LR numbers in the response to sucrose supplied to shoot in the presence of osmotic stress. (C) Decreased ≤ stage 3 LRP in the response to sucrose supplied to shoot in the presence of osmotic stress. (D) Increased > stage 3 LRP in the response to sucrose supplied to shoot in the presence of osmotic stress. (E) ≤ stage 3 LRP densities in the presence of osmotic stress. (F) Increased > stage 3 LRP densities in response to sucrose supplied to shoot in the presence of osmotic stress. (G) (H) Progression of LRPs from stage ≤3 to stage >3 is increased in the presence of osmotic similar to (Figures 3.4A, 3.4B & 3.4G). * p < 0.05,** p< 0.01, ****p<0.00001, ns >0.05 from 1% sucrose by a two-sided Welch's t-test; n =72. Data were pooled from three independent experiments. n = 24 for each independent experiment.

3.4.6 Progression of LRPs from stage 1 to later stages is reduced in the absence of exogenous sucrose.

The observation that the supply of sucrose to shoot promotes the progression of LRPs from ≤ stage 3 to > stage 3 led to experimental objective to determine the distribution of LRPs among the early stages (≤ stage 3) in response to exogenous sucrose supplied to the shoot. The distribution of LRPs among ≤ stage 3 in response to exogenous sucrose supplied via shoot may suggest from which stage sucrose may be acting to promote the progression of LRPs from the early stages to the later stages. To determine the distribution of early-stage LRPs in response to exogenous sucrose, 0%, and 1% sucrose were supplied to the shoot of 10-day-old *pGATA23::NLS-GFP* seedlings and grown for 6 days. The data showed higher numbers of stage 0 LRPs in the presence of exogenous sucrose compared to the absence of exogenous sucrose (Figure 3.10D). However, these higher numbers of stage 0 LRPs were due to longer PRs (Figure 3.10A) under 1% sucrose conditions as shown by the lack of statistically significant differences in stage 0 LRP density between 0% and 1% exogenous sucrose (Figure 3.10E).

Interestingly, there were significantly higher numbers of stage 1 LRPs and density under 0% exogenous sucrose conditions compared to 1% exogenous sucrose conditions (Figure 3.10D & 3.10E). Additionally, there were significantly higher numbers of stage >3 LRPs and densities under 1% exogenous sucrose conditions compared to 0% sucrose conditions (Figure 3.10D). This observation was also reflected in higher LR numbers and densities in the presence of 1% exogenous sucrose compared to statistically significant lower LR numbers and densities in the presence of 0% exogenous sucrose (Figures 3.10B & 3.10C). Taken together, these data suggest that the progression of LRPs from stage 1 to later stages is accelerated in the presence of 1% exogenous sucrose.

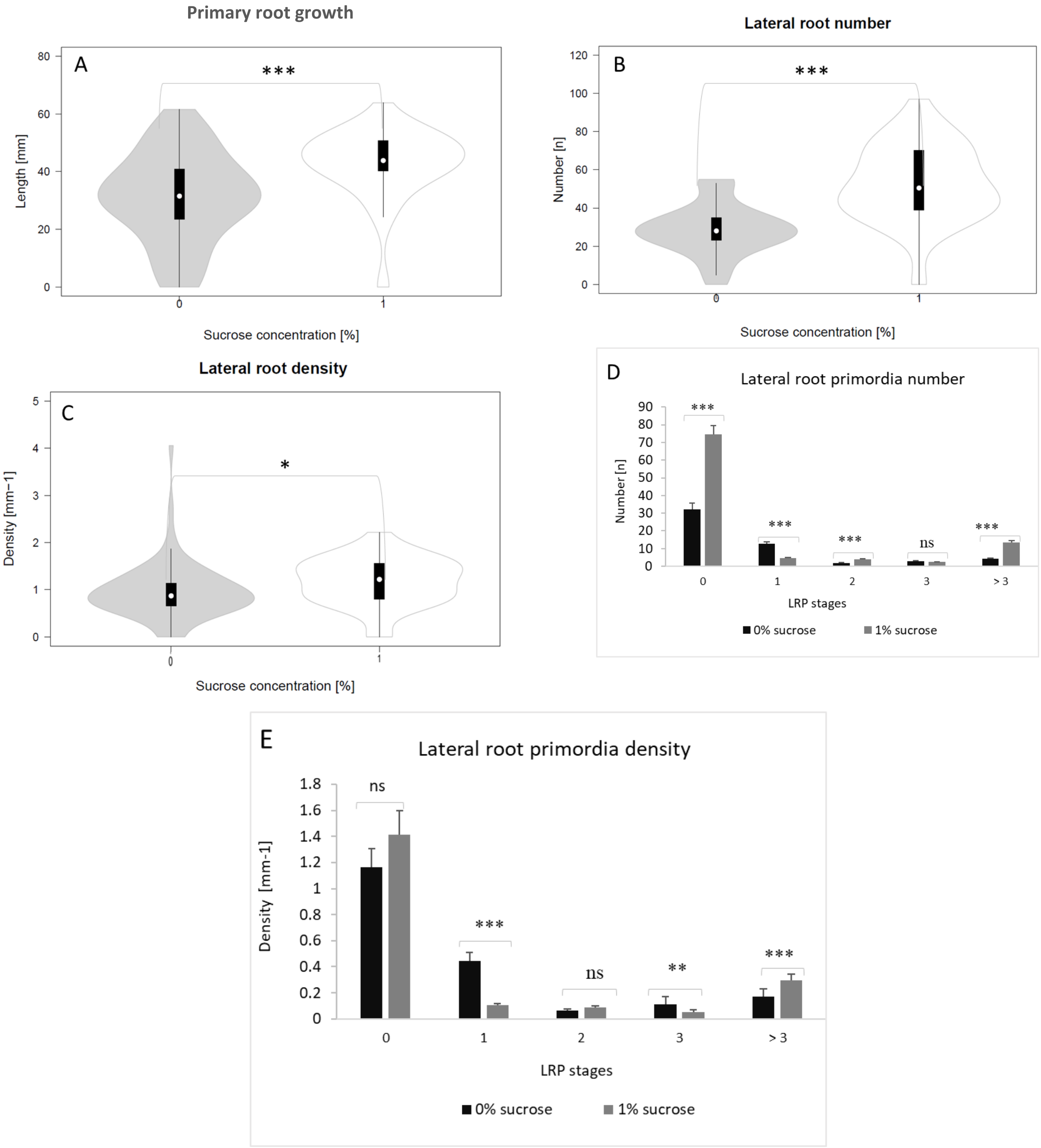

Primary root growth
A
***
Length [mm]
80
60
40
20
0
0
1
Sucrose concentration [%]
Lateral root number
B
***
Number [n]
120
100
80
60
40
20
0
0
1
Sucrose concentration [%]
Lateral root density
C
*
Density [mm−1]
5
4
3
2
1
0
0
1
Sucrose concentration [%]
D
Lateral root primordia number
Number [n]
90
80
70
60
50
40
30
20
10
0
***
***
***
ns
***
0
1
2
3
> 3
LRP stages
■ 0% sucrose
■ 1% sucrose
E
Lateral root primordia density
Density [mm-1]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ns
***
ns
**
***
0
1
2
3
> 3
LRP stages
■ 0% sucrose
■ 1% sucrose

Figure 3.10 **Progression of LRPs slowdown in the absence of sucrose.**

(A) Primary root growth in response to exogenous sucrose supplied to shoot. (B) Lateral root numbers in response to exogenous sucrose supplied to shoot. (C) Lateral root density in response to exogenous sucrose supplied to shoot. (D) Lateral root primordia numbers at the indicated stages in response to exogenous sucrose supplied via the shoot. (E) Lateral root primordia density at the indicated stages in response to exogenous sucrose supplied via the shoot. Error bars represent means ± standard error of the mean (SEM). **$p < 0.01$, ***$p < 0.001$, ns $p > 0.05$ by a two-sided Welch t-test; 0% sucrose, n = 72, 1% sucrose, n = 36. Data were pooled from 3 independent experiments. 0% exogenous sucrose, n = 24 for each independent experiment; 1% exogenous sucrose, n = 12 for each independent experiment.

3.5 Discussion

The *pGATA23::NLS-GFP* reporter line allowed stage 0 LRPs to be scored in the study presented in this chapter. The growth behaviour of the *pGATA23::NLS-GFP* plants was significantly different from the Col-0 in the absence of exogenous (Figure. 3.11) and significantly similar in the presence of exogenous sucrose (Figure 3.11). There was a lower PR growth and LR emergence and outgrowth (as determined by LR numbers (Figure 3.11B) and LR density (Figure. 3.11C) in *pGATA23::NLS-GFP* plants in the absence of exogenous sucrose and higher growth behaviour in the presence of exogenous sucrose (Figure 3.11). It is, however, not clear from this data why the growth behaviour of *pGATA23::NLS-GFP* plants is different from Col-0 in the absence of exogenous sucrose. However, the data suggest that the pattern of accelerated LRP progression and distribution of LRPs among early and later stages in response to exogenous sucrose supply may be the same in both the wild type Col-0 and *pGATA23::NLS-GFP* reporter line. This was, however not tested due to the purpose to use a GATA23-based reporter line to study LRP formation in this study. In future studies, the distribution of LRPs among developmental stages in response to exogenous sucrose could be compared between Col-0 plants and *pGATA23::NLS-GFP* plants.

The increase in LR numbers, LR density, and LRP numbers in response to exogenous sucrose as reported in this chapter (Figures 3.3 & 3.6) suggest a stimulatory role of sucrose in LRP morphogenesis. The data presented in this chapter suggest that exogenous sucrose stimulates LRP morphogenesis by promoting the developmental progression of LRPs. And that this stimulatory effect of exogenous sucrose is dependent on the supply and uptake of exogenous sucrose by the shoot. In this study, the supply of exogenous sucrose to the root via MS agar media did not produce the same stimulatory effect on the progression of LRPs as the supply of exogenous sucrose via the shoot. It is noteworthy that in this study, there was no knockout or knockdown of sucrose synthesis, transport, and/or hydrolysis in any of the plants used in this study. Such an approach would have either reduced or blocked the availability of endogenous sucrose to the root thereby decreasing the developmental progression of LRPs even in the presence of 1% exogenous sucrose.

It is, therefore, likely that there was a supply of endogenous sucrose to plants under both 0% and 1% exogenous sucrose conditions. Consequently, the developmental progression of LRPs was not blocked under 0% sucrose conditions. Additionally, the reduction in the LRP progression in roots under 0% sucrose conditions suggests there may be a reduction in LRP progression when one source of sucrose (either endogenous or exogenous) is ‘cut-off’.

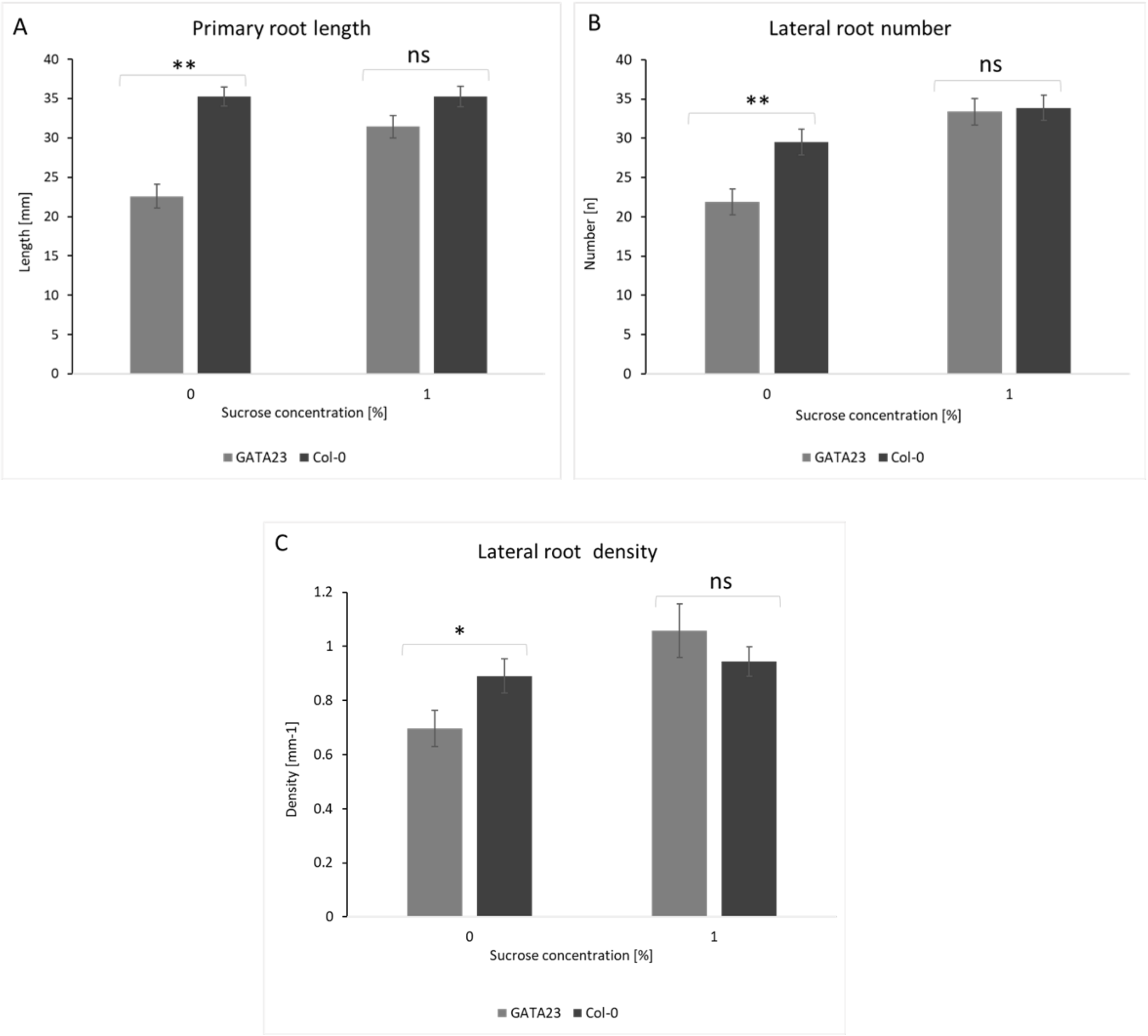


Figure 3.11 **Comparison between the growth behaviour of *pGATA23::NLS-GFP* and wild type Col-0 in response to exogenous sucrose.**

(A) Differences between PR growth of 16-day-old *pGATA23::NLS-GFP* and Col-0 plants in the presence and absence of exogenous.

(B) Differences between LR numbers in 16-day-old *pGATA23::NLS-GFP* and Col-0 plants in the presence and absence of exogenous.

(C) Differences between LR density in 16-day-old *pGATA23::NLS-GFP* and Col-0 plants in the presence and absence of exogenous.

Since endogenous sucrose and exogenous sucrose supplies were neither concurrently knocked-down nor knocked out in this thesis, to determine the downstream effect on LRP formation, it is, therefore, impossible to conclude from the data shown in this chapter whether or not sucrose availability is limiting for the developmental progression of LRPs.

Uptake of exogenous sucrose from media by the shoot has been reported to increase LR lengths (Macgregor et al., 2008). Takahashi et al (2003) showed that 1% sucrose promotes adventitious root inductions only if the hypocotyl is in contact with sucrose in the media. As adventitious roots are formed from the hypocotyl, contact and uptake of sucrose into the hypocotyl stimulated adventitious root formation in the hypocotyl and contact and uptake of sucrose into the PR was not necessary (Takahashi et al., 2003). However, the data showed in this chapter has shown that contact of the PR with sucrose via the media promotes PR growth and increases LR numbers and densities similar to the effect of the supply of exogenous sucrose to shoot via filter paper (Figure 3.6). The fact that adventitious roots are formed from the hypocotyl, may, however, explain why contact of the PR with sucrose was found not to be necessary in the study by Takahashi et al (2003) (Takahashi et al., 2003).

It has been suggested that under plate-grown conditions and in experimental frameworks where the root is in contact with sucrose via the media; exogenous sucrose is taken up via transpirational pull into the xylem and transported from the root to the shoot (Lalonde et al., 2004). Sucrose may then be transported back to the root apoplastically to promote PR growth and to increase LR lengths (Macgregor et al., 2008). However, under conditions where sucrose is supplied to the root, if sucrose were taken up from the media by the root into the xylem stream and then transported through the phloem via bulk flow back to the root similar to what is expected to happen under the conditions where exogenous sucrose is supplied to the shoot; then it would have been expected that the response of LRP progression to exogenous sucrose supplied by the same methods would be the same.

However, the results presented in (Figures 3.4A, 3.4B, 3.4C & 3.4D) show otherwise. A similar pattern of LRP distribution among early stages and later stages was observed under the two conditions where exogenous sucrose was supplied to the root. This suggests a similar mechanism of sucrose uptake and transport. Under *in vitro* conditions when both the shoot and the root are in contact with sucrose, the root is predicted to take up sucrose and transport it to the shoot, thereby becoming a carbon source instead of sucrose (Papaioannou et al., 2018). This might have happened in this study, suggesting that the transport of a shoot-derived factor or signal from the shoot to the root may be controlling the developmental progression of LRPs. Therefore, in the absence of shoot transport, LRP progression would then be reduced as was shown in this study (Figure 3.4).

The shoot-derived factor that may be transported from the shoot to the root to promote the developmental progression of LRPs is likely to be auxin (IAA). Auxin is the master regulator of LRP formation (De Smet et al., 2007; Swarup et al., 2008; De Rybel et al. 2010). Auxin is also synthesised in the shoot and transported via the same bulk flow as sucrose (Swarup et al. 2001; Vanneste and Friml 2009). The auxin efflux transporter, AUX1 is known to be involved in the phloem transport of shoot-derived auxin through the protophloem in the PR, AUX1 mediates the acropetal transport of auxin to the PR tip, and its subsequent transport into XPP cells (Swarup et al., 2001; Marchant et al., 2002).

Sucrose is the driver of phloem transport (Münch, 1930; Knoblauch and Peters, 2017), it is, therefore, possible that exogenous sucrose taken by shoot will supplement the endogenous sucrose levels, and both sucrose pools would drive the transport of auxin to the root. AUX1 would then transport it to the XPP where auxin would promote the developmental progression of LRPs. Sucrose may also regulate AUX1 activity at the unloading site in the protophloem because both shoot-derived auxin and sucrose are unloaded at the same site (Swarup et al. 2001; Ross-Elliott et al., 2017). Although there is no known evidence of the effect of sucrose on AUX1 activity, the transcription of the genes that encode other auxin transporters such as *PIN1*, *PIN2,* and *PIN3* has been reported to be induced by sucrose (Zhang et al., 2020), this may also be the case for the *AUX1* gene.

This study has shown that the progression from stage 1 to later stages is sucrose-dependent (Figure 3.10E). AUX1 has been implicated in mediating the auxin transport requirement for the progression of LRPs from stage 1 to later stages as evidenced by a delay in the progression of LRPs from stage 1 to stage 2 in *aux1* loss-of-function mutants (Marchant et al., 2002). It is noteworthy that the lateral root phenotype of *aux1* was restored completely by the addition of 1-Naphthylacetic acid (1-NAA)—a very membrane permeant auxin which does not require carrier-mediated transport (Marchant et al., 2002). Taken together, it is possible that sucrose may promote the developmental progression of LRPs in an AUX1-dependent manner.

Sucrose taken up by the shoot may be metabolised at the shoot to promote shoot growth and development. Exogenous sucrose has been reported to increase leaf area (Eckstein et al., 2012). An increase in shoot growth may then have a downstream effect on LR emergence and outgrowth. In this study, supply of exogenous sucrose to the shoot led to increases in LR numbers (Figure 3.6C). A correlation between leaf size and LR lengths has been reported (Macgregor et al., 2008), suggesting an effect of exogenous sucrose on LR outgrowth. In that study, the effect of exogenous sucrose on the shoot (e.g., size and area of leaves) was not studied. Leaves with larger surface areas may take up more exogenous sucrose (than leaves with smaller surface areas) thereby promoting LRP progression. In this thesis, a correlation between exogenous sucrose supply and leaf area, as well as the correlation between leaf area and LRP progression were not determined, therefore an indirect effect of exogenous sucrose on LRP progression via stimulation of leaf growth cannot be concluded.

It has been shown in this chapter that there are similar effects of exogenous sucrose on PR growth, LR numbers, and densities under conditions where it was supplied: (i) only to the shoot, (ii) only to the root via media, and (iii) to both shoot and root. However, there are differences in the effect of exogenous sucrose on LRP progression under the aforementioned approaches (Figure 3.6). This suggests that the uptake of exogenous sucrose by the root from the media and by the shoot from the filter paper has different downstream effects on LRP morphogenesis. However, due to the unnatural nature of sucrose uptake by roots, this prediction was not explored experimentally, as that would have defeated the purpose of developing a method that would enable sucrose supply to recapitulate sucrose supply via sucrose transport under soil conditions.

This study has shown the requirement of sucrose uptake by the shoot and not the root for the developmental progression of LRPs. This study, as already discussed in this section suggests a role of sucrose phloem transport or sucrose transport (as sucrose drives phloem transport). To test the hypothesis of the involvement of sucrose transport in promoting the developmental progression of LRPs, mutants with a defect in sucrose transport, *atsweet11/12* and *atsuc2* (Chen et al., 2012; Gottwald et al., 2000; Wippel and Sauer, 2012) can be used to study the distribution of LRPs among early and late stages in the presence and absent of exogenous sucrose. Since the wild types of these mutants are involved in sucrose transport from the shoot to the root, their mutants show reduced endogenous sucrose levels which may lead to a reduction in root growth and development (Chen et al., 2012). The results from this study suggest that reduced availability of sucrose to the root will lead to a reduction in the developmental progression of LRPs. Therefore, the aforementioned mutants could be crossed with the *pGATA23::NLS-GFP* reporter line or another GATA23-based reporter line (e.g., *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4),* to allow for the scoring of LRPs using epifluorescence microscopy*.* The resultant hybrid line could then be used to study LRP progression in response to exogenous sucrose supply.

Crosses of *atsweet11/12* double mutant and *atsuc2* single mutant with the *pGATA23:NLS-GFP* and the *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4 reporter* lines, respectively were generated. However, they could not be successfully used to understand how a defect in sucrose transport as mediated by AtSWEET11/12 and SUC2 would affect the developmental progression of LRPs. These mutants of Arabidopsis sucrose transporters were crossed with the aforementioned fluorescence reporter lines to allow the quantification of LRPs using epifluorescence microscopy due to the reasons discussed in section 3.1 of this chapter. The cross of the *atsweet11/12* double mutants with the *pGATA23:NLS-GFP* failed to produce a triple homozygote line in time to be used to perform follow-up experiments for this thesis.

This was because during the genotyping of seedlings in the F6 generation, a wild type band was found from the atsweet11 gene specific primer/ TDNA primer PCR reaction, and troubleshooting could not be done due to the need to complete this thesis and submit it in time for examination.

An F2 generation of the cross with the at*suc2-1* (heterozygote) single mutant and the *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4* reporter line was generated, however, there was no way a double homozygous hybrid line could have been generated because the suc2-1 homozygous line is smaller, small in development and produces no viable seeds (Gottwald et al., 2000). Additionally, a hybrid line from a heterozygous suc2-1 mutant and a *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4* reporter line, would require genotyping to confirm homozygosity before every experiment, which due to time and resources constraints was not feasible.

Higher concentrations of exogenous sucrose may cause osmotic stress (Cui et al., 2010). Osmotic stress is known to repress PR growth by repressing PIN3 expression which leads to a reduction in auxin levels in the PR (Yuan et al., 2021). Although the study by Yuan et al (2021) was done entirely on PR tips and does not discuss the effect of osmotic stress in LRs, it has been reported that even mild osmotic in the PR leads to a reduction in LR outgrowth (Deak & Malamy, 2005; Macgregor et al., 2008). As such, it could be predicted that osmotic stress in the PR could equally lead to repression in the expression and/or activity of PIN3 in LRPs which could inhibit LR emergence since *PIN3* works co-ordinately with LIKE-AUX3 (LAX3) to modulate the emergence of LRPs through the overlaying tissues of the PR (Péret et al., 2013). Consequently, the study presented in this chapter sought to control for the osmotic effect of sucrose by using sorbitol. There were similar responses of LRP progression to sorbitol-treated roots and in control roots, and similar PR growth and LR numbers in sorbitol-treated roots and control roots suggesting the plants studied were not stressed.

3.6 Conclusions

The data presented in this chapter has shown that under *in vitro* conditions, and in physiological experiments aimed at establishing a causal relationship between sucrose availability and LRP formation, sucrose must be supplied to the shoot, without contact of the root with sucrose via the media to attempt to recapitulate sucrose supply under soil conditions. This study showed the supply of sucrose this way allows for an effect of sucrose on the developmental progression of LRPs to be observed.

This study has also shown that LRP progression from early stages to later stages (1 to stages > 1) is promoted by the supply of exogenous sucrose via the shoot. This suggests the role of the transport of a shoot-derived factor, likely auxin in promoting the developmental progression of LRPs.

Additionally, the filter paper/Parafilm strip system developed for this study, not only turned out to be successful based on the physiological data but provide a simple and elegant system for supplying shoot-derived resources in developmental and physiological studies. This system provides a means for generating accurate and biologically meaningful data from developmental studies that seek to establish a relationship between shoot-derived resource availability and plant growth *in vitro*.

These results have implications for understanding the relationship between carbon resource availability and LRP formation, and further studies are needed to understand the phloem or sucrose, as well as the auxin dynamics involved in regulating the developmental progression of LRPs.

Chapter 4 Supply of exogenous sucrose increases the rate of LRP progression.

4.1 Introduction

Lateral root (LR) formation in Arabidopsis is a tightly coordinated multi-stage process. This multi-stage nature of LR formation provides opportunities for several control points. For example, LR initiation is known to be dependent on a stringent developmental window (Dubrovsky et al., 2006). Within a period of 10.4h and 15.7h, 56% and 82% of LRPs were found at stage1 respectively in 8-day-old Col-0 and C24 Arabidopsis accessions grown on 0.2 x MS media containing 1% sucrose and 0.8% agar. An undisclosed percentage of LRPs were found at stages 2 and 3 in both accessions (Dubrovsky et al., 2006). This estimated developmental window was calculated by dividing the PR into zones: zone 1 which extended from the base of the PR to the most distal LR (about 0.5 mm), and zone 2 which extended from the youngest LRP observed in the most distal region of the PR to the first emerged LR (region closest to the PR tip) (Dubrovsky et al., 2006).

Based on these delimitations of regions on the PR, Dubrovsky, and co-authors observed the earliest LRPs at a range of distances from the PR tip (excluding the root cap): from 2.05 to 5.42 mm, and from 2.68 to 6.20 mm (Dubrovsky et al., 2006). A combination of these distances and the known rate of PR growth as recorded by Dubrovsky et al (2006) led to the estimation of the 10.4h and 15.7h developmental window within which LR initiation is predicted to begin (Dubrovsky et al. 2006). However, the presence of LRPs at stages > 1 within the same regions of the PR suggests that the developmental window for LR initiation is shorter than estimated (Dubrovsky et al., 2006). Delay in the emergence of LRPs has been observed in Arabidopsis (Dubrovsky et al., 2006; Napsucialy-Mendivil and Dubrovsky, 2018), and other plants (Akhiyarova et al., 2023).

The fact that the progression of some LRPs is delayed suggests a regulatory mechanism and checkpoints of regulation for the developmental progression of LRPs. Such mechanisms of regulation may be growth factors such as hormones and nutrients which may serve as resources (e.g., sucrose) that fuel the developmental progression of LRPs. Or act as signals to activate aspects of the LRP developmental machinery. It is, therefore, vital to study factors that could potentially limit the developmental progression of LRPs and to provide a mechanistic understanding of how they regulate the developmental progression of LRPs.

LRP initiation can be induced mechanically by root bending either by the application of gravistimulus by rotating the plates on which plants grow by a particular angle (e.g., 90°) or by direct manipulation (e.g., the formation of J hooks on the PR using sterile forceps) (Ditengou et al., 2008). When roots of 10-day-old *pDR5::GUS* seedlings (auxin response reporter line) were manually bent by 135° (using sterile forceps) and returned to their original position, *DR5:GUS* activity was observed at the bend site 5 hours after bending indicating auxin response at the bend sites; emerged LRs were observed 72 hours after bending (Ditengou et al., 2008). *DR5* activity is known to (i) precede the formation of prebranch sites (which are sites at which future LRs will be formed), (ii) be expressed at prebranch sites, and (ii) be expressed in LRPs (De Smet et al., 2007; Moreno-Risueno et al., 2010). A *DR5* activity at the bend site, therefore, indicates: whether a prebranch site is about to be formed or has already been formed, or the presence of an LRP. Taken together, these observations show that manual bending of roots induces LR formation. Gravitropically induced curvatures, in the PR lead to the formation of LRPs at the convex side of the curve (Figure 4.1; (Lucas et al. 2008; Ditengou et al. 2008; Richter et al. 2009), and an LRP is induced as early as 6h after gravitropic bending (Lucas et al. 2008; Richter et al. 2009).

Gravitropic bending of the PR causes re-localisation of auxin influx transporters which leads to the accumulation of auxin at the bend site, thus, inducing an LRP (Ditengou et al., 2008; Gala et al., 2021). 3 hours after gravitropic bending to 135°, *PIN1* localisation was observed at both the apical and basal regions of a single protoxylem cell at the convex side of the elongation zone (Ditengou et al., 2008) (Figure 4.2A). *PIN1* localisation was only observed at the apical side of the basal protoxylem cells on the convex side of the root after 5 hours of gravitropic bending (Figure 4.2B) (Ditengou et al., 2008). 2 hours before changes in the localisation of *PIN1*, there was an asymmetric *pDR5rev::3XVENUS-N7* (an auxin response reporter line) fluorescence at the protoxylem poles of the bend site in the gravitropically bent roots compared to the unbent controls (Figures 4.2B & 4.2C) (Ditengou et al., 2008). This asymmetric fluorescence of *pDR5rev::3XVENUS-N7* suggests a gravity-induced auxin flow towards the protoxylem poles of the bend sites, which would lead to the accumulation of auxin and a concomitant bending of the root (Kleine-Vehn and Friml, 2008).

Additionally, 3 hours after gravitropic bending, in the protoxylem on the convex side of the curve, a diffused cytosolic *PIN1* localisation was observed at the cell immediately above the cell in which PIN1 is observed on both sides (Figure 4.2A). After 5 hours, the observed diffused cytosolic PIN1 localisation was lost completely from the (Figure 4.2B [asterisk]). Taken together, these observations suggest that gravitropic bending induces positional cues in protoxylem cells, and these cues cause the relocalisation of PIN1. The relocalisation of PIN1 at the bend site suggests the role of PIN1 in maintaining auxin maximum at the bend site which is crucial for the initiation of LRPs (Ditengou et al., 2008).

The gravistimulus applied from gravitropic bending does not induce LRPs independent of the endogenously controlled regulatory mechanism of LRP formation (Moreno-Risueno et al., 2010). Roots that are either agravitropic due to defects in auxin signalling and/or transport or due to the removal of gravity-sensing tissues (i.e the root cap) were able to form LRs outside of curvatures.

This suggests that gravistimulus may not be exactly required for LR formation and that LR formation is endogenously controlled, for example by an oscillation in auxin response and auxin-responsive genes which lead to the formation of prebranch sites (Lucas et al., 2008; Richter et al. 2009; Moreno-Risueno et al., 2010).

The frequency of bending (the left-right orientation) that characterises PR growth in Arabidopsis grown on inclined impenetrable agar surfaces (vertical plates) is reported to have a periodicity of ~ 6 hours which is similar to the periodicity of prebranch site formation as reported by *DR5:Luciferase* (Moreno-Risueno et al. 2010). This left-right orientation of PR growth in plate-grown Arabidopsis, which is often referred to as skewing (from the expected vertical orientation of the PR), and a sinusoidal pattern of PR growth is referred to as waving (Okada and Shimura 1990; Simmons et al., 1995). This waving and skewing pattern of PR growth on vertical plates is regulated by several factors *inter alia,* gravitropism, and plant-gel surface interaction (of which are well outside the scope of this chapter and thesis as a whole) (Oliva and Dunand, 2007). Although gravitropism is a contributing factor to root waving and skewing (bending), the periodicity of bending due to plate rotation is much earlier as a bend site is formed as early as 3h after gravitropic bending (Moreno-Risueno et al., 2010).

The periodicity of gravity-induced bending does not reflect in the formation of prebranch sites, even though the expression of *DR5:Luciferase* pulsed as early as 4h under gravitropic bending conditions (bent to 90°), just a few static points created from DR5 expression formed prebranch sites (Moreno-Risueno et al. 2010). Additionally, there is still prebranch site formation under conditions that do not favour bending (e.g., growing roots through the media, an experimental condition that results in the formation of straight roots). Taken together, these observations suggest that the formation of prebranch sites which are sites from which future LRPs will be formed is independent of gravity-induced bending. Instead, the gravistimulus applied as a result of gravitropic bending may simply promote the development of prebranch sites into LRPs and then into LRs (Moreno-Risueno et al., 2010). Notwithstanding these predictions, the gravitropic induction of LRPs provides an excellent method for synchronising LRP formation under experimental systems (Xun et al., 2020; Qian et al., 2022).

In chapter 3, LRPs were scored along the length of an approximately 40 mm long PR. Such a PR contains LRPs at distinct stages which suggest that they were initiated at different times. This is problematic because, it is impossible to track the developmental trajectory of individual LRPs within a given period in response to a particular treatment (e.g., exogenous sucrose). This makes it difficult to unequivocally determine the effect of a treatment on the developmental progression of LRPs if certain LRPs are more advanced than others. To address this challenge, the gravitropic bending assay can be used to induce individual LRPs at a bend site; since only one bend site is formed per plant in an experiment, there may potentially be a uniform start time for the initiation of individual LRPs in response to a given treatment across the different plant samples in an experiment. Additionally, just one or two LRPs are formed per bend site, as such, the scoring of these LRPs is not only more rapid compared to scoring over 20 LRPs per root; per plant, but quantification of LRPs under the gravitropic bending conditions can be easily streamlined to reduce over- and/or under-allocation of LRPs to specific stages which may create artifacts.

The whole process of LR initiation and LRP morphogenesis is dependent on cell proliferation dynamics (Torres-Martínez et al., 2019). In Arabidopsis, the duration of LR initiation to emergence ranges from 38.4 to 52.5 hours (Napsucialy-Mendivil et al., 2014; Von Wangenheim et al., 2016), this suggests the formation of a fully mature LR within a short time. The duration of the cell cycle during the overall process of LR formation in combination with mechanical constraints imposed by the overlaying tissues of the growing LRPs (e.g., the Casparian strip of the endodermis) (Lucas et al. 2013; Vermeer et al., 2014; Von Wangenheim et al., 2016) likely affects the rates of LRP developmental progression. Before LRP initiation occurs, space must be created to accommodate imminent radial growth of the XPP cells, this space is created by an increase in the width of the founder cells, which consequentially requires loss in the volume of the endodermis that overlays it (Vermeer et al., 2014).

Endodermal volume loss is reported to be crucial for LRP initiation, as demonstrated by the inability of XPP cells to form an LRP when endodermal cells that overlay them were eliminated via cell ablation (Marhavý et al., 2016). Ablation of endodermal cells that overlay the so-called naïve pericycle cells (XPP cells that were not specified as LR founder cells) was still able to undergo periclinal division. It is noteworthy that these naïve pericycle cells are no auxin responsive, thus, do not have the auxin maximum that is required for founder cell identity specification and an eventual LRP initiation. However, the removal of their overlaying endodermal cells still induced cell divisions in them (Marhavý et al., 2016). In a follow-up experiment, the authors ablated xylem and cortical cells overlaying XPP cells, however, these ablations did not induce cell divisions in the adjacent pericycles as was observed from the endodermal cell ablation (Marhavý et al., 2016). Altogether, these observations suggest that the endodermis restrains cell division and that their removal stimulates cell division in adjacent pericycle cells—auxin response and signalling in the endodermis is hypothesised to coordinate the release of the endodermal constraints on XPP cells, thereby allowing them to undergo the cell divisions required to initiate LRPs (Marhavý et al., 2016).

Auxin is known to promote cell division in plants (Hobbie and Estelle, 1994; Gray and Estelle, 2000). The division of pericycle cells at the differentiation zone (where LRPs are initiated) of the Arabidopsis root is arrested at the G2 phase and re-enters into the cell cycle at the G2-M phase by responding to a local auxin maximum (Blakely and Evans 1979; Beeckman et al. 2001; Casimiro et al. 2003). Blocking polar auxin transport using naphthylphthalamic acid (NPA) at concentrations of 10 µM completely inhibits LRP initiation by blocking the asymmetric division that underpins (Himanen et al. 2002; Casimiro et al. 2001) as no LRP or LR was observed at this concentration (Casimiro et al., 2001). Additionally, disruption of auxin response in mutants (e.g., *solitary-root* (*slr-1)*) completely blocks the cell division-driven LR initiation (Fukaki et al., 2002; Vanneste et al., 2005;).

LRP developmental progression is underpinned by cell division and auxin is likely to drive it via its chief role in cell division. In developing seedlings, the removal of the aerial tissues, which are auxin sources led to the concentration of LRPs in early stages (≤ stage 4) compared to the control (Bhalerao et al., 2002). This observation, therefore, suggests that the developmental progression of LRPs is dependent on auxin. However, the observation from chapter 3 of this thesis poses the question of where sucrose fits into the LRP developmental progression programme if the programme is very auxin-centric. Sucrose is likely to be upstream of the auxin in the LRP development programme; Sucrose is hypothesised to promote various aspects of auxin homeostasis, for example, biosynthesis and transport by inducing the expression of the genes that encode the proteins involved in these processes (e.g., YUCCAs and PINs) as discussed in chapters 1 and 3 (Lilley et al., 2012; Sairanen et al., 2012; Zhang et al., 2020). Sucrose could also have a direct role in promoting LRP developmental progression via its role as a signal, probably by inducing the expression of some LRP formation-related genes.

To separate these two possibilities, it is vital to identify whether there are checkpoints at which sucrose could be acting to promote LRP development, and the potential regulation of these by checkpoints are directly or indirectly controlled by sucrose. This question could certainly take years of research to answer, however as a start, understanding the temporal dynamics and rates of LRP progression in response to exogenous sucrose supply could help identify some of these checkpoints. This could be useful in allowing for the identification of potential arrest points along the developmental trajectory of LRPs.

Although stage 1 was concluded as a likely checkpoint for a sucrose effect on LRP development, LRP initiation in those experiments was not synchronised as explained earlier in this section. It is, therefore, difficult to draw solid conclusions on checkpoints of regulation or control by sucrose. Therefore, as a follow-up to the results presented in chapter 3 of this thesis, the current chapter describes the effect of exogenous sucrose on the temporal dynamics and rates of the developmental progression of LRPs for the reasons explained earlier.

4.2 Objective and Hypothesis

4.2.1 Objectives

The objectives of the study presented in this chapter were: (i) to determine the temporal dynamics of LRP progression in response to exogenous sucrose supplied directly to the shoot, and (ii) to determine whether exogenous sucrose (supplied directly to the shoot) promotes LRP progression from early stages (stages ≤ 3) to later stages (stages > 3) by increasing the rate of LRP progression from the early stages to the later stages.

4.2.2 Hypothesis

It was concluded in chapter 3 that the supply of exogenous sucrose directly to the shoot promotes the progression of LRPs from the early stages (stages ≤ 3) to later stages (stages > 3). This was evidenced by higher numbers of stages ≤ 3 and higher numbers of stages > 3 in the absence of exogenous sucrose supplied to the shoot and the presence of exogenous sucrose supplied to the shoot respectively. This observation suggests that the developmental progression of LRPs is accelerated by sucrose supplied to the shoot. Therefore, supplying exogenous sucrose to shoot will increase the rate of LRP progression from early stages to later stages. It is likely sucrose directly promotes the progression of LRP development by indirectly promoting cell division during LRP initiation and morphogenesis by promoting auxin biosynthesis (Lilley et al. 2012; Sairanen et al. 2012), auxin will then regulate and promote cell division (Himanen et al. 2002; Beeckman et al. 2001). Alternatively, sucrose may directly promote via its role as a signal through an unknown mechanism.

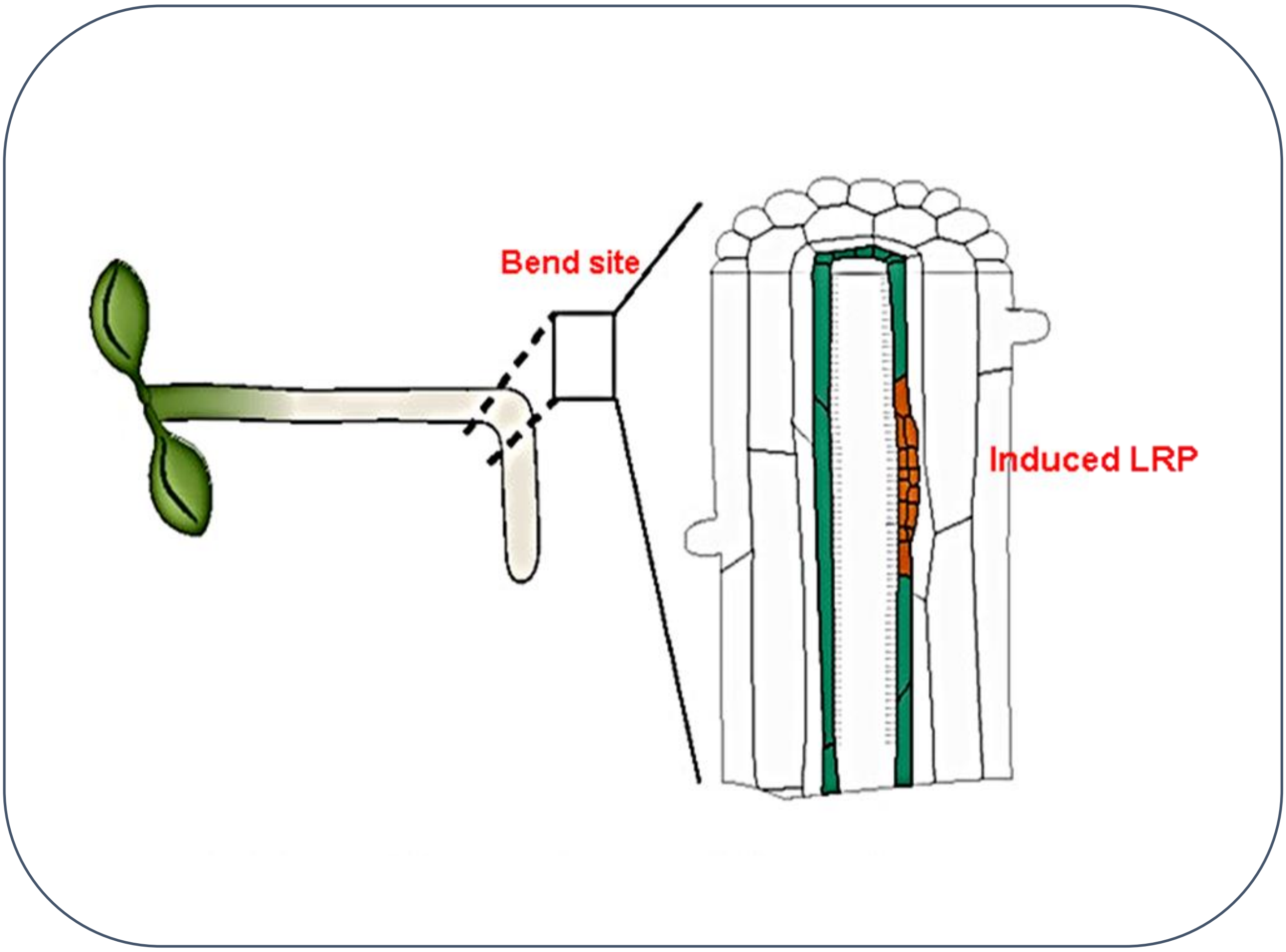


Figure 4.1 **Induction of LRPs due to root bending**. Bending of the PR leads to the formation of LRPs at the convex side of the mechanically-induced curvature. Image was adapted from (Gala et al. 2021).

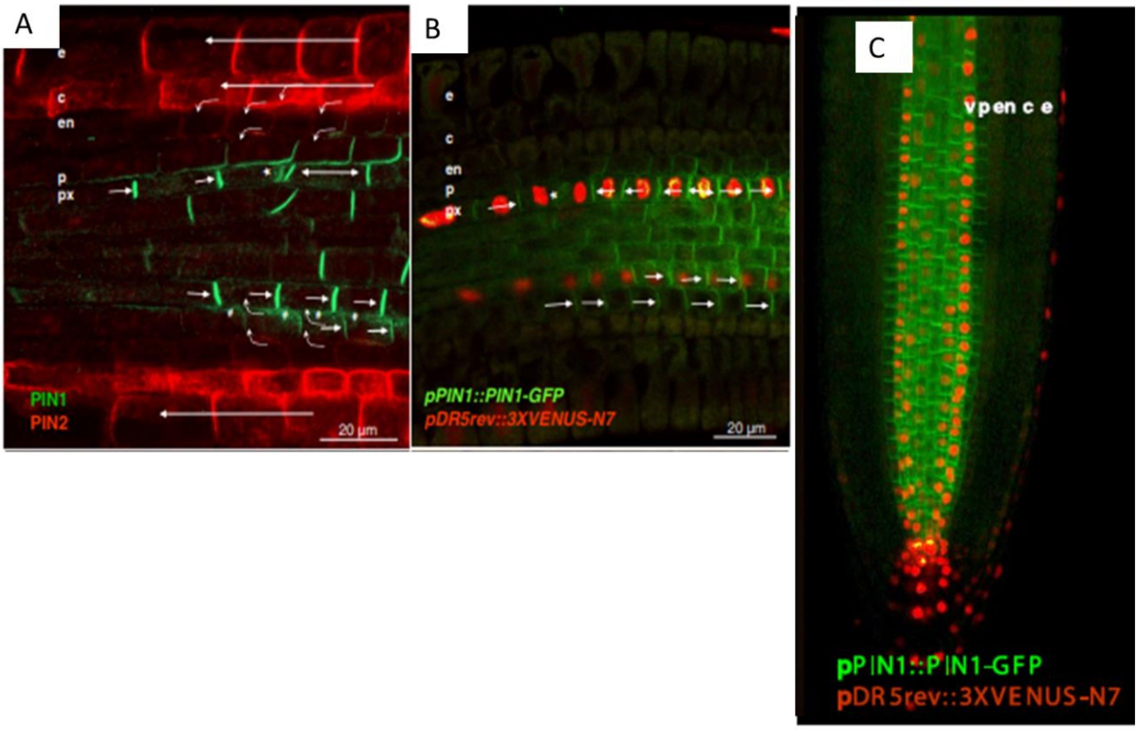


Figure 4.2 **PIN protein localization roots of wild-type and pPIN1::PIN1-GFP, pDR5rev::3XVenus-N7 transgenic lines after gravitropic bending.**

(A) root elongation zone 3 h after gravistimulation. Immunolocalization with anti-PIN1 (green) and anti-PIN2 (red), arrows indicate inferred auxin fluxes, asterisk (*) and hash (#) indicate diffuse cytosolic PIN1 localization. (B) PIN1-GFP (green) and DR5- Venus (pink), 5 h after gravistimulation, DAPI labelled nuclei (blue), asterisk (*) indicates protoxylem cell with diffuse PIN1-GFP signal (C).pPIN1::PIN1-GFP, pDR5rev::3XVenus-N7. PIN1-GFP (green) and DR5-Venus (red).Epidermis (e), cortex (c), endodermis (en), pericycle (p), protoxylem (px), vascular bundle (v). Bar is 20 µm. Image was taken from (Ditengou et al. 2008).

4.3 Methods

4.3.1 Seed sterilisation and pre-growth

pGATA23::NLS-GFP seeds were used in all experiments in this chapter. Seeds were sterilised, cold stratified and pre-grown as described in chapters 2 and 3.

4.3.2 Placement of Parafilm and filter paper strips on media

Parafilm and filter paper strips for all experiments in this chapter were placed diagonally (Figure 4.3).

4.3.3 Transfer and post-transfer growth conditions

- **Acclimation of plants**: Plants were allowed to acclimatise for 2 days to allow for adjustment to post-transfer conditions (Figure 4.3B).

- **Gravitropic bending**: Plates were rotated to a 90º to allow plants to grow perpendicular to the media surface (Figure 4.3C).

- **Post-gravitropic bending growth**: Plants were grown at various time points (0h (no rotation), 6h, 12h, 18h, 24h, 36h, (48h, and 72h).

4.3.4 Bend site analysis.

The bend site was excised and the LRP stages were scored (Figure 4.3D). LRPs in plants under 0h time points were scored at approximately 2.2 mm from the root tip. The region to score LRPs under 0h conditions was determined by measuring the average distance from the PR tip (marked after acclimation) to the bend sites. The 2.2 mm section was measured using a 30 cm ruler because there is no 0.2 mm on a 30 cm ruler, the distance from the PR tip to the bend site was between the 2 mm and 3 mm mark. The 24h time point was used as a baseline time point by choice because the distance from the tips of PRs (marked after acclimation; Figure 4.4) is independent of the time point studied in the study presented in this thesis a bend site is formed as early as 3h after gravitropic bending (Moreno-Risueno et al. 2010). Distance from PR tips to bend sites was measured using the segmented line feature in the Fiji Image J software (https://imagej.nih.gov/ij/) to ensure accurate measurements of distance.

4.3.5 Calculation of rate of LRP progression.

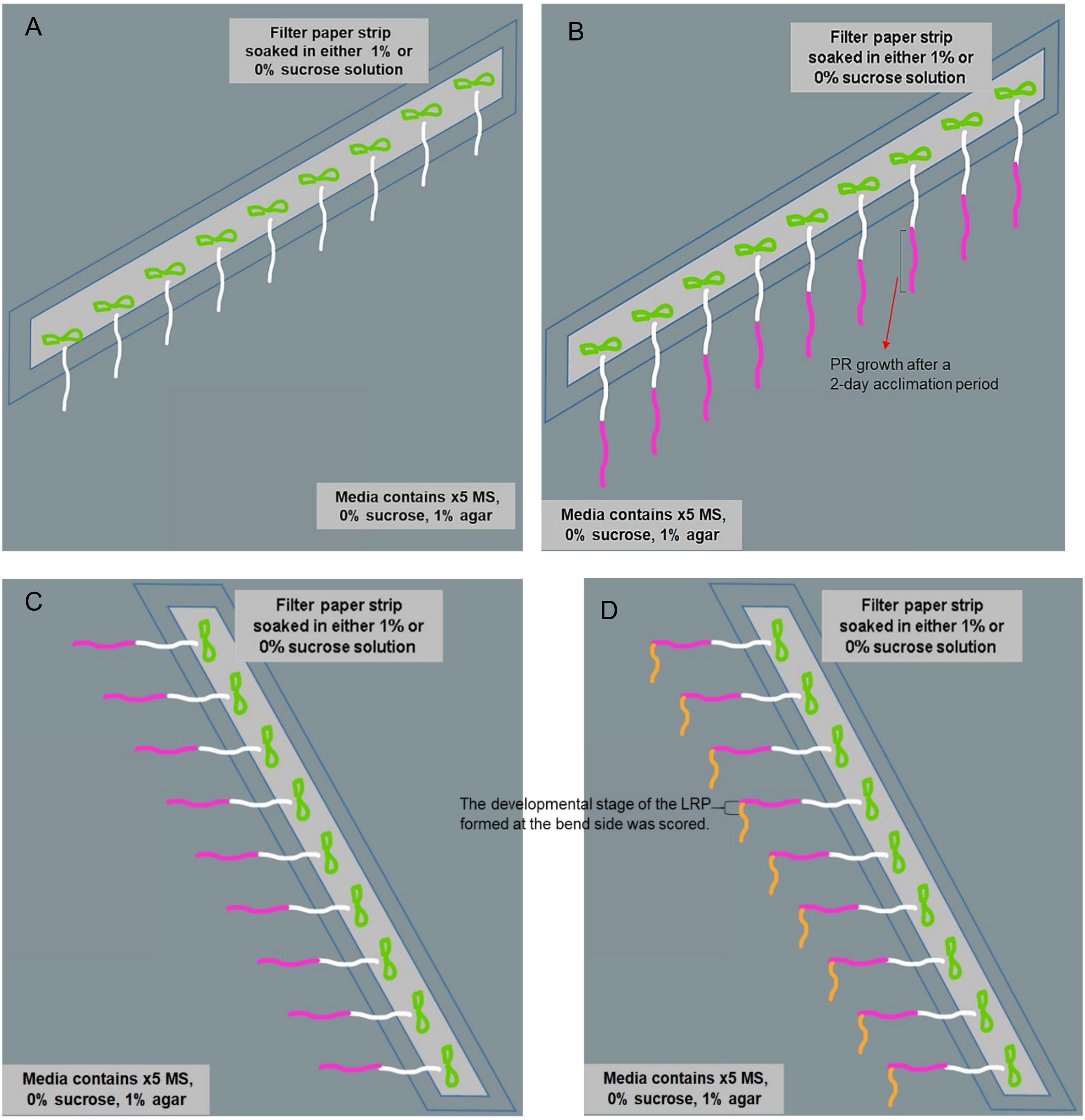
The rate of LRP progression was calculated by first assigning numerical values to the various LRP stages and finding the differential between LRP stages (as numerical values) at two successive time points. These differentials were then divided by the difference between the two successive times (e.g., 12h -18h = 6h). The formula for the rate of LRP progression was as follows:

$$Rate\ of LRP\ progression = \frac{LRP\ stage\ at\ current\ time\ point\ (n) -\ LRP\ stage\ at\ previous\ time\ point (n)}{current\ time\ point\ (h) - previous\ time\ point\ (h)}$$

For example, the rate of progression of LRPs in response to exogenous sucrose supplied via shoot at 12h after gravitropic bending (rotation) following a preceding time point of 6h was calculated as follows:

$$Rate\ of LRP\ progression = \frac{LRP\ stage\ at\ 12h\ (after\ gravitropic\ bending) - LRP\ stage\ at\ 6h}{12h - 6h}$$

The unit for the rate of LRP progression was number/hour (n/h)



PR growth after a 2-day acclimation period

The developmental stage of the LRP formed at the bend side was scored.

Figure 4.3 **Experimental design for the adaptation of the gravitropic bending assay to the filter paper/Parafilm experimental system.**

(A) Transfer of seedlings onto treatments plates. 10-day old seedlings of pGATA23::NLS-GFP reporter line were transferred onto bend assay plates. Plates were prepared as described in section 2.3.2.3 of chapter 2.

(B) Acclimation of plants for 2 days. Plants were acclimatised for 2 days to allow for adjustment to post-transfer conditions before gravitropic bending.

(C) Rotation of experimental plates. Experimental plates were rotated at 90º after a 2-day acclimation period.

(D) Analysis of the bend sites. Bend sites were excised and LRP stages and were scored as described in section 2.8 of chapter 2.

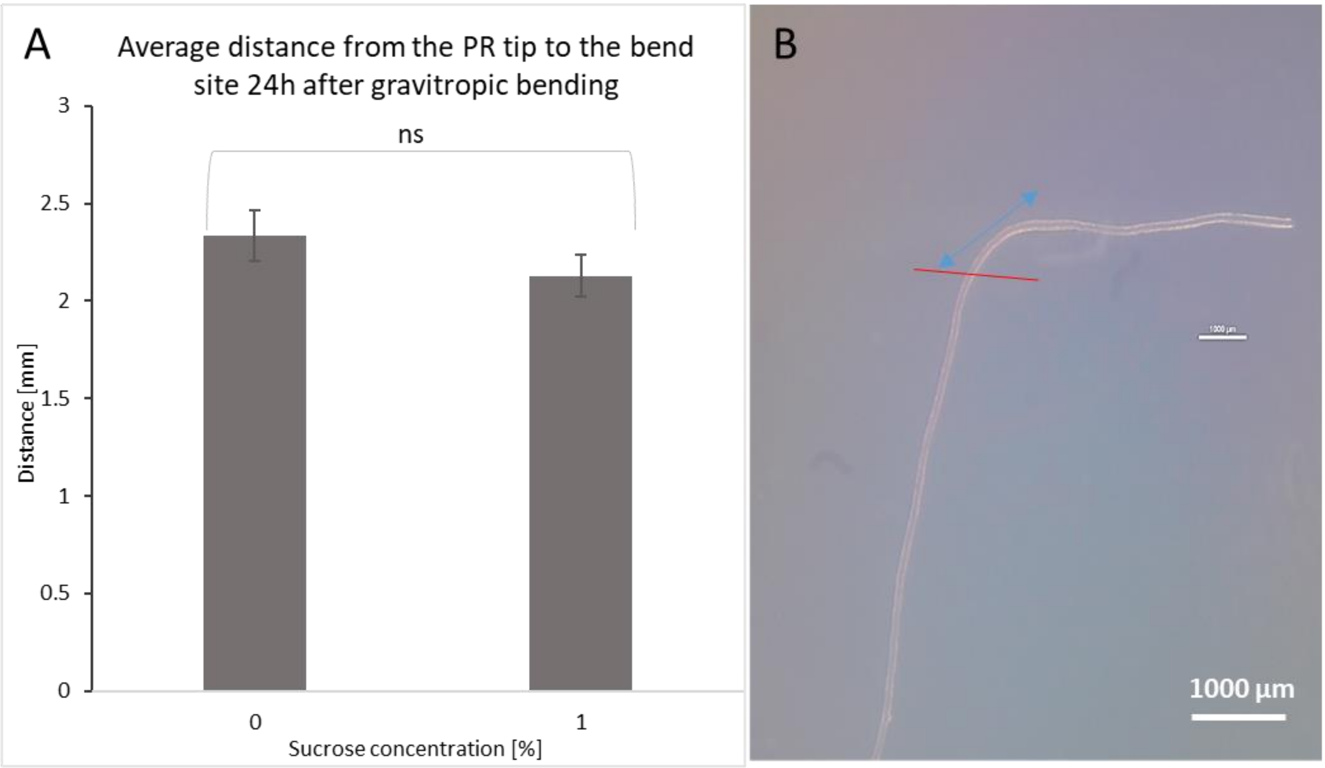


Figure 4.4 **Measurement of distance from PR tip to bend site.**

(A) Average distance from the PR tip to the bend site 24 h after gravitropic bending. LRPs in plants under 0h time points were scored at approximately 2.2 mm from PR tip marked after acclimation. The 2.2 mm section was measured using a 30 cm ruler, because there is no 0.2 mm on a 30 cm ruler, the distance from the PR tip to the bend site was between the 2 mm and 3 mm mark; Error bars represent means ± standard error of the mean (SEM). ns $p > 0.05$ by two-sided Welch t-test; 0% sucrose, n = 28, 1% sucrose, n = 26. (B) Representative of images used to calculate distance from PR tip (marked after acclimation) to bend site in order to determine which region on the PR to score LRPs under 0h (no rotation) conditions. Red line is reiteration of the PR tip marked after acclimation; double arrowed line is the distance from the PR tip after acclimation to the bend site. Bar is 1000 µm.

4.4 Results

4.4.1 Temporal dynamics of the distribution of LRPs among stages in response to exogenous sucrose supplied via shoot after gravitropic bending.

LRPs can be mechanically induced by gravitropic bending, this is done either by directly bending the PR with sterile forceps or rotating the plates on which they grow by 90° (Ditengou et al., 2008; Gala et al., 2021). The plate rotation approach was used and the gravitropic bending assay was adapted to the filter paper/Parafilm strip system described in chapter 3 and (Figure 4.3) to study the temporal dynamics of LRP progression in response to exogenous sucrose supply. To do this, 0% and 1% sucrose were supplied to the shoots of 10-day-old *pGATA23::NLS-GFP* seedlings, and plants were allowed to acclimatise to treatment conditions for 2 days. Plates were rotated by 90° and allowed to grow at various time points (6h, 12h, 18h, 24h, 36h, 48h, and 72h). Control plates designated 0h were not subjected to gravitropic bending, this was to provide a basis for understanding: (i) whether an LRP has been formed at the bend site, (ii) the extent of LRP progression under the various exogenous sucrose conditions at distinct time points.

The data presented in this section were obtained from three independent experiments for 0h, 6h, 12h, 24h, and 36h time points (Figure 4.7) and 2 independent experiments for later time points (48h and 72h). The data from these experiments showed that, without gravitropic bending (0h), 100% of LRPs are found in stage 0 in the absence of exogenous sucrose (Figures 4.5A & 4.5B). However, 26.58% of LRPs were found in stage 1 in the presence of exogenous sucrose, while > 70% (73.43%) of LRPs were found in stage 0 (Figures 4.5A & 458B). The observation of stage 1 LRPs in response to 1% exogenous sucrose supply under no bend conditions suggests LRP initiation in some of the plants studied under 1% exogenous sucrose conditions. There was a statistically significant difference in the distribution of LRPs at the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.1).

At 6h after gravitropic bending, > 90% of LRPs were found at stage 1 (91.36%), 6.17% of LRPs were found at stage 2, and < 3% of LRPs were found at stages 0 and 4 in the absence of exogenous sucrose (Figures 4.5C & 4.5D). In the presence of exogenous sucrose, almost 90% (88.89%) of LRPs were found at stage 2, 3.70% were found at stage 1 and 7.41% were found at stage 3 (Figures 4.5C & 4.5D). This suggests that the majority of LRPs (> 80%) progressed one stage further (Figure 4.5C) in the presence of exogenous sucrose 6h after gravitropic bending. Additionally, there was a statistically significant difference in the distribution of LRPs among the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.2).

At 12h after gravitropic bending, about 80% (80.24%) of LRPs were found at stage 3, 11.11% of LRPs were found at stage 2, 6.17% of LRPs were found at stage 4, and < 3% of LRPs were found at stages 1 and 5 in the absence of exogenous sucrose (Figures 4.5E & 4.5F). In the presence of exogenous sucrose, > 60% (64.19%) of LRPs were found at stage 4, 24.69% were found at stage 3, 9.87% were found at stage 5 and < 3% were found at stage 6 (Figures 4.5E & 4.5F). This data shows that > 60% of LRPs progressed one stage further in the presence of exogenous sucrose compared to the absence of exogenous sucrose as similarly observed at 6h after gravitropic bending.

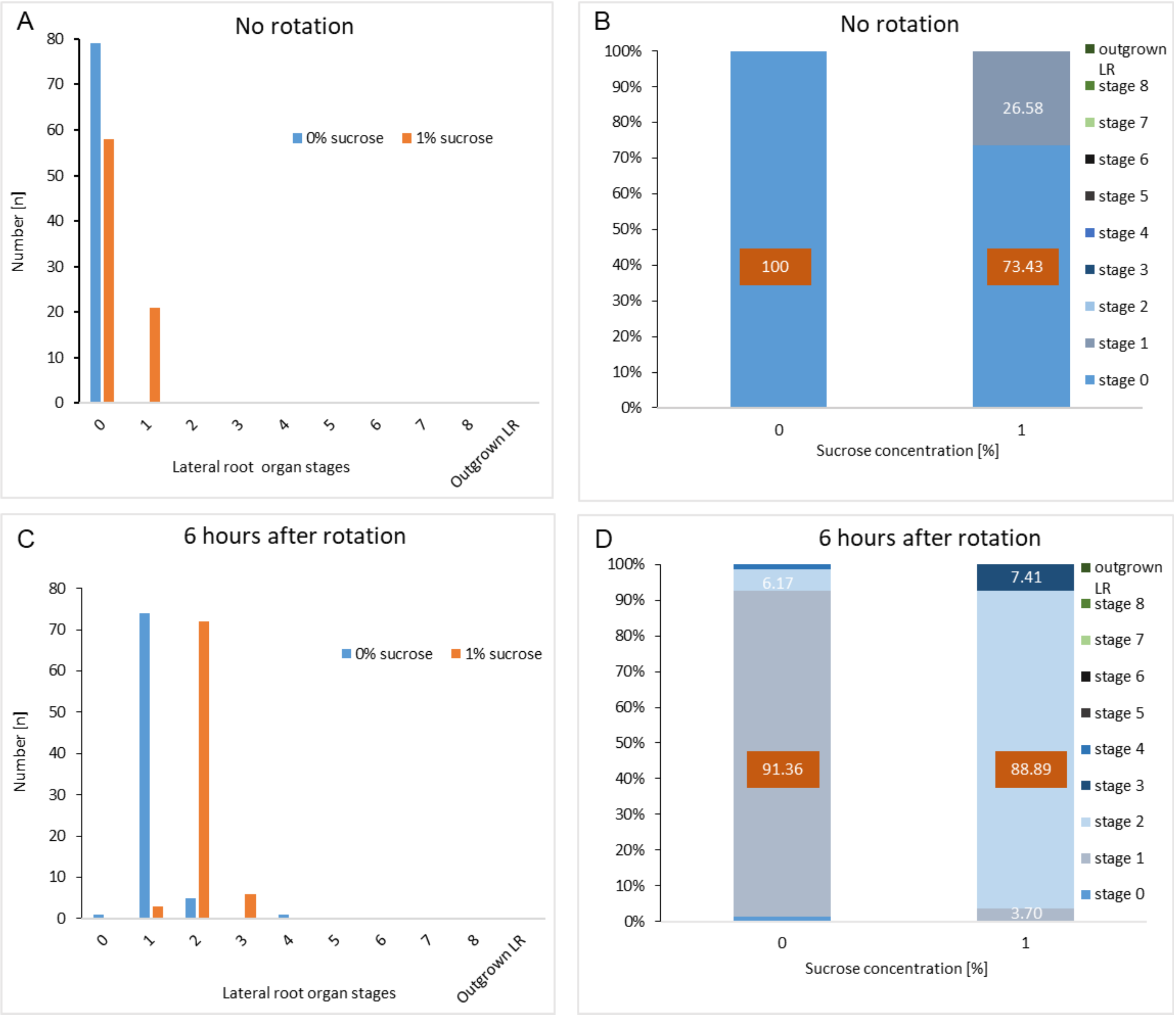
There was, similar to the 6h timepoint, a statistically significant difference in the distribution of LRPs among the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.3). Taken together, these data suggest that while LRP progression is faster in the presence of exogenous sucrose compared to the absence of exogenous sucrose, the acceleration speed of LRP progression remains constant after 6h and 12h of gravitropic bending. At 18h after gravitropic bending, almost 90% (86.42%) of LRPs were found at stage 4, 3.70% of LRPs were found at stage 3, 8.64% of LRPs were found at stage 5, and < 3% of LRPs were found at stage 6 in the absence of exogenous sucrose (Figures 4.5G & 4.5H). In the presence of exogenous sucrose, almost 60% (59.26%) of LRPs were found at stage 6, 34.57% were found at stage 5, 3.74% were found at stage 4, and < 3% were found at stages 2 and 7 (Figures 4.5G &

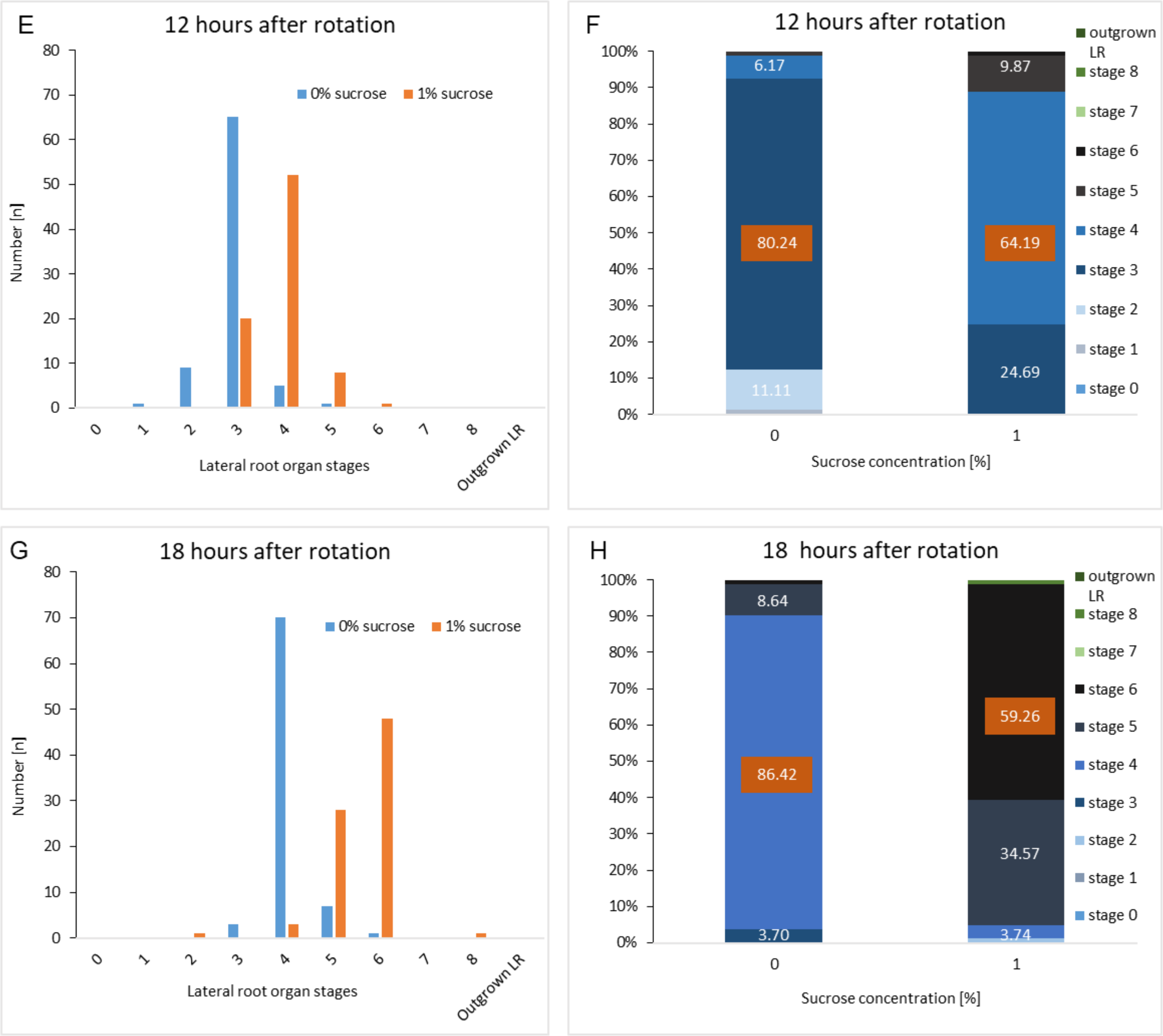
4.8H). This data suggest that > 50% of LRPs progressed two stages further (Figure 4.5G) in the presence of exogenous sucrose compared to the absence of exogenous at 18h after gravitropic bending. Similar to previous time points, there was a statistically significant difference in the distribution of LRPs among the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.4).

At 24h after gravitropic bending, almost 70% (66.24%) of LRPs were found at stage 5, 27.50% of LRPs were found at stage 6 and 6.24% of LRPs were found at stage 4 in the absence of exogenous sucrose (Figures 4.5I & 4.8J). In the presence of exogenous sucrose, 70.37% of LRPs were found at stage 7, 22.22% of LRPs were found at stage 6, and < 3% of LRPs were found at stages 3, 4, 5, and the outgrown LR stage (Figures 4.5I & 4.5J). This data suggests that > 60% of LRPs progressed two stages further (Figure 4.5I) in the presence of exogenous sucrose compared to the absence of exogenous sucrose 24h after gravitropic bending. There was, similar to previous time points, a statistically significant difference in the distribution of LRPs among the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.5).

At 36h after gravitropic bending, > 40% (42.50%) of LRPs had emerged and become LRs (stage 8), 27.50% were outgrown LRs (designated stage 9 elsewhere in this chapter; as described in chapter 2), 16.25% of LRPs were found at stage 7, 11.25% of LRPs were found at stage 6 and < 3% were found at stage 5 in the absence of exogenous sucrose (Figures 4.5K & 4.5L). In the presence of exogenous sucrose, almost 90% of emerged LRPs had become outgrown LRs, 11.11% of LRPs had become newly emerged LRs (stage 8), and < 3% of LRPs were found at stage 7 (Figures 4.5K & 4.5L). There was also found a statistically significant difference in the distribution of LRPs among the indicated stages between 0% sucrose-treated plants and 1% sucrose-treated plants (Table 4.6). At 48h and 72h, almost all LRPs had become outgrown LRs both in the absence and presence of exogenous sucrose (Figures 4.6A & 4.6B). LRP distribution at 48h and 72h were not considered for statistical analyses due to the little to no variations in the distribution of LRP among stages.

Taken together, these data show that LRPs still progress in the absence of exogenous sucrose, possibly due to the effect of endogenous sucrose. The results also corroborate the observation that the supply of exogenous sucrose accelerates the developmental progression of LRPs.




E
12 hours after rotation
Number [n]
80
70
60
50
40
30
20
10
0
0% sucrose
1% sucrose
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral root organ stages
F
12 hours after rotation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
6.17
80.24
11.11
9.87
64.19
24.69
0
1
Sucrose concentration [%]
outgrown LR
stage 8
stage 7
stage 6
stage 5
stage 4
stage 3
stage 2
stage 1
stage 0
G
18 hours after rotation
Number [n]
80
70
60
50
40
30
20
10
0
0% sucrose
1% sucrose
0
1
2
3
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5
6
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8
Outgrown LR
Lateral root organ stages
H
18 hours after rotation
100%
90%
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70%
60%
50%
40%
30%
20%
10%
0%
8.64
86.42
3.70
59.26
34.57
3.74
0
1
Sucrose concentration [%]
outgrown LR
stage 8
stage 7
stage 6
stage 5
stage 4
stage 3
stage 2
stage 1
stage 0

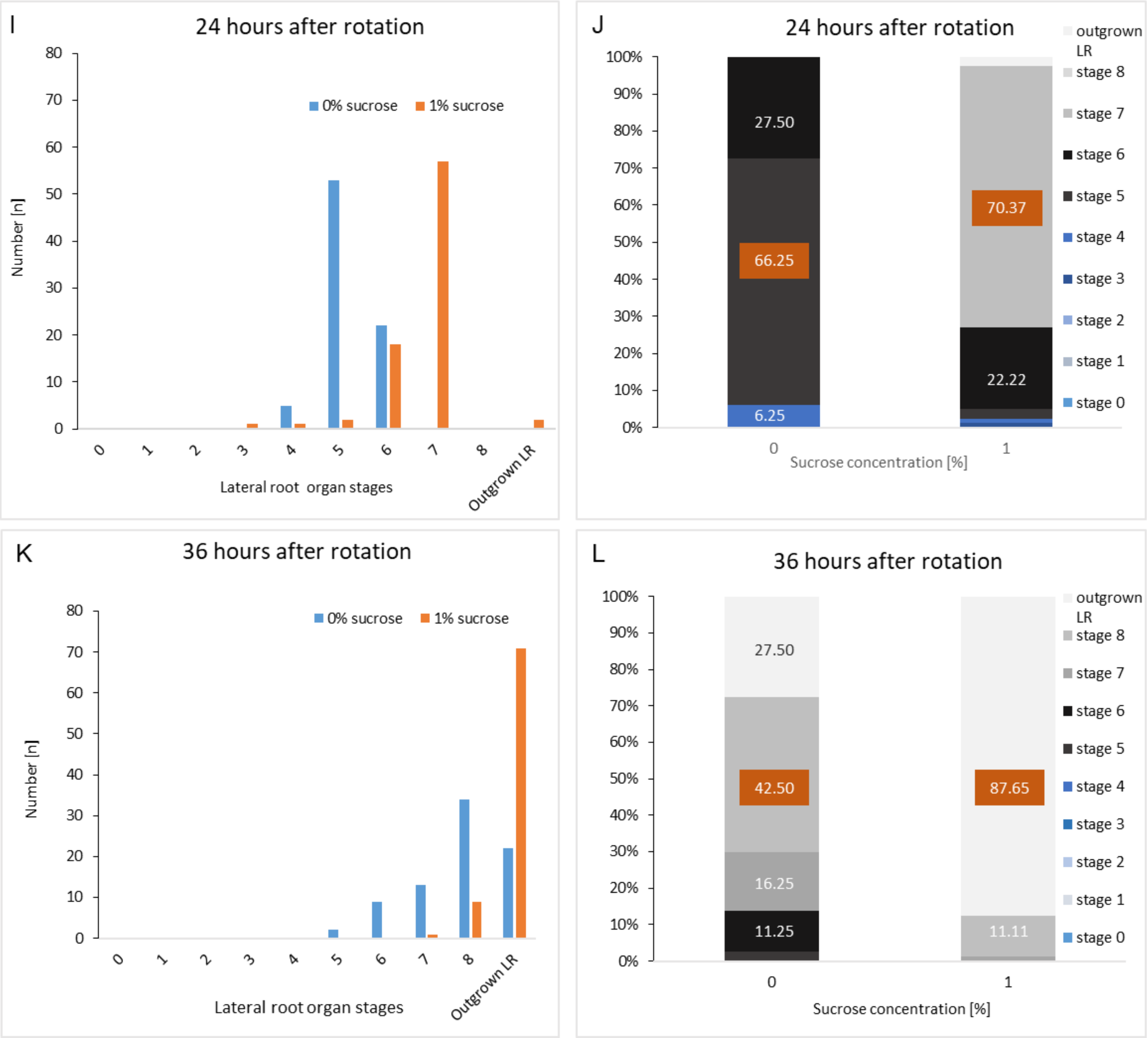

I
24 hours after rotation
Number [n]
80
70
60
50
40
30
20
10
0
0% sucrose
1% sucrose
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral root organ stages
J
24 hours after rotation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
27.50
66.25
6.25
70.37
22.22
0
1
Sucrose concentration [%]
outgrown LR
stage 8
stage 7
stage 6
stage 5
stage 4
stage 3
stage 2
stage 1
stage 0
K
36 hours after rotation
Number [n]
80
70
60
50
40
30
20
10
0
0% sucrose
1% sucrose
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral root organ stages
L
36 hours after rotation
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
27.50
42.50
16.25
11.25
87.65
11.11
0
1
Sucrose concentration [%]
outgrown LR
stage 8
stage 7
stage 6
stage 5
stage 4
stage 3
stage 2
stage 1
stage 0

Figure 4.5 **Distribution of LRPs stages in response to exogenous sucrose at indicated time points after gravitropic bending.**

(A-B). Distribution of lateral root primordia among stages without gravitropic bending. Dark orange highlights represent stages with the highest percentages of LRP. Percentages of LRPs < 3% are not shown numerically on column bars. n = 79; datasets were pooled from 3 independent experiments. 25 < n < 27 for each independent experiment.

(C-D). Distribution of lateral root primordia among stages at 6h after gravitropic bending. Dark orange highlights represent stages with the highest percentages of LRP. Percentages of LRPs < 3% are not shown numerically on column bars. ; n = 81; datasets were pooled from 3 independent experiments. n = 27 for each independent experiment. Percentages of LRPs < 3% are not shown numerically on column bars. n = 81; datasets were pooled from 3 independent experiments. n = 27 for each independent experiment

(G-H). Distribution of lateral root primordia among stages at 18h after gravitropic bending. Dark orange highlights represent stages with the highest percentages of LRP. Percentages of LRPs < 3% are not shown numerically on column bars. n = 81; datasets were pooled from 3 independent experiments. n = 27 for each independent experiment.

(I-J). Distribution of lateral root primordia among stages at 24h after gravitropic bending. Dark orange highlights represent stages with the highest percentages of LRP. Percentages of LRPs < 3% are not shown numerically on column bars. 0% sucrose, n = 80; 1% sucrose, n= 81; datasets were pooled from 3 independent experiments. 26 < n < 27 for each independent experiment

(K-L). Distribution of lateral root primordia among stages at 36h after gravitropic bending. Dark orange highlights represent stages with the highest percentages of LRP. Percentages of LRPs < 3% are not shown numerically on column (A) Distribution of lateral root primordia among stages at 48h after gravitropic bending; 0% sucrose, n = 39; 1% sucrose, n= 43. Datasets were pooled from 2 independent experiments. 19 < n < 22 for each independent experiment. (B) Distribution of lateral root primordia among stages at 72h after gravitropic bending; 0% sucrose, n = 44; 1% sucrose, n= 42. Datasets were pooled from 2 independent experiments. 21 < n < 22 for each independent experiment.

Table 4.1 Statistical analyses of the distribution of LRPs among indicated stages in response to exogenous sucrose under no gravitropic bending (0h) conditions.

Number of roots per indicated LRP stage

LRP stage	0% sucrose	1% sucrose	
0	79	58	
1	0	21	

Chi-Squared tests

Rows, columns:	2, 2	df:	1
Chi2 value	24.219	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom.

Table 4.2 Statistical analyses of the distribution of LRPs among indicated stages in response to exogenous sucrose at 6h after gravitropic bending.

Original table

Number of roots per indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0	1	0	
1	74	3	
2	5	72	
3	0	6	
4	1	0	

Table used for chi-Squared tests

Number of roots per indicated LRP stages			
LRP stage	0% Sucrose	1% sucrose	
0 to1	75	3	
2 to 3	5	78	
4	1	0	

Chi-squared tests

Rows, columns	2, 3	Df	2
Chi2 value	131.67	p (no assoc.)	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom.

Table 4.3 Statistical analyses of the distribution of LRP stages in response to exogenous sucrose at 12h after gravitropic bending.

Original table

Number of roots per indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0	0	0	
1	1	0	
2	9	0	
3	65	20	
4	5	52	
5	1	8	

Table used for Chi-Squared tests

Number of roots per indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0-1	1	0	
2 to 3	74	20	
4 to 5	6	60	
Chi-squared tests			
Rows, columns:	2, 3	df	2
Chi2 value	76.2	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded pink indicate statistical significance ($p<0.05$). df is the degree of freedom.

Table 4.4 Statistical analyses of the distribution of LRP stages in response to exogenous sucrose at 18h after gravitropic bending.

Original table

Number of roots at the indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0	0	0	
1	0	0	
2	0	1	
3	3	0	
4	70	3	
5	7	28	
6	1	48	
7	0	0	
8	0	1	

Table used for Chi-Squared tests

Number of roots at the indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0 to 2	0	1	
3 to 5	80	31	
6 to 8	1	49	

Chi-squared tests

Rows, columns:	2, 3	df:	2
Chi2 value	68.711	p (no assoc.):	<0.000001
Monte Carlo p:	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom.

Table 4.5 Statistical analyses of the distribution of LRP stages in response to exogenous sucrose at 24h after gravitropic bending.

Original table

Number of roots at the indicated stages			
LRP stage	0% sucrose	1% sucrose	
0	0	0	
1	0	0	
2	0	0	
3	0	1	
4	5	1	
5	53	2	
6	22	18	
7	0	57	
8	0	0	
9	0	2	

Table used for chi-square tests

LRP stage	0% sucrose	1% sucrose	
0 to 3	0	1	
4 to 6	80	22	
7 to 9	0	59	
Chi-squared tests			
Rows, columns:	2, 3	df:	2
Chi2 value	92.97	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom.

Table 4.6 Statistical analyses of the distribution of LRP stages in response to exogenous sucrose at 36h after gravitropic bending.

Original table

Number of roots at the indicated LRP stages			
LRP stage	0% sucrose	1% sucrose	
0	0	0	
1	0	0	
2	0	0	
3	0	0	
4	0	0	
5	2	0	
6	9	0	
7	13	1	
8	34	9	
9	22	71	

Table used for chi-square tests

LRP stage	0% sucrose	1% sucrose	
5 to 6	11	0	
7 to 8	47	10	
9	22	71	

Chi-squared tests

Rows, columns:	2, 3	df:	2
Chi2 value	60.831	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact	<0.000001		
p (no assoc.):			

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded blue indicate normal distribution (p>0.05). The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom.

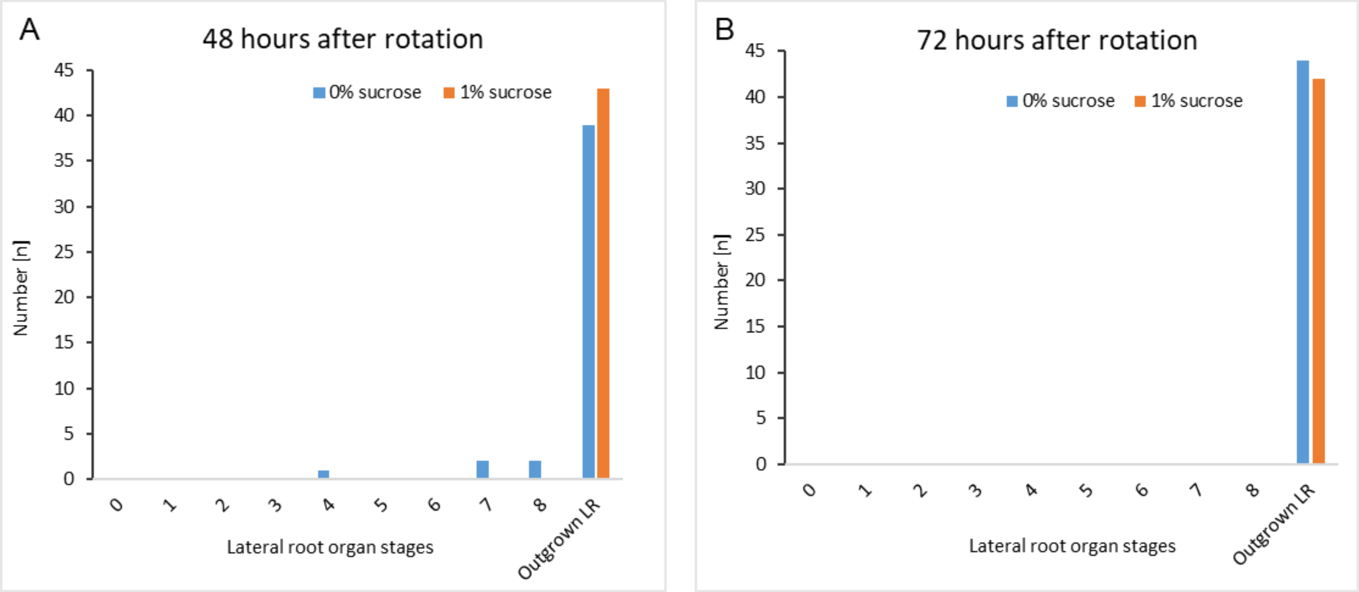
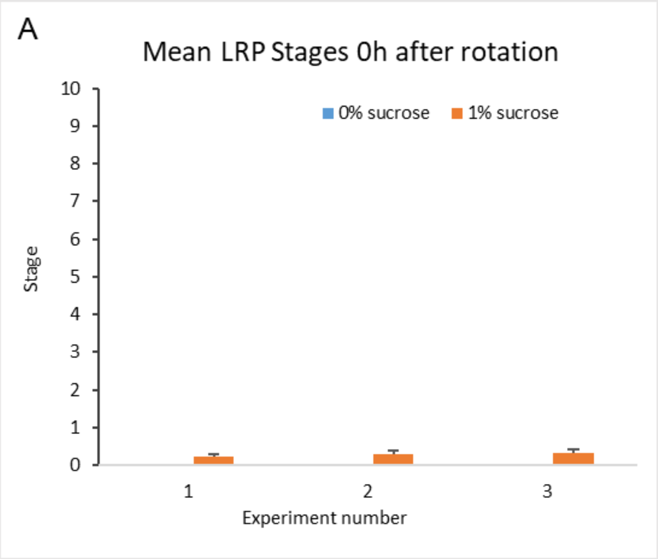
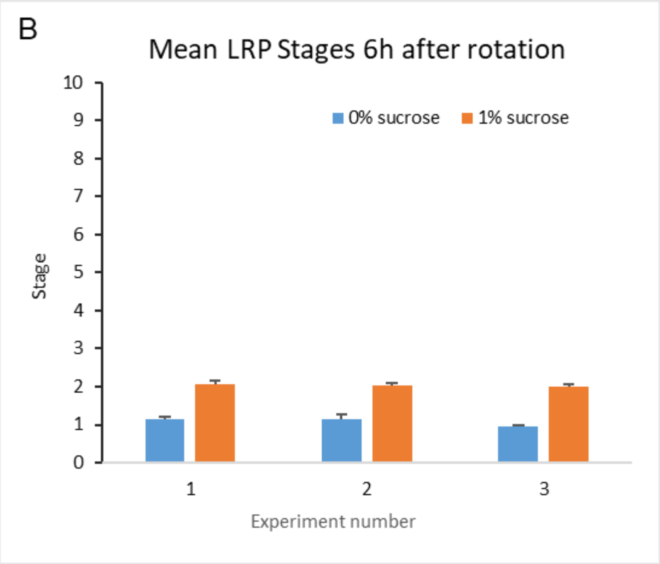
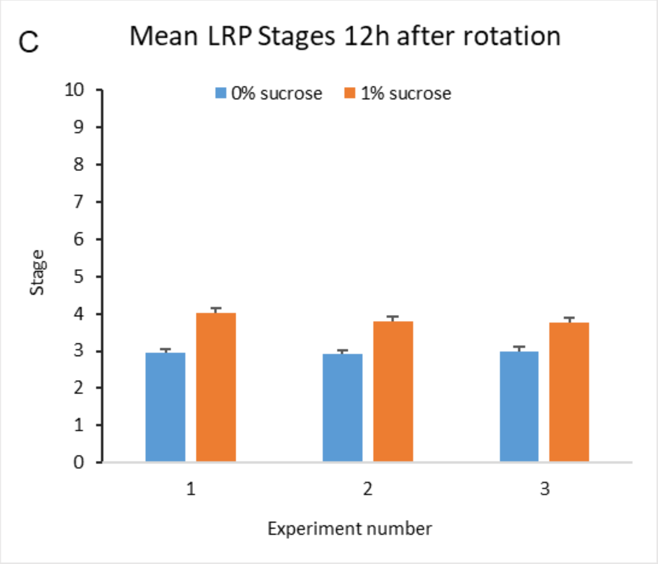
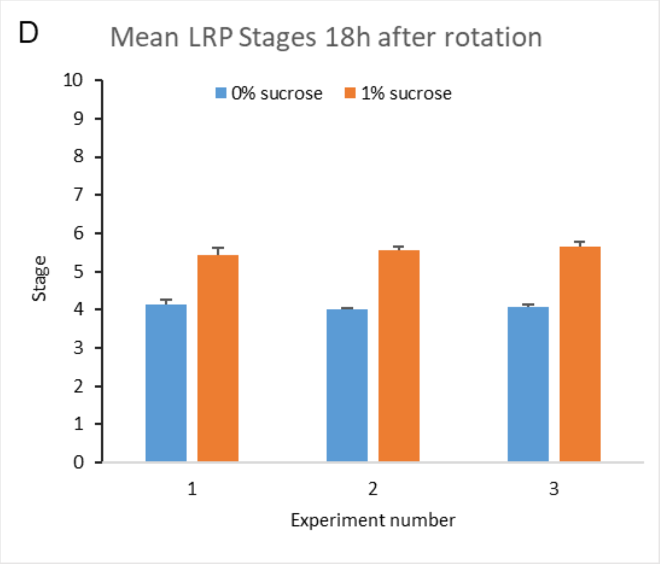


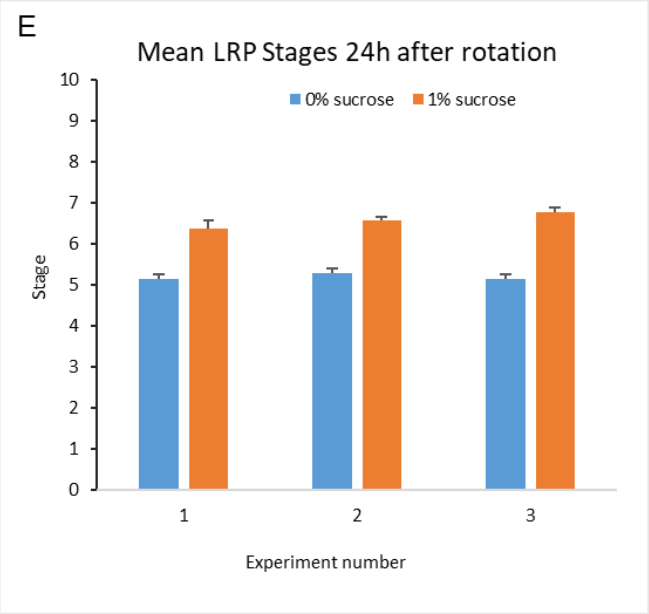
Figure 4.6 **Distribution of LRP stages in response to exogenous sucrose supplied via shoot at later time points after gravitropic bending**. (A) Distribution of lateral root primordia among stages at 48h after gravitropic bending; 0% sucrose, n = 39; 1% sucrose, n= 43. Datasets were pooled from 2 independent experiments. 19 < n < 22 for each independent experiment. (B) Distribution of lateral root primordia among stages at 72h after gravitropic bending; 0% sucrose, n = 44; 1% sucrose, n= 42. Datasets were pooled from 2 independent experiments. 21 < n < 22 for each independent experiment.


Mean LRP Stages 0h after rotation


Mean LRP Stages 6h after rotation


Mean LRP Stages 12h after rotation


Mean LRP Stages 18h after rotation


Mean LRP Stages 24h after rotation

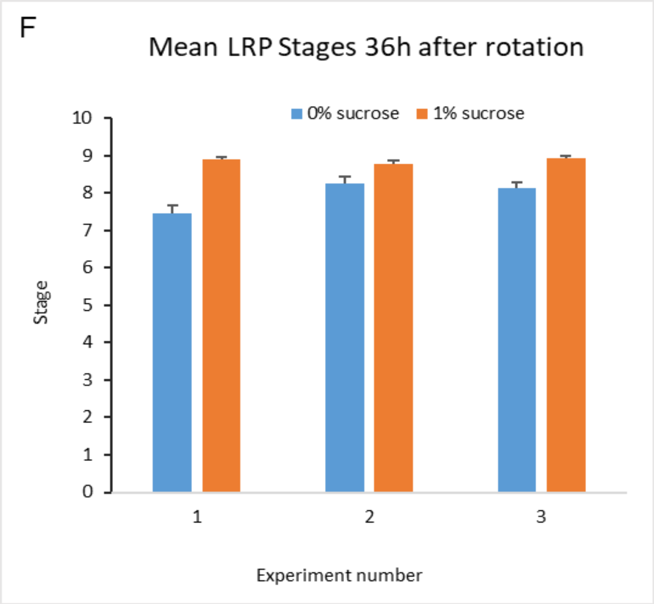

Mean LRP Stages 36h after rotation

Figure 4.7 **Reproducibility of distribution of LRP stages in response to exogenous sucrose via shoot after gravitropic bending in 3 independent experiments**.

(A) Mean LRP stages in response to exogenous sucrose without gravitropic bending for 3 independent experiments; 25 < n < 27 for each independent experiment. Error bars are the standard error of the mean (SEM).

(B) Mean LRP stages in response to exogenous sucrose supplied shoot 6h after gravitropic bending for 3 independent experiments; n = 27 for each independent experiment. Error bars are the standard error of the mean (SEM).

(C) Mean LRP stages in response to exogenous sucrose supplied shoot 12h after gravitropic bending for 3 independent experiments; n = 27 for each independent experiment. Error bars are the standard error of the mean (SEM).

(D) Mean LRP stages in response to exogenous sucrose supplied via shoot 18h after gravitropic bending for 3 independent experiments; n = 27 for each independent experiment. Error bars are the standard error of the mean (SEM

(E) Mean LRP stages in response to exogenous sucrose supplied via shoot 24h after gravitropic bending for 3 independent experiments; 26 < n < 27 for each independent experiment. Error bars are the standard error of the mean (SEM

(F) Mean LRP stages in response to exogenous sucrose supplied via shoot 36h after gravitropic bending for 3 independent experiments; 26 < n < 27 for each independent experiment. Error bars are the standard error of the mean (SEM).

4.4.2 Exogenous sucrose supplied via shoot increases the rate of LRP progression.

The conclusion that exogenous sucrose accelerates the progression of LRPs necessitated the calculation of the rate of LRP progression. The rate of progression of LRP progression data based on the distribution of LRPs at the various time points discussed earlier will help to determine whether there is a particular stage or checkpoint at which sucrose accelerates the progression of LRPs or the acceleration of LRP progression by sucrose occurs across all developmental stages. The question then is: is sucrose a driver of the developmental progression of LRPs or does it merely fuel a factor (e.g., auxin) to drive the developmental progression of LRPs? As discussed earlier in section 4.1 of this chapter, the study discussed in the chapter 3 of this thesis identified stage 1 as a potential checkpoint of the regulation by sucrose, however, a more synchronised initiation of LRPs under the current experimental conditions and a time series data were needed to attempt to unequivocally identify potential checkpoints from which sucrose promotes LRP progression.

The rate of LRP progression is the differential of average LRP stages (number [n]) at two consecutive time points per hour [h] in response to exogenous sucrose. The unit of calculation of the rate of LRP progression is, therefore, n/h. The rate of LRP progression indicates how fast or slow LRPs progress from one stage to the other in the presence or absence of exogenous sucrose. To calculate the rates of LRP progression in response to exogenous sucrose supply at the various time points stated in section 4.4.1, the individual LRP stages were assigned numerical values (Table 4.7), these numerical values were the same as the numbers that indicate the various stages (Table 4.7). It is noteworthy that the allocation of stages to LRPs as pioneered by Malamy and Benfey (1997) (Malamy and Benfey, 1997) is purely artificial and it is by no means indicative of any ‘numbering’ of LRPs *in planta*, but only to categorise and characterise the various stages of LRP development based on their morphological features for research purposes.

It is also noteworthy that the data collected from LRP distribution in the study presented in this chapter and elsewhere in this thesis is categorical, in the sense that they represent distinct morphological features of the LRPs at each development stage as explained earlier thus the assignment of numerical values to represent the stages was merely done to aid in mathematical calculations from which statistical analyses could be run to make predictions on the developmental progression of LRPs in response to sucrose availability *in planta*.

The time points studied as stated in section 4.4.1 were categorised into three different interval classes: 6h interval (0h, 6h, 12h, and 18h),;12h interval (0h, 12h, 24h, 36h), and 24h interval (0h, 24h, 48h, and 72h). These interval classes of the time points were chosen to understand the temporal dynamics of the effect of exogenous sucrose on the progression of LRPs at discrete time points, and to also understand whether there was a potential checkpoint of regulation by sucrose that was dependent on a ‘developmental time window’. Averages of LRPs at specific time points were calculated (Table 4.8), and these values were used to calculate the differential between two successive points. The differential of the averages of LRPs (n) at two successive time points were divided by the difference between the time points (h) to determine the rates of progression as described in section 4.3 of this chapter.

The data showed that the median and modal LRP stage at 0h was 0 in both the absence and presence of exogenous sucrose (Table 4.8). The mean LRP stage in the presence of exogenous was 0.25, this was due to the presence of some (26.58%) LRPs at stage 1 (Table 4.8 and Figure 4.5B). The mean LRP stage in the absence of sucrose was 0 (Table 4.8). At 6h after gravitropic bending, the modal and median LRP stage was 1 and the mean LRP stage was 1.09 (Table 4.8). In the presence of sucrose, the median and modal LRP stages were 2, and the mean LRP stage was 2.09 (Table 4.8). This suggests a progression of LRP one stage further in the presence of exogenous sucrose compared to the absence of exogenous sucrose.

There were statistically significant differences in the progression of LRPs between 0% sucrose-treated roots and 1% sucrose-treated roots 6h after gravitropic bending (Table 4.9). The median of progression from stage 0 (at 0h) to LRP stages observed at 6h after gravitropic bending in the absence of exogenous sucrose was 0.1667 n/h (Table 4.9). There was a two-fold increase in the rate of progression in the presence of exogenous compared to the absence of exogenous sucrose (median rate of progression: 0.3333 n/h (Table 4.9). This result is supported by the observation of 88.89% LRPs at stage 2 at 6h after gravitropic bending in the presence of exogenous sucrose compared to the 91.36% of LRPs observed at stage 1 in the absence of exogenous sucrose (Figure 4.5D). There were > 70% of LRPs observed at stage 0 at 0h without rotation (Figure 4.5B). This suggests that LRPs progressed two stages further in response to exogenous sucrose supply compared to the one-stage further progression in the absence of exogenous sucrose supply, corroborating the already established conclusion that the supply of exogenous accelerates the progression of LRPs.

At 12h after gravitropic bending, the median and modal LRP stages were 3, and the mean LRP stage was 2.95 in the absence of exogenous sucrose (Table 4.8). In the presence of exogenous sucrose, the median and modal LRP stage were 4 and the mean LRP stage was 3.87 (Table 4.8). This suggests a progression of LRP one stage further in the presence of exogenous sucrose from the average LRP stage observed in the absence of exogenous sucrose. The rate of progression of LRP from stages observed at 6h after gravitropic bending to the stages observed at 12h after gravitropic bending in the absence of exogenous sucrose was 0.3333 n/h (Table 4.9). The rate of progression of LRPs remained significantly constant even in the presence of exogenous sucrose 0.3333 n/h (Table 4.9). This observation was supported by a progression of LRPs two stages further from their previous stages at 6h under both conditions. In the absence of exogenous sucrose, > 90% of LRPs were found at stage 1 at 6h after gravitropic bending, and > 80% of LRPs were found at stage 3 at 12h after gravitropic bending (Figures 4.5D & 4.5F).

However, in the presence of exogenous sucrose, > 80% of LRPs were found at stage 2 after 6h of gravitropic bending and > 60% of LRPs were found at stage 4 after 12h of gravitropic bending (Figures 4.5D & 4.5F). These data suggest that at 12h after gravitropic bending, LRPs progress two stages further from the average LRP stages observed at 6h after gravitropic bending in the absence of exogenous sucrose.

At 18h after gravitropic bending, the median and modal LRP stage was 4 and the mean LRP stage was 4.08. In the presence of exogenous sucrose, the median and modal LRP stage were 6 and the mean LRP stage was 5.55. This suggests a progression of two stages further in the presence of exogenous sucrose compared to the average LRP stages observed in the absence of exogenous sucrose. The rate of progression of LRP from stages at 12h to stages at 18h in the absence of exogenous sucrose was 0.1667 n/h (Table 4.9). There was a statistically significant increase (two-fold) in the rate of progression in the presence of exogenous at this time point (0.3333 n/h; Table 4.9), This two-fold increase in the rate of progression in the presence of exogenous sucrose at 18h after gravitropic bending was supported by a progression of LRPs two stages further from the average stages observed at the previous time point, 12h after gravitropic bending (Figure 4.5F). These data show that at 18h after gravitropic bending, > 50% of LRPs progressed two stages further from their previous stages observed at 12h after gravitropic bending further confirming the hypothesis that exogenous sucrose accelerates the progression of LRPs. Although the progression of LRPs in the plants studied at the 6h interval was accelerated in the presence of exogenous sucrose compared to the absence of exogenous sucrose, the speed of progression remained constant in plants studied at the 6h interval class (Figure 4.8B). Taken together, these data suggest that the developmental progression of LRPs is accelerated by sucrose, but it may not be the main factor driving the developmental progression.

4.4.3 The method for calculating rate of progression is more suitable for early timepoints.

The rate of progression of LRPs from observed stages at 0h to the observed stages at 12h (12h time interval class) in the absence of exogenous sucrose was 0.25 n/h (Table 4.10). The rate of progression of LRPs from observed stages at 0h to stages at 12h in the presence of exogenous sucrose was 0.3333 n/h (Table 4.10). There was a statistically significant increase in LRP progression from observed stages at 0h to observed stages at 12h in response to the supply of exogenous sucrose (Table 4.10).

At 24h after gravitropic bending, the median and modal LRP stage was 5, and the mean LRP stage was 5.19 in the absence of exogenous sucrose (Table 4.10). In the presence of exogenous sucrose, the median and modal LRP stage were 7 and the mean LRP stage was 6.63 (Table 4.10). This suggests a progression of LRPs two stages further from the observed stages 12 hours earlier (12h after gravitropic bending) in the presence of exogenous sucrose compared to the absence of exogenous sucrose. The rate of LRP progression at 24h after gravitropic bending in the absence of exogenous sucrose was 0.167 n/h (Table 4.10). There was a 1.5-fold increase in the rate of LRP progression in the presence of exogenous sucrose (0.25 n/h; Table 4.9) compared to the absence of exogenous sucrose (0.167n/h; Table 4.10). There was a statistically significant increase in the progression of LRPs from observed stages at 12h to observed stages at 24h in response to exogenous sucrose supply (Table 4.10).

At 36h, the median and modal LRP stage was 8 and the mean was 7.81 (Table 4.8) in the absence of exogenous sucrose. In the presence of exogenous sucrose, the median and modal were 8.86 and the mean was 9 (Table 4.8). The rate of LRP progression from stages observed at 24h to the observed stages at 36h after gravitropic bending in the absence of exogenous sucrose was 0.25 n/h (Table 4.10).

There was a statistically significant decrease in the rate of LRP progression in the presence of exogenous sucrose (0.1667 n/h; Table 4.9). This was because > 80% of LRPs had emerged and become outgrown LRs at 36h after gravitropic bending (Figure 4.5L). At later time points (36h onwards; Figure 4.8; Table 4.11), in the presence of exogenous sucrose, and at 48 to 78h in the absence of exogenous sucrose, as the number designating an LRP stage for two successive time points get bigger, the differential of the average LRPs at the two successive time points gets smaller, making the rate of LRP progression small (as the rate of LRP progression is directly proportional to the differential of the LRP stages between the two successive time points under comparison) (Table 4.10; Figure 4.8).

This observation is a glaring mathematical artifact as the rates of LRP progression at time points after 24h make it seem as though LRPs stop growing or developing at some developmental times, especially after emergence *in planta.* The median LRP stage in the presence of exogenous sucrose at 36h was 9 which is the post-emergence stage (outgrown LR) (Table 4.8; Figure 4.8A), the same median stages were observed at 48 and 72h in the presence and of exogenous sucrose (and the absence of exogenous sucrose). The rates of LPR progression data, therefore, suggest that LRs stop growing after emergence which is physiologically inaccurate. Of course, under non-optimal physiological conditions or soil conditions (e.g., drought stress) LR development may be inhibited (e.g., Inhibition of LR elongation under drought stress conditions) (Xiong et al. 2006). However, the conditions used for the experiments discussed in this chapter and elsewhere in this thesis were non-stress conditions as was shown in chapter 3 of this thesis. This mathematical artifact likely explains why the rate of LRP progression from stages observed at 24h to stages observed at 36h after gravitropic bending in the presence of exogenous sucrose is smaller compared to the rate of LRP progression in the absence of exogenous sucrose (Table 4.10).

This mathematical artifact is even more pronounced at 48 to 72h (the 24h time interval). The rate of LRP progression in the absence of exogenous sucrose was 0.125 n/h at 24h (24h time interval) after gravitropic bending (Table 4.11), and the rate of LRP progression increased to 0.2083 n/h in the presence of exogenous sucrose (Table 4.10). However, at 48h after gravitropic bending, the rate of LRP progression was lower (0.167 n/h; Table 4.11) in the presence of exogenous sucrose. The median LRP stages at 24h after gravitropic bending were 5 and 7 in the absence and presence of exogenous sucrose, respectively (Table 4.8), while the median LRP stage at 48h was 9 under both exogenous sucrose conditions (Table 4.8). This shows that at 48h after gravitropic bending, LRPs progressed 4 stages further from the average LRP stages observed at 24h after gravitropic bending in the absence of exogenous sucrose and merely 2 stages further from the average observed stages at 24h in the presence of exogenous sucrose, this together with a lower rate of LRP progression reported seems to suggest a reduced progression of LRPs in response to exogenous sucrose supply at 48h, but this is inaccurate as discussed earlier. This seeming reduction in LRP progression was much pronounced at 72h after gravitropic bending when the rates of LRP progression had become zero because all LRPs had emerged and outgrown (stage 9) in both the presence and absence of exogenous sucrose (Table 4.11; Figures 4.8). Taken together, these data show that the method for calculating the rates of LRP progression is not useful for the analyses of time series data on LRP stages at time points later than 24h. Already in the root biology community, the lengths of LRs are measured to study the effects of treatments on LR elongation and outgrowth in response to exogenous sucrose treatment (Freixes et al. 2002; Macgregor et al. 2008), however, this was not done in the study presented in this chapter because the focus was LRP formation, and not LR elongation and outgrowth as already explained in chapters 1 and 3.

Table 4.7 Numerical values assigned to LRP stages to aid in the calculation of rates of LRP progression.

LRP STAGE	**ASSIGNED NUMERICAL VALUE**
0	0
1	1
2	2
3	3
4	4
5	5
6	6
7	7
8	8
9	9

Table 4.8 Mean, median, and modal LRP stages at designated time points in response to exogenous sucrose supply.

	0% SUCROSE		**1% SUCROSE**		
		LRP stages			
Timepoint/h	**Mean**	**Median**	**Mean**	**Median**	**Mode**
0	0	0	0.25	0	0
6	1.09	1	2.29	2	2
12	2.95	3	3.87	4	4
18	4.08	4	5.55	6	6
24	5.19	5	6.63	7	7
36	7.81	8	8.86	9	9
48	8.36	9	9	9	9
72	9	9	9	9	9

$79 < n < 81$; datasets were pooled from 3 independent experiments. $25 < n < 27$ for each independent experiment.

Table 4.9 Mann-Whitney U tests on the progression of LRPs in response to exogenous sucrose supply in the 6h interval class of time points.

6h intervals							
0% sucrose				**1% Sucrose**			
Intervals	(0-6h)						
Rate of LRP progression [n/h]							
Median	0.167				Median	0.333	
Mann-Whitney test for "equal medians"							
0h		6h		0h		6h	
Mann-Whitn U :	40			Mann-Whitn U :	84		
z :	12.321	p (same med.):	6.96E-35	z :	11.969	p (same med.):	5.16E-33
Intervals	(6-12h)						
Rate of LRP progression [n/h]							
Median	0.167				Median	0.333	
Mann-Whitney test for "equal medians"							
6h		12h		6h		12h	
Mann-Whitn U :	143.5			Mann-Whitn U :	661.5		
z :	11.584	p (same med.):	4.96E-31	z :	10.08	p (same med.):	6.77E-24
Intervals	(12-18h)						
Rate of LRP progression [n/h]							
Median	0.167				Median	0.333	
Mann-Whitney test for "equal medians"							
12h		18h		12h		18h	

Mann-Whitn U :	362			Mann-Whitn U :	356.5			
z :	10.746	p (same med.):	6.16E-27	z :	10.339	p (same med.):	4.68E-25	

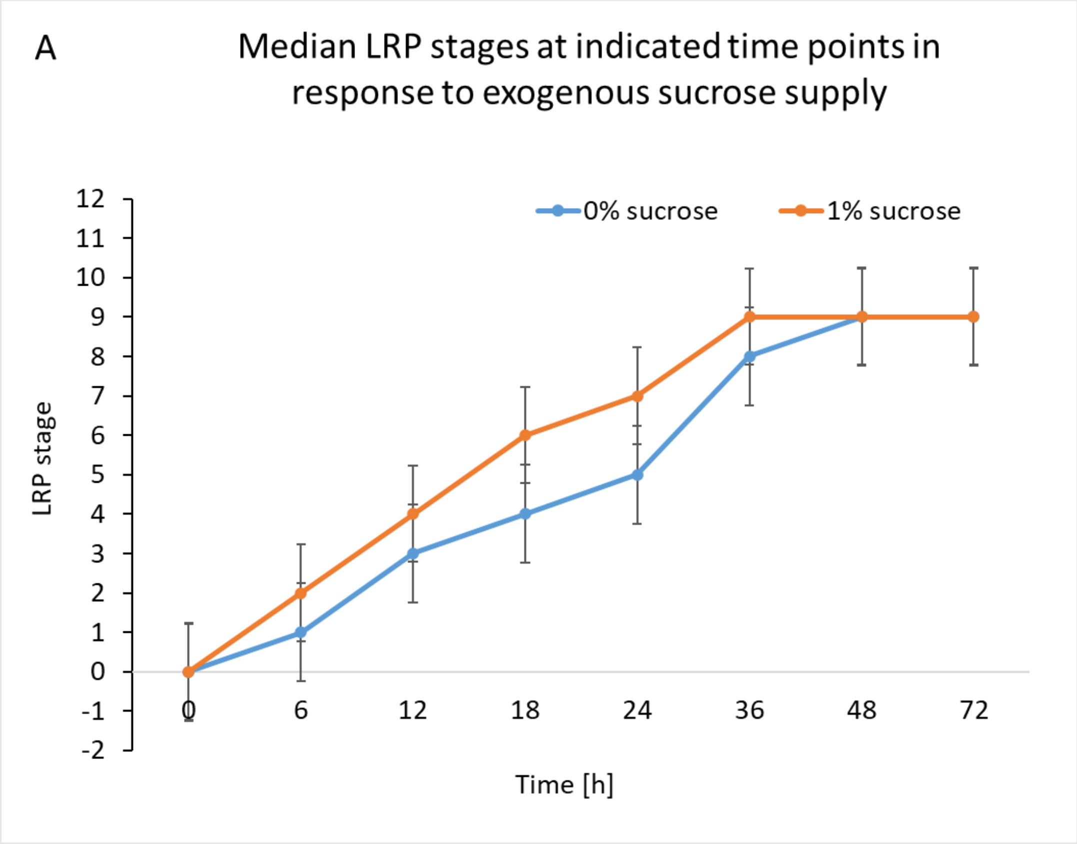
79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded grey indicate statistical significance (p<0.05). Mann Whit U is the Mann-Whitney U test (Mann and Whitney 1947). Same med signifies the same median.

Table 4.10 Mann-Whitney U tests on the progression of LRPs in response to exogenous sucrose supply in the 12h interval class of time points.

12h intervals								
0% sucrose				**1% Sucrose**				
Intervals	(0-12h)							
Rate of LRP progression [n/h]								
Median	0.25				Median	0.333		
Mann-Whitney test for "equal medians"								
0h		12h		0h		12h		
Mann-Whitn U :	0			Mann-Whitn U :	0			
z :	12.194	p (same med.):	3.36E-34	z :	11.656	p (same med.):	2.14E-31	
Intervals	(12-24h)							
Rate of LRP progression [n/h]								
Median	0.167				Median	0.25		
Mann-Whitney test for "equal medians"								
12h		24h		12h		24h		
Mann-Whitn U :	83			Mann-Whitn U :	125.5			
z :	11.382	p (same med.):	5.17E-30	z :	10.955	p (same med.):	6.26E-28	
Intervals	(24-36h)							
Rate of LRP progression [n/h]								
Median	0.25				Median	0.167		
Mann-Whitney test for "equal medians"								
24h		36h		24h	36h			

Mann-Whitn U :	196			Mann-Whitn U :	28			
z :	10.651	p (same med.):	1.73E-26	z :	11.609	p (same med.):	3.73E-31	

79 < n < 81; datasets were pooled from 3 independent experiments. The cells shaded grey indicate statistical significance (p<0.05). Mann Whit U is the Mann-Whitney U test (Mann and Whitney 1947). Same med signifies the same median.


A
Median LRP stages at indicated time points in response to exogenous sucrose supply
0% sucrose
1% sucrose
LRP stage
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
0
6
12
18
24
36
48
72
Time [h]

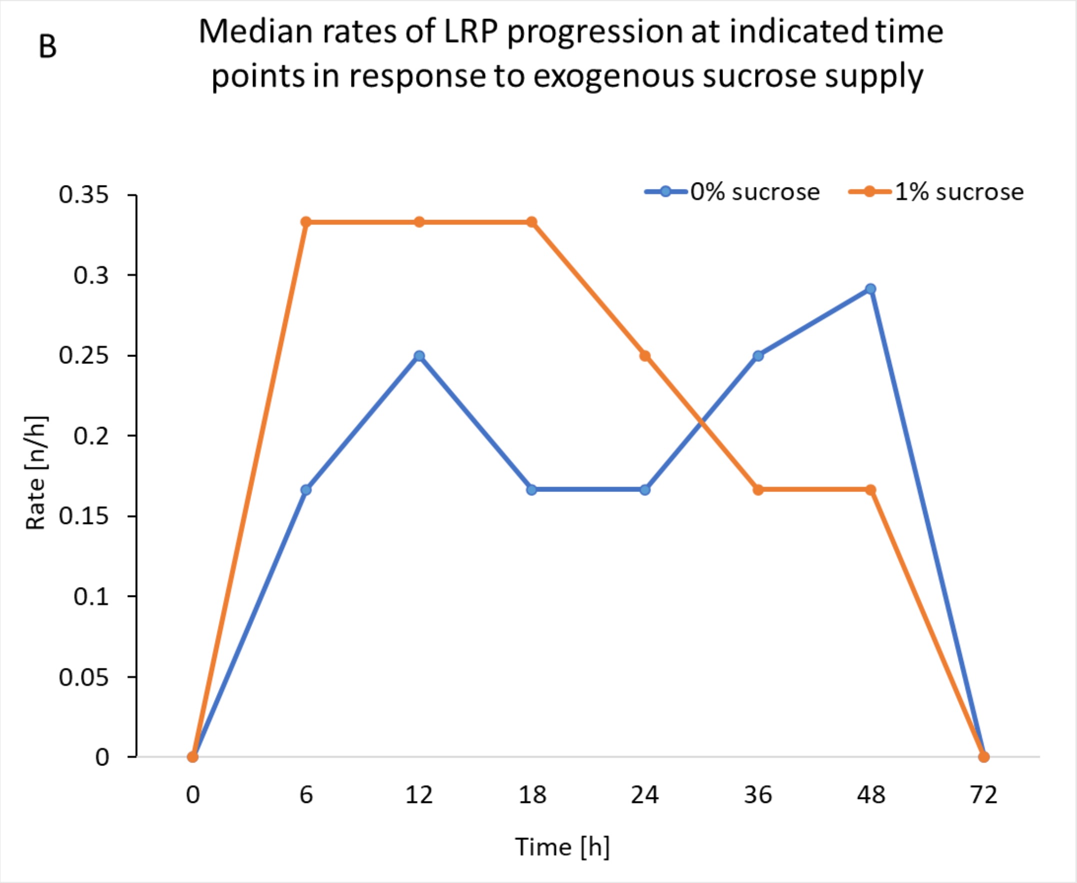

B
Median rates of LRP progression at indicated time points in response to exogenous sucrose supply
0% sucrose
1% sucrose
Rate [n/h]
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
6
12
18
24
36
48
72
Time [h]

Figure 4.8 **Progression of LRPs in response to exogenous sucrose supply.**

(A) Median LRP stages at time points (6-72h) show the progression of LRPs in reference to the 0h under each exogenous sucrose condition. Error bars are the standard error of the mean, 79 < n < 81 for 0 to 36h; 25 < n < 27 for 48 to 72h.

(B) Median rates of LRP progression in response to exogenous supply. 79 < n < 81 for 0 to 36h; 25 < n < 27 for 48 to 72h.

Table 4.11 Median rates of LRP progression at 24h time interval class.

Rates of LRP progression				
	0% sucrose		1% sucrose	
Intervals	**per 24h**	**per h**	**per 24h**	**per h**
1 (0-24h)				
	3	0.125	5	0.2083
2 (24-48h)				
	6	0.25	4	0.1667
3 (48-72)				
	0	0	0	0

Datasets pooled from two independent experiments;25 < n < 27; for each independent experiment.

4.5 Discussion

Exogenous sucrose and factors that promote the synthesis of endogenous sucrose (e.g., increased light intensities and increased CO_2) are known to increase LR numbers and outgrowth (Freixes et al., 2002; Takahashi et al. ,2003; Lee-Ho et al., 2007; Macgregor et al., 2008). However, in the absence of data on the effect of sucrose both endogenous and exogenous on the LR initiation and LRP morphogenesis as determined by the numbers, densities, and distribution among stages, a mechanistic understanding of how sucrose promotes LR formation cannot be determined. This is because the determination of checkpoints of potential regulation by sucrose cannot be determined in the absence of LRP distribution data as explained in detail in chapters 1 and 3. Chapter 3 of this thesis showed that exogenous sucrose promotes the progression of LRPs from early stages (≤ stage 3) to later stages (> stage 3), and the current chapter has shown that the supply of exogenous sucrose promotes the progression of LRPs by increasing the rate of their progression from the early stages to later stages - this was evidenced by the concentration of LRPs at more advanced stages in response to 1% exogenous sucrose compared to 0% exogenous sucrose. The study presented in this chapter has, therefore, corroborated the results in chapter 3, by showing that LRP progression is indeed accelerated in response to exogenous supply (Figures 4.5 & 4.6).

Upon gravitropic bending, LRPs are induced as early as 6 hours (Ditengou et al., 2008). In the study presented in this chapter, a majority of LRPs were found at stage 0 under both 0% and 1% exogenous sucrose conditions at 0h (no rotation), however, the scoring of the stage 0 LRPs in this study may have been an over quantification as some LRPs considered as stage 0 may have been at the ground state. This was potentially due to the lack of distinction between the ground state and the nuclear migration stage (stage 0) (Figure 4.9) at the time the experiments presented in this chapter were conducted and the resulting data analysed.

Both the ground state and the characterised by two nuclei (there are single nuclei in the ground state sometimes; white arrowhead; Figure 4.9A), this may have caused an over-allocation of LRPs to stage 0 in the study presented in this chapter. Similar to the results by Ditengou et al (2008), in the study presented in this chapter, at as early as 6 hours, LR initiation (stage 1) had already occurred in over 90% of the roots studied under 0% exogenous sucrose conditions and over 80% of LRPs had progressed to stage 2 under 1% sucrose conditions.

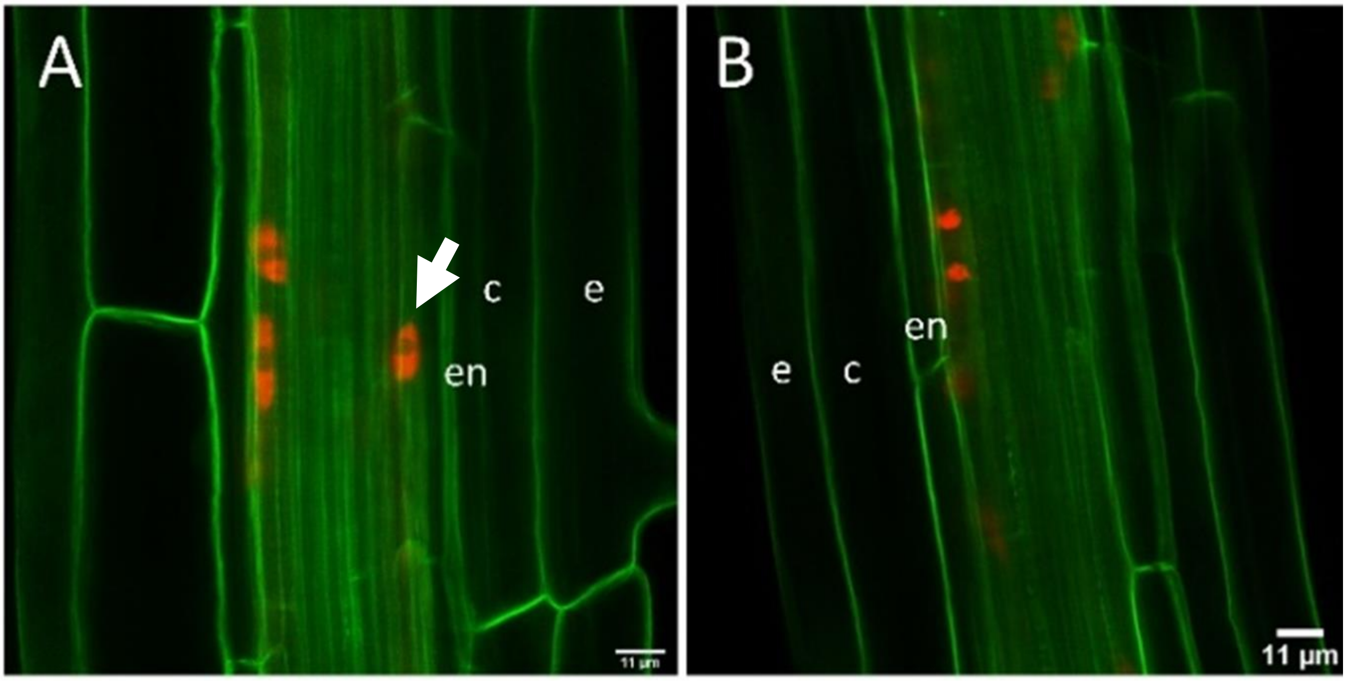


Figure 4.9 **Distinction between the ground state and stage 0**

Confocal images of xylem pole pericycle layer and LRP stages in roots of 7-day old seedlings of *GATA23:H2B:3xmCherry/pUBI: 3xmCherry:3xGFP:PIP1,*4 reporter line. (A) Ground state xylem pole pericycle cell layer. The nuclei of the ground state pericycle cells are lens-shaped compared to the round nuclei of stage 0. White arrowhead represents a ground state XPP cell with a single nucleus (B) Stage 0 LRP characterised by the migration of two adjacent nuclei toward their common cell wall. en: endodermis, c: cortex, e: epidermis. Bar is 11 µm. Images were taken by Frank Machin.

The study described in this chapter has shown that exogenous sucrose increases the rate of LRP progression (Tables 4.9 & 4.10). Although LRPs still progressed under 0% exogenous sucrose conditions, due to the effect of endogenous sucrose availability to the root, as discussed in section 3.5 of chapter 3 of this thesis, the progression of LRPs under 0% exogenous was slower, compared to 1% exogenous sucrose. This shows that sucrose is an accelerator of the developmental progression of LRPs from early stages (≤ stage 3) to later stages (> stage 3). LRP morphogenesis is underpinned by cell division, therefore a stimulatory effect of 1% exogenous sucrose could be its effect on cell division during LRP morphogenesis, either directly or indirectly. Sucrose could indirectly promote cell division during LRP development by inducing some or all of the components of the auxin homeostasis pathway (Lilley et al., 2012; Sairanen et al., 2012). Sucrose could directly promote LRP developmental progression by inducing the expression of key factors involved in the regulation of cell cycle progression (e.g., *CYCLIN D* (*CYCD*) (Gaudin et al. 2000; Riou-Khamlichi et al., 2000; Gutierrez, 2009).

The visualisation of the rates of LRP progression data in response to exogenous sucrose supply showed that while the supply of exogenous sucrose to accelerates the progression of LRPs, the acceleration (speed) remained constant at the 6h time interval class (Figure 4.8B). The acceleration seemed to decline after 18h, however, this is likely a mathematical artifact as discussed in section 4.4.3 of this chapter. This mathematical artifact arose due to the non-allocation of stages to lateral root organs beyond stage 9 in this thesis. In the broader literature and the root biology community, the allocation of stages to LRPs has been strictly limited to the pioneering categorisation by Malamy and Benfey (1997), which stages LRPs from stages 1 to 8 (Malamy and Benfey, 1997). Although in this thesis LRPs have been staged from stages 0 to 9 (as explained earlier in chapters 1 and 2) of this thesis, the numerical values for calculating the rates of progression for more advanced stages after 18h (later time points, hereafter) begin to cancel out as LRP stages become similar at later time points.

This, therefore, makes it seem as though lateral root organs do not progress or continue to develop after emergence—they do. Therefore, the formula used to calculate the rates of LRP progression in this chapter is not useful at later time points and therefore must only be used for the calculations of rates of LRP at early time points (0h to 18h). Nevertheless, to use the formula devised in this chapter for studies on LRP developmental progression after emergence, further characterisation of the morphology of emerged and outgrown LRs should be done, and stages allocated accordingly to avoid mathematical artifacts which would muddle biological interpretation of the rates of LRP progression calculated. For example, the lengths of LRs could be measured at later time points, and therefore the calculation of rates of LRP progression could be could restricted to studies on the progression of LRPs from stages 0 to stage 6. Stage 6 was the most advanced LRP stage between the presence and absence of exogenous sucrose conditions at the early time points studied (0 to 18 h) (Table 4.2; Figure 4.8A).

The observation that the supply of exogenous sucrose accelerates LRP progression, but the acceleration potentially remains constant across developmental times suggests that sucrose may not be the driver of LRP development. And that it stimulates or induces, or fuels a factor that drives LRP development, and this factor is likely to be auxin as explained earlier in this chapter. In the gravitropic bending assay, LRPs are induced due to the relocalisation of auxin transporters (e.g., PIN1) at the bend site, leading to the accumulation of auxin at the bend site which induces LRPs (Lucas et al., 2008; Ditengou et al., 2008). It has also been hypothesised that the gravistimulus applied during gravitropic bending do not promote the formation of prebranch sites themselves, but promote the development of LRPs, certainly via auxin (Moreno-Risueno et al., 2010). it is, therefore, likely to be where sucrose comes in to promote auxin transport to the bend site (probably via upregulation of PIN expression (Zhang et al., 2020) where LRPs are developed from prebranch sites and their developmental progressions accelerated. Or, sucrose may promote the synthesis of an unknown factor which will then accelerate LRP progression, or, sucrose itself will directly promote LRP progression via an unknown mechanism. Further experiments are needed to separate these possibilities; more crucially, to determine the role of sucrose in the transport dynamics at the bend site, especially by PIN1, the activity of the PIN1 protein in response to exogenous supply can be studied. These experiments could not be done due to time constraints.

4.6 Conclusion

The study presented in this chapter has corroborated the results presented in chapter 3 of this thesis and has further suggested that while sucrose accelerates developmental progression, the speed remains constant across developmental times. This, therefore, suggests that sucrose may not be driving LRP development, instead, it must be fuelling a factor, potentially auxin, which drives the LRP developmental machinery. The method used for the study presented in this chapter has also shown that the filter paper/Parafilm strip experimental method can be adapted to the gravitropic bending assay to serve as a vital tool for studies aimed at understanding the relationship between shoot-derived resources and LR formation. Further studies are needed to understand the molecular underpinnings of how sucrose accelerates the developmental progression of LRPs, especially to establish a link between auxin transport and sucrose under the experimental conditions described in this chapter.

Chapter 5 Investigating the effect of extended darkness on LRP progression.

5.1 Introduction

Light quality and quantity regulate photosynthesis which is the major process from which sucrose is synthesised (Smith, 2000). Photosynthesis is light wavelength-dependent (Yavari et al., 2021). The light wavelengths within which photosynthesis occurs, the so-called photosynthetically active radiation (PAR) are a part of the electromagnetic radiation between the wavelengths of 400 to 700 nm (McCree, 1971, 1972; Shibles, 1976; Tibbitts, 1993; Mõttus et al., 2012). The measurement of PAR has been described as the driver of photosynthesis in Plant Biology (Yavari et al., 2021). The intensity of photosynthesis is determined by the absorption of photons other than the reception of radiant energy by plant leaves (McCree 1965; McCree, 1972). Consequently, PAR is often measured as the flux of photons which is commonly termed the photosynthetic photon flux density (PPFD). PPFD is also known as light intensity with a unit of μmol photons m^{-2} s^{-1}, that is also simplified to μmol m^{-2} s^{-1} (Retkute et al. 2015; Yavari et al. 2021), and sometimes, mol $d^{-1}m^{-2}$ (Freixes et al., 2002).

Low light intensities lead to a low rate of photosynthesis as shown in the decreased expression of genes and the activity of Calvin cycle enzymes involved in CO_2 fixation and regeneration of Ribulose 1,5-bisphosphate (RuBP). This, therefore reduces the potential for carbon assimilation in plants (Sharkey et al., 2007). Variations in light intensities (e.g., low light intensity at 120 μmol m^{-2} s^{-1} and high light intensity at 460 μmol m^{-2} s^{-1}) are expected endogenous sucrose levels (Freixes et al., 2002), which is expected to affect PR growth and LR formation. Reduced lengths and outgrowth of LRs are the results of growing Arabidopsis plants under low light intensities. Conversely, high PR growth rates and longer LRs are the results of the growth of Arabidopsis seedlings under higher light intensity (Freixes et al., 2002).

In the absence of sucrose in the medium, the elongation rate of PRs doubled from 5.2 to 10.7 mm d−1(per day) when daily PPFD (light intensity) increased from 5·2 mol $m^{-2}d^{-1}$ (120 µmol m^{-2} s^{-1}) to 19·8 mol $m^{-2}d^{-1}$ (460 µmol m^{-2} s^{-1}). Reduced PR elongation rate under low light intensity (120 µmol m^{-2} s^{-1}) was restored by the addition of 0.5% exogenous sucrose (Freixes et al., 2002). A high PR elongation rate could be achieved either under high light intensities without exogenous sucrose in the medium or at low light intensities with sucrose in the medium (Freixes et al., 2002). A similar pattern was observed in the elongation rates of LRs (Freixes et al., 2002). Additionally, increases in light intensities caused an increase in the concentrations of endogenous hexose and sucrose in the elongation zone of the PR (Freixes et al., 2002).

Measurement of hexose (fructose and glucose) concentrations in the elongation zone of the PR as well as the differentiation zone (where LRPs initiate and develop) at varying light intensities (120, 290, and 460 µmol m^{-2} s^{-1}) showed higher hexose concentration in PRs of plants grown at high PPFD or in the presence of sucrose than plants grown without sucrose and at low PPFD (Freixes et al., 2002). Higher hexose concentrations were found at the differentiation zone of the PRs of plants grown at high light intensity (460 µmol m^{-2} s^{-1}) than in plants grown at low light intensity (20 µmol m^{-2} s^{-1}) with a two-fold and three-fold difference between both conditions for hexoses and sucrose, respectively (Freixes et al. 2002b). Similarly in another study, 8-day-old Arabidopsis seedlings grown for longer (up to 5 days) under low light intensity (15µmol·m^{-2}·s^{-1}) and returned to control light conditions (70 µmol·m^{-2}·s^{-1}, 16h/light, 8h/dark cycle) showed reduced LR densities compared to plants grown only under control light conditions and those grown for up to 3 days under low light conditions and returned to control conditions (Muralidhara et al., 2021).

Taken together, these data suggest that light availability promotes PR growth and LR outgrowth, likely via its role as an energy source for sucrose synthesis. It is worth noting that light also plays a role in root growth and development via its role as a signal. For example, the *ELONGATED HYPOCOTYL 5* (*HY5*) which is a major light-regulated transcription factor moves from the shoot to the root to promote root growth and development (Oyama et al., 1997; Lee et al., 2016). *HY5* has been reported LR emergence in a Red: Far-red (R: FR ratio) dependent manner (van Gelderen et al., 2018), however, this relationship between components of light signalling and LR formation is not the focus of this chapter.

In plants, there is a diurnal alternation and changes in carbon supply (Gibon et al., 2009). During the day under soil conditions or light cycles under experimental conditions, photosynthesis provides the carbon (sucrose) needed for plant growth and development. Some of the sucrose is converted to transitory starch during the day or light cycles to be metabolised into sucrose to support plant growth and development at night/dark cycles when photosynthesis is impossible (Sulpice et al. 2014; Zeeman et al. 2010). Starch metabolism is linear, such that starch is almost used up, but not completely during the night/dark periods (Gibon et al., 2004; Smith and Stitt, 2007; Graf et al., 2010).

An extension of the dark periods could lead to carbon starvation that could impact plant growth and development (e.g., reduce PR growth and LR densities) (Miotto et al., 2021). The Extension of the dark periods, termed unexpected darkness or simply extended darkness has been used to study the effect of the perturbation in carbon levels or energy on LR formation (Muralidhara et al., 2021). For example, growth of plants under short-term extended darkness during the light period, starting 2h after onset of light for a period (0.5h, 1h, 2h, and 4h) and then returned to control conditions (70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) resulted in significant increases in LR numbers and densities already as early as 0.5h of treatment. Additionally 4h of extended darkness led to significant increases in LRP numbers compared to control light conditions, and these LRPs could not emerge out of the PR until after return to light, suggesting an effect of extended darkness on LR emergence (Muralidhara et al., 2021).

The aforementioned authors did not study the effect of extended darkness over long periods on LR numbers and densities, but most importantly the effect of extended darkness on the developmental progression of LRPs. This, therefore, raises the question of whether long periods of extended darkness (without exogenous sucrose supply) would lead to carbon starvation which would affect the distribution of LRP stages and reduce their developmental progression. Based on the conclusions made in chapter 4 of this thesis on the lower rates of LRP progression, especially in the early stages under 0% exogenous sucrose conditions, it is expected that long periods of extended darkness treatments would lead to either low rates of LRP progression or no progression at all (arrest of LRPs at specific stages). The study presented in this chapter, therefore, set out to determine the effect of extended darkness on developmental progression.

Dark treatment is expected to reduce cell division, due to its reported effect on reducing the size of the RAM (Sassi et al., 2012; Miotto et al., 2021). The reduction in the size of the RAM is reported not to be due to changes in meristem patterning, but rather an inhibition in cell proliferation (Sassi et al., 2012; Miotto et al., 2021). This inhibition in cell proliferation was confirmed by a reduced GUS activity in the *CYCB1;1:GUS* reporter line; additionally, the inhibition in the cell proliferation due to a reduction in RAM size was reversed when dark-grown plants were exposed to light (Sassi et al., 2012; Miotto et al. 2021). Dark-induced inhibition of cell division is due to its effect on the PIN2 transporter (Laxmi et al. 2008). Dark treatments lead to the vacuolar targeting of PIN2, disrupting the expected subcellular localisation of PIN2 in the plasma membrane, this is evidenced by a reduction in PIN2 abundance in the plasma membrane (Laxmi et al. 2008). Because PIN2 is a membrane transporter, the absence of its expression in the plasma membrane leads to defects in auxin transport. This defect in auxin transport and availability explains the inhibition in cell division under dark conditions (Laxmi et al., 2008; Sassi et al., 2012)

Based on the understanding of the crucial role of cell division in LRP development, especially the very first formative divisions required for LRP initiation, it is expected that extended darkness treatments could lead to an inhibition of LRP initiation. The question becomes whether, the supply of exogenous sucrose would restore a potential inhibition in LRP initiation in extended darkness-treated roots. Exogenous sucrose and light availability are reported to act synergistically to promote root growth and development under experimental conditions and that exogenous sucrose alone is not sufficient to override the requirement of light for root growth and development (Miotto et al., 2021).

In the study by Miotto et al (2021) 4dag seedlings were grown in complete darkness with or without an exogenous sucrose supply, the shoots of the plants were exposed to light for 8 hours and then returned to complete darkness for an additional 6 days (Miotto et al., 2021). After 6 days of growth, the data revealed that seedlings grown with or without exogenous sucrose conditions grew short PRs. The authors, therefore concluded that although the supply of exogenous could induce root growth, it is not sufficient to bypass the need for a continuous shoot illumination probably as a signal for root growth (Miotto et al., 2021). In that study, LRP formation was not studied, and the conditions under which the study was conducted defer from the conditions used in chapter 4 which are the same conditions used in this chapter. Miotto and co-authors supplied exogenous sucrose to both shoot and root and not to the shoot only, and based on the results from chapter 3, there will be a differential response of LRP development to exogenous sucrose under their conditions and the conditions of this study. In that respect, the current study set out to also ask whether the supply of exogenous sucrose to dark-treated plants would restore any dark-induced phenotype that may be observed.

Although green light is a part of the photosynthetically active radiation (PAR) (Mõttus et al., 2012), it is speculated not to be absorbed by the plant and instead reflects it, explaining the pleasant green color of plants, however, a recent study disputes this long-held knowledge that plant leaves absorb green light (Hao et al., 2023). Additionally, green does not affect phytochromes because they are red/far-red light-absorbing (Chen and Chory, 2011; Arsovski et al., 2012; Hao et al., 2023), therefore it is often used in illuminating dark rooms in photobiology studies. With the understanding that LRPs cannot be scored in total darkness, and with the further understanding of the responsive of LRP (Freixes et al., 2002; van Gelderen et al., 2018; van Gelderen et al., 2021) development to light, a green light lamp was used for scoring LRPs in this study.

5.2 Objectives, Questions and Hypotheses.

5.2.1 Objectives

The objectives of the study presented in this chapter were to determine the effect of extended darkness on the developmental progression of LRPs and to understand whether the supply of exogenous sucrose is sufficient to promote LRP progression independent of light availability.

5.2.2 Questions

Below were the questions asked in the study presented in this chapter:

I. Does extended darkness (carbon starvation) affect the developmental progression of LRPs?

II. Can the supply of exogenous sucrose restore LRP progression in extended darkness-treated plants?

III. Can the return of extended darkness-treated plants to light conditions restore LRP progression?

5.2.3 Hypothesis

The developmental progression of LRPs will be reduced in response to extended darkness treatment due to a reduction in endogenous sucrose levels as discussed in section 5.1 and based on the conclusions from chapters 3 and 4 of this thesis. This reduction in the developmental progression of LRPs will be reflected in reduced rates of LRP progression under extended darkness conditions compared to control light conditions. The effect of extended darkness on LRP developmental progression of LRPs would be due to its effect on cell division (Sassi et al., 2012; Miotto et al., 2021). The return of dark-treated plants to light would restore their LRP progression phenotype due to the reasons explained earlier, additionally, the supply of exogenous sucrose to dark-treated plants would restore their LRP progression phenotype due to the reported role of sucrose on PIN2 expression (Zhang et al., 2020), and the prominent role PIN2 plays in a dark-induced inhibition of cell division which drives LRP development.

5.3 Methods

5.3.1 Generic experimental design of chapter

- **Seeds sterilisation**: *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1* seeds, where indicated were ethanol sterilised and cold stratified for 2 days (Figure 5.1).
- **Pe-growth**: Seeds were pre-grown on 0.6% sucrose for 10 days (Figure 5.1).
- **Transfer**: Seedlings were transferred onto various sucrose plates as described in chapter 4 (Figure 5.1).
- **Root analyses**: LRP numbers were scored using Fiji ImageJ and epifluorescence microscopy, respectively (Figure 5.1).

5.3.2 Dark treatment

Plants were subjected to dark treatments by placing them in light-tight boxes. Plants were subjected to dark treatment at various time points: 6h and 12h, (Figure 5.1)

5.3.3 Green light treatment

Dark-treated plants were exposed to green light for 5 minutes and returned to dark light-tight boxes for an additional 6h.

5.3.4 Light restoration treatment

Dark-treated plants were returned to light conditions at 16h/day, and 8h/night, with a temperature of 22°C/day, 21°C/night, and light intensity of 150 $\mu mol/m^2/s$ as described in section 2.5 of chapter 2.

5.3.5 Sucrose restoration treatment

Using a green lamp as a source of light, 100 µl of 1% sucrose was added to the shoot of individual seedlings of dark-treated plants. Plants were returned to light-tight boxes for an additional 6h.

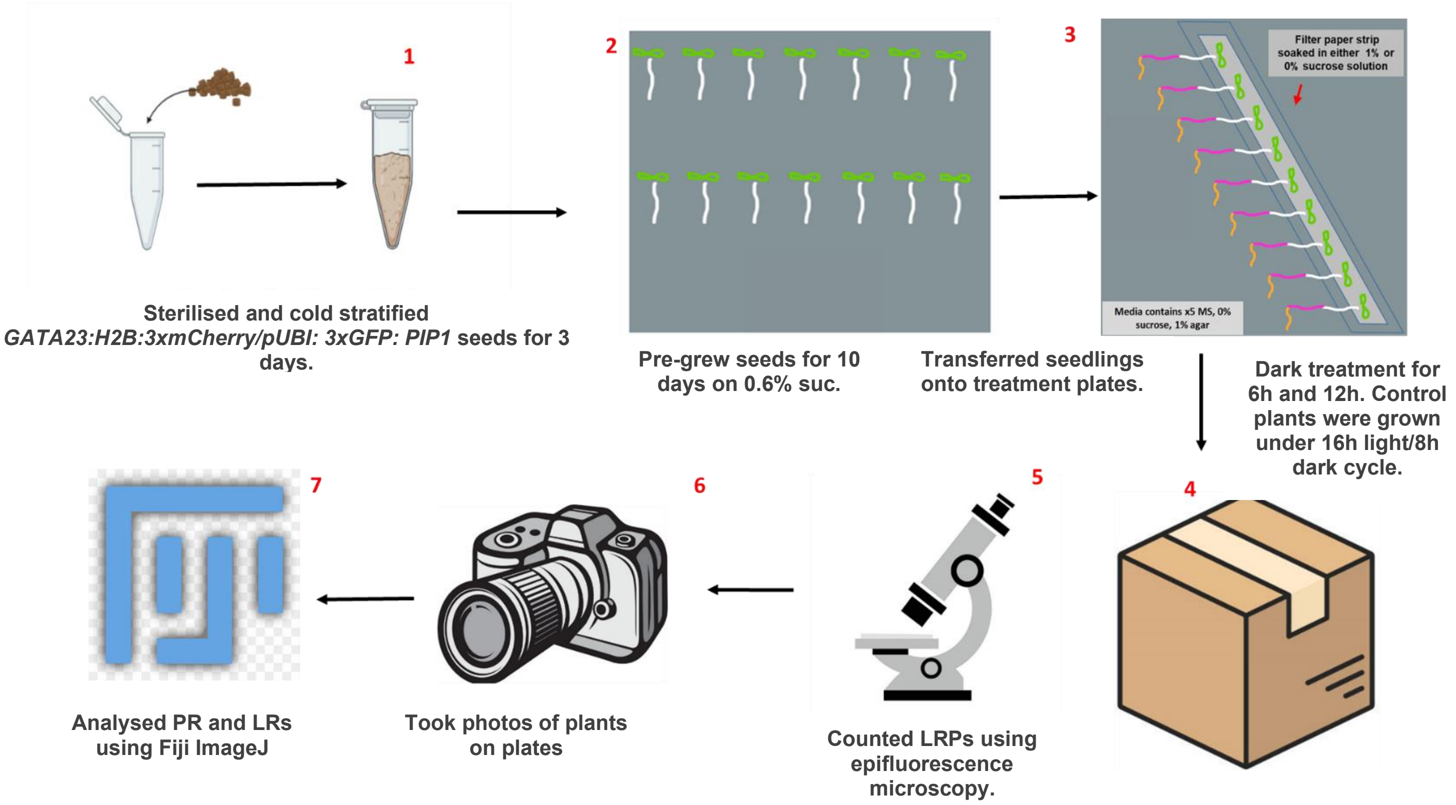


Figure 5.1 **Generic experimental design for chapter.**

(1) *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1* seeds were sterilised as described in section 3.3 of chapter 3 and cold stratified for 2 days. (2) Seeds were pre-grown on 0.6% sucrose for 10 days as described section 4.3 of chapter 4, (3) plants were transferred onto filter paper strips soaked in 0% sucrose and allowed to acclimatise for 2 days as described in section 4.3 of chapter 4, (4) plants earmarked for dark treatment were placed in light-tight boxes as described in section 5.3 of this chapter. (5) LRPs were scored as described in sections 2.6.2.3 and 2.7 of chapter 2, (6) to avoid exposure of dark-treated plants to white light, tips of PRs were marked with a scalpel; images of plates were taken using a Nikon D60 digital camera as described in section 2.6.1 of chapter 2 after LRP quantification and, (7) PR growth after a 2-day acclimation period were measured where indicated as described in section 4.3 of chapter 4.

5.4 Results

5.4.1 Exposure to green light does not affect LRP progression in extended darkness-treated plants.

Light is an essential energy source for the photosynthetic apparatus from which sucrose is produced. Therefore, controlling the intensity of light and/or its availability is expected to control endogenous sucrose levels. Low light intensity is expected to lead to low endogenous sucrose levels and potentially reduced sucrose availability to the root for LR formation (Freixes et al., 2002). Similarly, the unavailability of light should lead to the unavailability of endogenous sucrose, thereby inhibiting LR formation, and in the context of this thesis, the developmental progression of LRPs. Therefore, to understand how the unavailability of light may affect LRP progression, extended darkness experiments were conducted. To execute these experiments, light-tight boxes, as described in section 5.3 of this chapter were used. Seeds of *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4* were pre-grown for 10 days. Using the gravitropic bending assay adapted to filter paper/ Parafilm strip experimental method as discussed in chapters 4, 10-day old seedlings were transferred onto filter papers soaked in 0% exogenous sucrose. The 0% exogenous sucrose conditions were used to completely starve treatment plants of carbon, except the control plants which were grown under the long day regime (16h/light, 8h/dark) conditions in the growth cabinet (in which the experiments were conducted) throughout the experiments. There were two main experimental conditions: the extended darkness condition and the control conditions. Experiments under both conditions were conducted at two main time points: 12h and 18h after gravitropic bending. Plants for all treatments were allowed to acclimatise under the long-day regime and rotated as described in section 5.3 of this chapter. Experiments under control conditions were as described in section 4.3 of chapter 4, however, the duration of the experiments was extended to match the duration of the extended darkness treatments.

Under the16h/light, 8h/dark cycle in the growth cabinet in which these experiments were executed, plants are programmed during the pre-growth period (Figure 5.1) to expect darkness at the beginning of day/light during which endogenous sucrose is not synthesised and transitory starch is converted into sucrose to be used as an energy source (Graf et al., 2010; Zeeman et al., 2010; Sulpice et al., 2014). Consequently, these dynamics do not impact the plant's physiology, as plants are already anticipating darkness (Green et al., 2002; Dodd et al., 2005). However, when darkness is extended (extended darkness) into the expected light periods, then some developmental processes (e.g., LR formation), if not all will be inhibited. In the study presented in this chapter, extended darkness was administered by placing plates into light-tight boxes which were covered with aluminium foil. Lightboxes were then sealed with opaque brown Sellotape to prevent the entry of light into the box as described in section 5.3 of this chapter.

Both the control and extended darkness plates were rotated 2 hours into the dark phase of the 16h/ 8h, day/night (light/dark) cycle, to enable the plants under extended darkness conditions to adjust to dark conditions. Additionally, the plants earmarked to be under both conditions were allowed to grow for 6 more hours under expected darkness conditions, this was especially to allow the plants earmarked for extended darkness treatment to form a bend site, before subjecting them to the treatment. This was particularly important because, PRs were not expected to grow much, if at all under dark conditions (Miotto et al., 2021; Muralidhara et al., 2021). After the designated duration of the extended darkness treatment, LRPs at the bend sites were analysed as described in section 4.3 of chapter 4. However, because LRPs are expected to progress when exposed to white light, bend sites were analysed in a dark room illuminated with a green lamp. It is unknown whether green light would have any effect on LRP progression, therefore, the effect of green light exposure was studied by conducting a carbon starvation experiment as described earlier, and after 6h and 12h of extended darkness, plates containing extended darkness-treated plants were taken out from the lightbox and opened to allow for exposure to green light for 5 mins.

It is noteworthy that the green light was at a distance from the plants and plants were not directly exposed to the green light. Plates were put back into the lightbox and allowed to continue growing under extended darkness conditions for 6h. The data showed that there were no statistically significant differences in the distribution of LRPs stages under green light and carbon starvation conditions at 12h of extended darkness (Table 5.1). Under the green light conditions, about 80% of LRPs were found at the ground state, while 20% of the LRPs were found at stage 0 at 12h of extended darkness and 18 hours after gravitropic bending (Figures 5.2B & 5.2C). At 18h of extended darkness and 24 hours after gravitropic bending, about 83% of LRPs were found at the ground state, while about 16% were found at state 0 (Figures 5.2D & 5.2E). Comparatively, almost 60% of LRPs were found at the ground state, and over 30% were found at stage 0 under carbon starvation conditions at 12h of extended darkness and 18 hours after gravitropic bending (Figures 5.2B & 5.2C). At 18h of extended darkness and 24 hours after gravitropic bending, almost 40% of LRPs were found at the ground state, while a little over 60% were found at stage 0 (Figures 5.2D & 5.2E). These data suggest the green light treatment did not have any effect on the developmental progression of LRPs, and that it was safe to be used to illuminate the room designated for analysing the bend sites of extended darkness-treated plants.

5.4.2 Extended darkness inhibits LRP progression.

In the extended darkness experiments, bend sites were analysed at the end of the designated experimental duration. The data showed that LRPs were arrested at the ground states and stage 0 after both 6h and 12h of extended darkness (Figures 5.3B& 5.3C; 5.3D & 5.3E). In reference to the distribution of LRP stages under the no-bend (no gravitropic bending) conditions, LRPs would have progressed just one stage further as shown by 90% of LRPs at the ground state and 10% at stage 0 under no-bend conditions, compared to the 40% of LRPs at both the ground state and stage 0 under extended darkness conditions (Figures 5.3C & 5.3E). The progression of LRPs under extended darkness conditions is possibly due to residual carbon in the plant at the end of the expected darkness cycle, as a pre-emptive measure to avoid carbon starvation. The starch degradation is potentially readjusted to avoid this until expected light comes on (Graf et al., 2010). In comparison to control conditions LRPs progressed from the ground state and stage 0 to later stages as expected (Figures 5.3B & 5.3C; 5.3D & 5.3E). Taken together these data suggest, light availability is essential for the developmental progression of LRPs.

Analyses of the distribution of the LRP stages revealed statistically significant differences in the distribution of LRP stages under darkness and control conditions under both 6h and 12h of extended darkness conditions (Table 5.3). The rate of LRP progression was also calculated from the data from the extended darkness experiments, to do this, numerical values were assigned (reassigned to stages other than the ground state, from the assignments in chapter 4) to all the stages of LRP (ground state = 0; stage 0 = 1; stage 1 = 2; stage 3 = 4; stage 5 = 6; stage 6 = 7; stage 7 = 8; stage 8 = 9; outgrown LR = 10). This assignment of numerical values was to ensure that a resultant numerical value was achieved for the rates of progression. The rate of LRP progression was calculated as described in section 4.3 of chapter 4. The rates of progression were calculated as the differential of LRP stages between the no bend and either the control or the extended darkness conditions. These differentials were then divided by the number of hours after gravitropic bending. This denominator was chosen because the no-bend condition was the basis for determining whether LRPs were at an advanced upon a particular treatment.

The averages of the rates of progression showed a mean rate of progression of 0.04 n/h and 0.02 n/h under 6h and 12h extended darkness conditions respectively (Table 5.2). The modal and median rates of LRP progression were 0.00 n/h under both 6h and 12h extended darkness conditions (Table 5.2). The Mean rates of LRP progression were 0.1486 n/h and 0.15 n/h for the control conditions at 12h and 18h after gravitropic bending respectively. The modal and the median rate of LRP progression were 0.1667 n/h and 0.17 n/h at 12h and 18h after gravitropic bending respectively (Table 5.2). Similarly, statistical tests revealed statistically significant differences in the distribution of LRPs among stages under all treatment conditions and at the two time points studied (Table 5.4). Taken together, these data suggest that the developmental progression of LRPs is inhibited under extended darkness conditions.

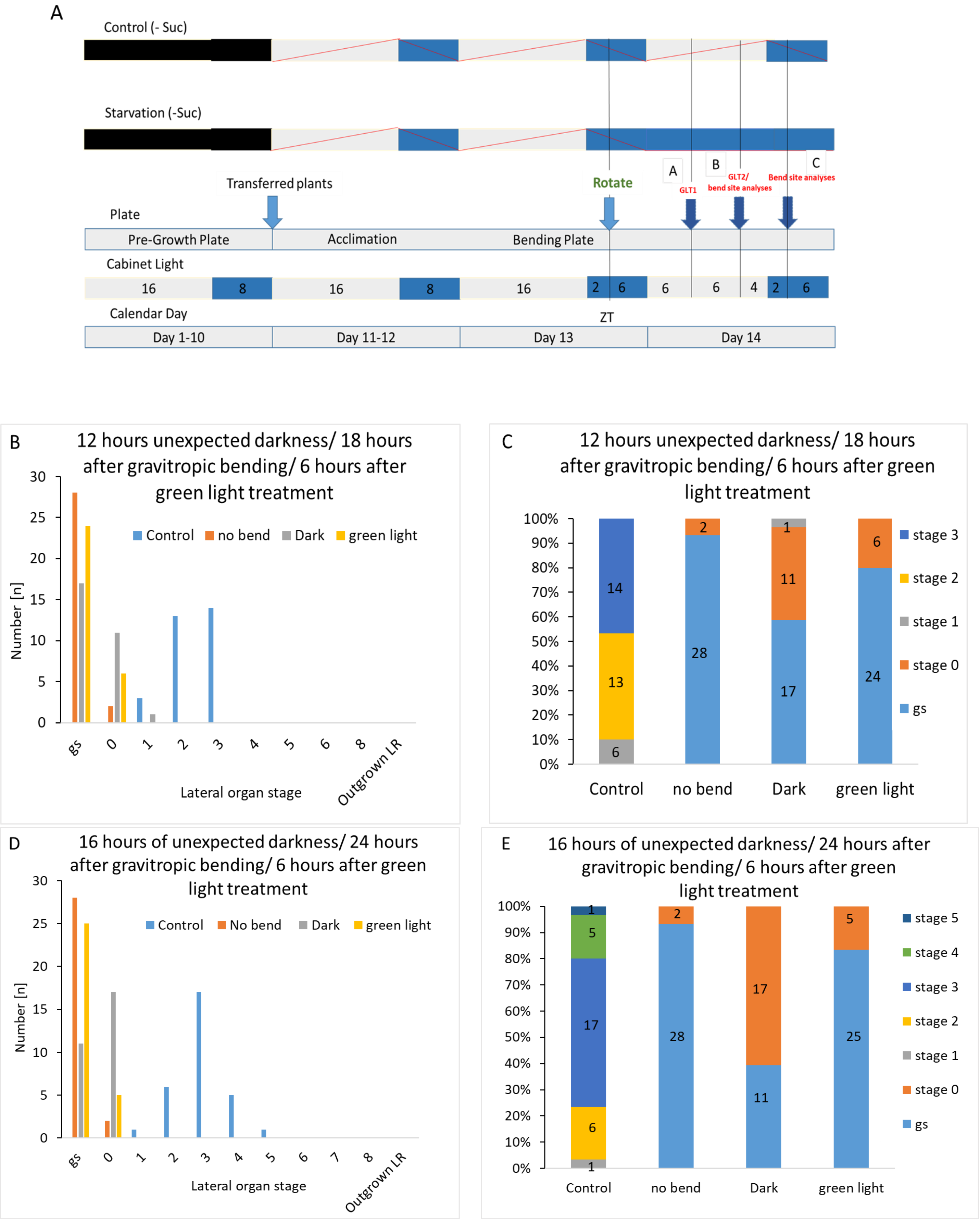
A
Control (- Suc)
Starvation (-Suc)
Transferred plants
Rotate
A
GLT1
B
GLT2/ bend site analyses
C
Bend site analyses
Plate
Pre-Growth Plate
Acclimation
Bending Plate
Cabinet Light
16
8
16
8
16
2
6
6
6
4
2
6
Calendar Day
ZT
Day 1-10
Day 11-12
Day 13
Day 14
B
12 hours unexpected darkness/ 18 hours after gravitropic bending/ 6 hours after green light treatment
Control
no bend
Dark
green light
Number [n]
30
25
20
15
10
5
0
gs
0
1
2
3
4
5
6
8
Outgrown LR
Lateral organ stage
C
12 hours unexpected darkness/ 18 hours after gravitropic bending/ 6 hours after green light treatment
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
14
13
6
2
28
1
11
17
6
24
Control
no bend
Dark
green light
stage 3
stage 2
stage 1
stage 0
gs
D
16 hours of unexpected darkness/ 24 hours after gravitropic bending/ 6 hours after green light treatment
Control
No bend
Dark
green light
Number [n]
30
25
20
15
10
5
0
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
E
16 hours of unexpected darkness/ 24 hours after gravitropic bending/ 6 hours after green light treatment
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
1
5
17
6
1
2
28
17
11
5
25
Control
no bend
Dark
green light
stage 5
stage 4
stage 3
stage 2
stage 1
stage 0
gs

Figure 5.2 **Response of LRP progression to green light treatment**

(A) Schematic for experimental design. **A**, 6 hour-extended darkness-treated plants were subjected to green light treatment as described in section 5.3 of this chapter and allowed to grow for 6 hours. GLT1 signifies the first (**1**) **G**reen **L**ight **T**reatment. **B**, Bend sites of plants subjected to the treatment described in (A) were analysed as described in section 4.3 of chapter 4; 12 hour-extended darkness-treated plants were subjected to green light treatment as described in section 5.3 of this chapter and allowed to grow for 6 hours. GLT1 signifies the second (**2**) **G**reen **L**ight **T**reatment. **C**, Bend sites of plants subjected to the treatment described in (B) were analysed as described in section 4.3 of chapter 4.

(B-C) The distribution of LRPs among stages in response to green light treatment to 6h extended-darkness-treated plants compared to dark control and white light (cabinet light) control conditions. gs represents the ground state stage, which is the stage where LRP formation has not begun. Dark, n =29; other treatments: n =30. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Dark signifies extended darkness treatment.

(D-E) The distribution of LRPs among stages in response to green light treatment to 12h unexpected-darkness-treated plants compared to dark control and white light (cabinet light) control conditions. gs represents the ground state stage, which is the stage where LRP formation has not begun.. Dark, n =29; other treatments: n =30. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Dark signifies extended darkness treatment.

Table 5.1 Chi-squared analyses of the distribution of LRPs among the indicated stages in response to green light treatment.

12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after green light treatment

Original table

	gs	**stage 0**	**stage 1**	**stage 2**	**stage 3**
green light	24	6	0	0	0
Dark	17	11	1	0	0

Table used for Chi-square tests

LRP stages		
Light condition	gs to 0	1
green light	30	0
Dark	28	1

Chi-Square tests

Rows, columns:	2, 2	df	1
Value:	1.0523	p (no assoc.):	0.30497
Monte Carlo p :	0.49		
Fisher´s exact			
p (no assoc.):	0.49153		

18 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after green light treatment

Original table

	gs	**stage 0**	**stage 1**	**stage 2**
green light	25	5	0	0
Dark	11	17	0	0

Table used for Chi-square tests

LRP stages		
	gs	0

green light	25	5	
Dark	11	17	

Chi-Square tests

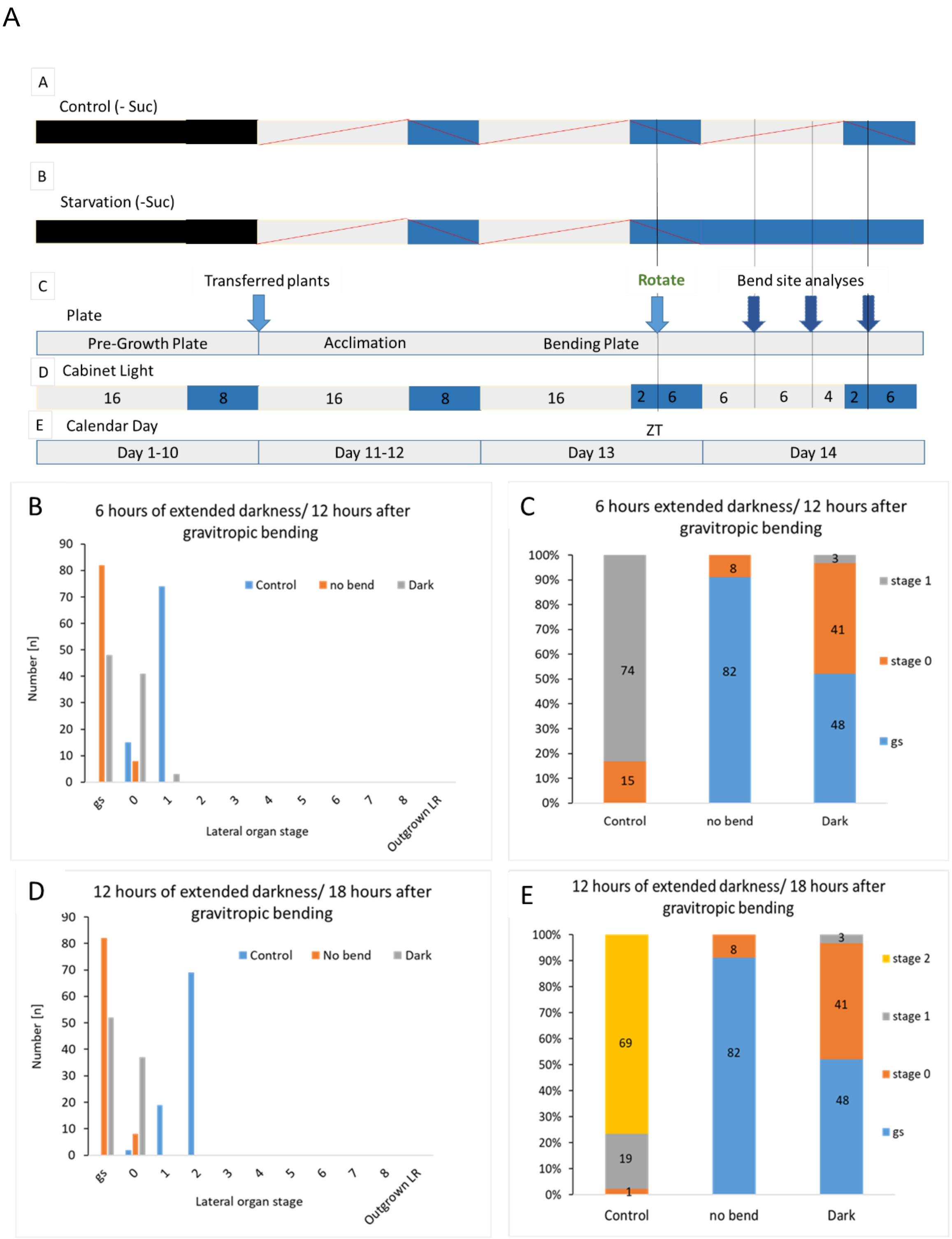
Rows, columns:	2, 2	df	1	
Value:	11.935	p (no assoc.):	0.000551	
Monte Carlo p :	0.0009			
Fisher´s exact				
p (no assoc.):	0.0009625			

Dark, n =29; other treatments: n =30. The cells shaded blue indicate no significant difference (p>0.05). The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom. gs represents the ground state stage.

Table 5.2 Average rates of LRP progression in response to extended darkness.

Control: n = 89; other treatments :n = 90. Rates of LRP progression were calculated by dividing averages of LRP stages at the indicated conditions by the number of hours after gravitropic bending at the indicated time points.

Average	**6 hours of extended darkness/12 hours after gravitropic bending**		**12 hours of extended darkness/ 18 hours after gravitropic bending**	
	per hour [h]		**per hour [h]**	
	Dark	**Control**	**Dark**	**Control**
	Rate of LRP progression			
Mean	0.04	0.1486	0.02	0.15
Median	0.00	0.1667	0.00	0.17
Modal	0.00	0.1667	0.00	0.17


A
A
Control (- Suc)
B
Starvation (-Suc)
C
Transferred plants
Rotate
Bend site analyses
Plate
Pre-Growth Plate
Acclimation
Bending Plate
D
Cabinet Light
16
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16
2
6
6
6
4
2
6
E
Calendar Day
ZT
Day 1-10
Day 11-12
Day 13
Day 14
B
6 hours of extended darkness/ 12 hours after gravitropic bending
Number [n]
90
80
70
60
50
40
30
20
10
0
Control
no bend
Dark
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
C
6 hours extended darkness/ 12 hours after gravitropic bending
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
74
15
8
82
3
41
48
Control
no bend
Dark
stage 1
stage 0
gs
D
12 hours of extended darkness/ 18 hours after gravitropic bending
Number [n]
90
80
70
60
50
40
30
20
10
0
Control
No bend
Dark
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
E
12 hours of extended darkness/ 18 hours after gravitropic bending
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
69
19
1
8
82
3
41
48
Control
no bend
Dark
stage 2
stage 1
stage 0
gs

Figure 5.3 **Effect of extended darkness on LRP progression**

(A) Schematic for experimental design. **A**, Plants were grown under normal cabinet light conditions as described in section 5.3 of this chapter. Black boxes represents pre-transfer periods. Red lines represent expected fluctuations in endogenous levels in the plant under 16h/light, 8h/dark cycle. **B**, Plants were subjected to unexpected dark treatments. Black boxes represents pre-transfer periods. Red lines represent expected fluctuations in endogenous sucrose levels in the plant under 16h/day, 8h/dark photoperiod. **C**, All plants were pre-grown, transferred onto treatments plates and allowed to acclimatise for 2 days, and rotated. Plates were rotated 2 hours into the expected dark period of the 16h/ 8h, day/night (light/dark) cycle, and allowed to grow throughout the expected darkness period for 6 hours. Plants were then subjected to 6 and 12 hours of unexpected darkness. Bend sites were analysed as described in section 4.3 of chapter 4. **D**, A breakdown of the light conditions in the growth cabinet where the carbon starvation experiments were conducted. **E**, A breakdown of the duration of the experiments.

(B-C) The distribution of LRPs among stages in response to 6h extended darkness treatment. gs represents the ground state stage, as described in chapter 2 of this thesis. Control: n = 89; other treatments :n = 90; data was pooled from 3 independent experiments. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Dark signifies extended darkness treatment.

(D-E) The distribution of LRPs among stages in response to 12h unexpected dark treatment. gs represents the ground state stage, as described in chapter 2 of this thesis. Control: n = 89; other treatments :n = 90; data was pooled from 3 independent experiments. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Dark signifies extended darkness treatment.

Table 5.3 Chi-squared analyses of the distribution of LRPs among the indicated stages in response to extended darkness.

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6 hours of extended darkness/ 12 hours after gravitropic bending

Original table

Treatment	gs	stage 0	stage 1	stage 0	
Control	0	15	74	0	
Dark	48	41	3	0	

Table used for Chi-squared tests

LRP stage				
Treatment	gs	0	1	
Control	0	15	74	
Dark	48	41	3	

Chi-squared tests

Rows, columns:	2, 3	df	2	
Value:	125.52	p (no assoc.):	<0.000001	
Monte Carlo p :	0.0001			
Fisher´s exact				
p (no assoc.):	<0.000001			

12 hours of extended darkness/ 18h after gravitropic bending

Original table

Treatment	gs	stage 0	stage 1	stage 2	stage 3
Control	0	2	19	69	0
Dark	48	41	3	0	0

Table used for Chi-squared tests

Treatment	gs to 0	1 to 2	
Control	2	88	

Dark	89	3		
Chi-squared tests				
Rows, columns:	2, 2	df	1	
Chi2:	162.57	p (no assoc.):	<0.000001	
Monte Carlo p :	0.0001			
Fisher´s exact				
p (no assoc.):	<0.000001			

Control: n = 89; other treatments:n = 90. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom. gs represents the ground state stage, as described in chapter 2 of this thesis.

Table 5.4 Kruskal Wallis tests on the distribution of LRPs among stages in response to extended darkness.

6 hours of extended darkness/ 12 hours after gravitropic bending

Kruskal-Wallis test for equal medians

H (chi2):	168.2	
Hc (tie corrected):	197.1	
p (same):	1.62E-43	

Dunn's post hoc test

	no bend	Control	Dark
no bend		5.24E-42	0.00019
Control	5.24E-42		3.01E-23
Dark	0.0001896	3.01E-23	

12 hours of extended darkness/ 18h after gravitropic bending

Kruskal-Wallis test for equal medians

H (chi2):	183.7	
Hc (tie corrected):	215.4	
p (same):	1.66E-47	

Dunn's post hoc test

	no bend	Control	Dark
no bend		1.21E-43	0.006374
Control	1.21E-43		9.42E-29
Dark	0.006374	9.42E-29	

Control: n = 89; other treatments: n = 90. The cells shaded blue indicate non-normal distribution ($p<0.05$). The cells shaded pink indicate statistical significance ($p<0.05$). H is the test statistic for the Kruskal-Wallis test. Hc signifies the corrected test statistic in the Kruskal-Wallis test.

5.4.3 The addition of 1% exogenous sucrose restores LRP progression in extended darkness-treated plants.

In a light/dark cycle, carbon is used up or almost used at the end of the dark cycle (via starch degradation) in anticipation of light, when the unmet expectation for light at the end of the dark cycle (extended dark periods) could lead to carbon starvation (Zeeman et al., 2010; Sulpice et al., 2014). Based on the conclusions from chapters 3 and 4, the unavailability of carbon is expected to affect the developmental progression of LRPs. It is, therefore, likely that the inhibition of LRP progression under extended darkness conditions reported earlier is due to carbon starvation. If the unavailability of a carbon source inhibits LRP progression, then it is expected that the supply of exogenous sucrose would restore the progression of LRPs.

Therefore, it was the aim of this study to determine whether the supply of exogenous sucrose to extended darkness-treated plants would restore their LRP progression phenotype. To do this, extended darkness experiments were conducted using the method and time points for both the extended darkness and control conditions as described earlier. For sucrose restoration treatments, 100 µl of 1% exogenous sucrose was added to individual plants after 6h and 12h of extended darkness. 100 µl of exogenous sucrose has been used in restoration treatments in a study by Macgregor et al (2008) to restore short LR length phenotype (Macgregor et al., 2008). It, therefore seemed that 100 µl of exogenous sucrose would be an appropriate volume to use. Additionally, that volume was sufficient to soak the filter papers on which the plants were laid without oversaturating it, which would lead to leakages of sucrose into the media, thereby defeating the purpose of using the filter paper/Parafilm strip experimental method—to avoid contact of the PR with the sucrose via the media.

Plants were allowed to grow for an additional 6h after sucrose restoration treatments at each time point and the bend sites were analysed as described earlier. There were also extended darkness and 1% exogenous sucrose controls which were executed as described earlier in this thesis. The difference between the extended darkness and the 1% exogenous sucrose controls in the sucrose restoration experiment and the extended darkness experiment (described earlier) is that the duration of extended darkness was 12h (18h after gravitropic bending) and 16h (24h after gravitropic bending) for the former and 6h (12h after gravitropic bending) and 12h (18h after gravitropic bending) for the latter. The sucrose restoration treatments for 12h extended darkness-treated plants coincided with the bend site analyses of the 6h extended darkness-treated plants (sucrose restored) in principle (Figure 5.4C). Therefore, sucrose treatments for 12h extended darkness-treated plants were conducted first, which took about 20 mins per experiment, and then the bend sites of 6h extended darkness-treated plants (sucrose restored) plants were analysed. It is noteworthy that at no point in the execution of the experiments were the extended darkness controls exposed to white light or even green light during the sucrose restoration treatments. Plates for extended darkness controls remained in the lightbox throughout the sucrose restoration treatments.

Analyses of the data from the bend site revealed statistically significant differences in the distribution of the LRP stages between the sucrose restoration and the controls (Table 5.6). The distribution of LRP stages revealed that the addition of 1% exogenous sucrose to 6h extended darkness-treated plants restored the progression of LRPs (Figures 5.4B & 5.4C). Under extended darkness conditions, over 60% of LRPs were found at the ground state while over 35% were found at stage 0 (Figures 5.4B & 5.4C), however, upon the addition of 1% exogenous sucrose, LRPs were nearly evenly distributed among stages 1, 2 & 3, although there was still a little over 10% of LRPs at stage 0 (Figures 5.4B & 5.4C). The analyses of the bend sites 6h after the addition of 1% sucrose and 12h of extended darkness-treated plants also revealed a surprisingly similar LRP distribution pattern (Figures 5.4D & 5.4E). This was surprising because plants under this condition had been carbon-starved longer than in the previous condition (6h extended darkness or extended darkness) and are therefore expected to be more carbon-starved.

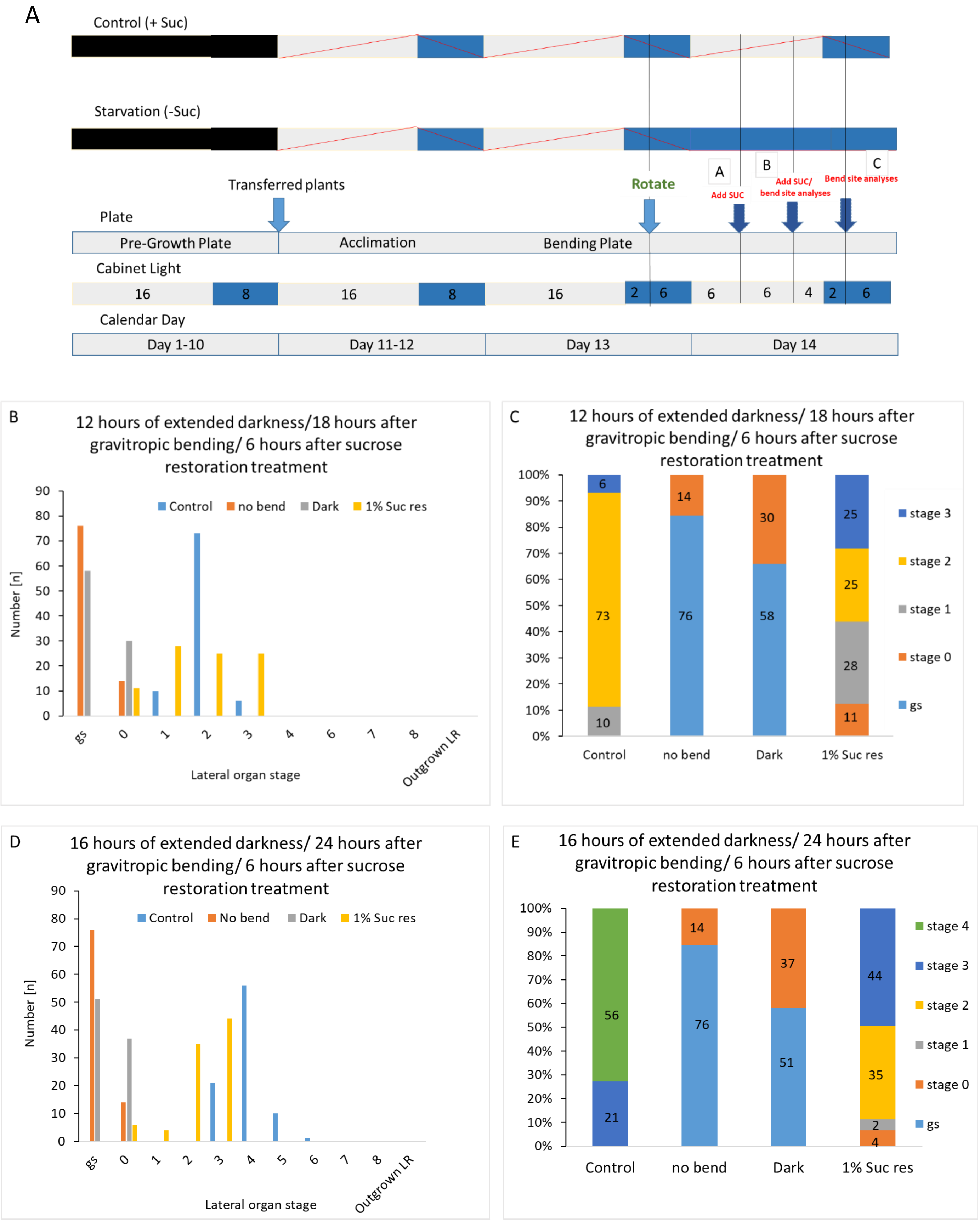
This longer carbon starvation should cause LRPs under this condition to be less advanced than plants carbon-starved for shorter periods. Although the pattern of LRP distribution under sucrose restoration conditions is not the same as that of the 1% exogenous sucrose control, the stages under both conditions were more advanced compared to the extended darkness control (Figures 5.4B, 5.4C, 5.4D & 5.4E). For example, at 18 hours after gravitropic bending (gravitropic bending), over 60% of LRPs were found at the ground state, and over 40% were found at stage 0, while <10% were found at stage 1 under the Extended darkness conditions, relatively, at the same time point, 80% of LRPs were found at stage 2, and 10% were found at stages 1 and 3 under the 1% sucrose control conditions (Figures 5.4B & 5.4C). Under sucrose restoration conditions, over 10% of LRPs were found at stage 0, over 30% were found at stage 1, and 30% were found at stages 2 and 3. At 24h after gravitropic bending, 25% of LRPs were found at stage 3, and 75% were found at stage 4 under 1% exogenous sucrose control conditions compared to the extended darkness conditions where over 60% of LRPs were found at the ground state, and over 40% were found at stage 0, while <10% were found at stage 1 (Figures 5.4D & 5.4E). Similarly, under sucrose restoration conditions, over 10% of LRPs were found at stage 0, over 30% were found at stage 1, and 30% were found at stages 2 and 3. Taken together, these data show that the addition of exogenous sucrose to extended darkness-treated plants restores inhibited LRP progression.

The rates of progression (Table 5.5) from these sucrose restoration experiments were calculated as described earlier, with the various hours after gravitropic bending used as the denominator and the differential between the LRP stages under no bend and rotated conditions used as the numerator. At 18h after gravitropic bending, average rates of LRP progression were: mean = 0.16 n/h; median and mode = 0.17 n/h under 1% exogenous sucrose control conditions (Table 5.5). Under the sucrose restoration conditions, the mean rate of LRP progression was 0.1553 n/h and the median and the modal rates of LRP progression were 0.1667 n/h compared to a mean rate of LRP progression of

0.02 n/h and modal and median rates of LRP progression of 0.00 n/h under extended darkness control conditions (Table 5.5).

At 24h after gravitropic bending, the mean rate of LRP progression was 0.20 n/h under 1% exogenous sucrose condition, and 0.14 n/h under sucrose restoration condition, while the modal and median rates of LRP progression were 0.21 n/h and 0.13 n/h under 0% exogenous sucrose condition and sucrose restoration condition respectively (Table 5.5). Statistical tests were also conducted on the distribution of LRPs among stages under all treatment conditions and at all time points studied (Table 5.7). The data showed statistically significant differences in the distribution of LRPs among stages under sucrose restoration conditions compared to the extended darkness control at all time points (Table 5.7).

There were no statistically significant differences in the distribution of LRPs among stages under the 1% exogenous sucrose control and sucrose restoration condition (Table 5.7) at 18h after gravitropic bending, as expected, there were statistically significant differences in the distribution of LRPs among all treatments (Table 5.7). Additionally, at 24h after gravitropic bending, there were statistically significant differences in the distribution of LRPs among stages under the 1% exogenous sucrose and sucrose restoration conditions, as well as among all three conditions (the extended darkness control included, and as expected) (Table 5.7). Taken together these suggest that the supply of 1% exogenous sucrose accelerates the progression to nearly control levels in plants starved of carbon over a relatively shorter period, however, when the duration of carbon starvation increases, LRP will still be increased by the addition of 1% exogenous sucrose, but not as similar as the control conditions.


A
Control (+ Suc)
Starvation (-Suc)
Transferred plants
Rotate
A
Add SUC
B
Add SUC/ bend site analyses
C
Bend site analyses
Plate
Pre-Growth Plate
Acclimation
Bending Plate
Cabinet Light
16
8
16
8
16
2
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6
6
4
2
6
Calendar Day
Day 1-10
Day 11-12
Day 13
Day 14
B
12 hours of extended darkness/18 hours after gravitropic bending/ 6 hours after sucrose restoration treatment
Number [n]
Control
no bend
Dark
1% Suc res
gs
0
1
2
3
4
6
7
8
Outgrown LR
Lateral organ stage
C
12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after sucrose restoration treatment
6
73
10
14
76
30
58
25
25
28
11
Control
no bend
Dark
1% Suc res
stage 3
stage 2
stage 1
stage 0
gs
D
16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose restoration treatment
Number [n]
Control
No bend
Dark
1% Suc res
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
E
16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose restoration treatment
56
21
14
76
37
51
44
35
2
4
Control
no bend
Dark
1% Suc res
stage 4
stage 3
stage 2
stage 1
stage 0
gs

Figure 5.4 **1% exogenous sucrose restores LRP progression in extended darkness-treated plants.**

(A) Schematic of experimental design. **A**, 100 µl of 1% exogenous sucrose was added to 6 hour-extended darkness-treated plants were returned to light conditions and allowed to grow for 6 hours. Add suc means addition of sucrose, which is referred to as sucrose restoration (suc res) in this chapter. **B**, Bend sites of plants subjected to the treatment described in (A) were analysed as described in section 4.3 of chapter 4;100 µl of 1% exogenous sucrose was added to 12 hour-extended darkness-treated plants were returned to light conditions and allowed to grow for 6 hours. Add suc means addition of sucrose, which is referred to as sucrose restoration (suc res) in this chapter. **C**, Bend sites of plants subjected to the treatment described in (B) were analysed as described in section 4.3 of chapter 4.

(B-C) The distribution of LRPs among stages in response to 1% exogenous sucrose treatment in 6h extended darkness treated-plants. 87 < n >89. Data were pooled from 3 independent experiments. gs represents the ground state stage, as described in chapter 2 of this thesis. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. 1% Suc res signifies 1% exogenous sucrose restoration treatment.

(D-E) The distribution of LRPs among stages in response to 1% exogenous sucrose treatment in 12h extended darkness treated-plants. 87 < n >89. Data were pooled from 3 independent experiments. gs represents the ground state stage, as described in chapter 2 of this thesis. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. 1% Suc res signifies 1% exogenous sucrose restoration treatment.

Table 5.5 Average rates of LRP progression in response to 1% exogenous sucrose restoration treatments.

	12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after sucrose restoration treatments			**16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose restoration treatments**		
	per hour [h]			**per hour [h]**		
Averages	**Dark**	**Control**	**Restoration**	**Dark**	**Control**	**Restoration**
	Rate of LRP progression (n/h)					
Mean	0.02	0.16	0.1553	0.02	0.20	0.14
Median	0.00	0.17	0.1667	0.00	0.21	0.13
Modal	0.00	0.17	0.1667	0.00	0.21	0.13

87 < n >89. Rates of LRP progression were calculated by dividing averages of LRP stages at the indicated conditions by the number of hours after gravitropic bending at the indicated time points.

Table 5.6 Chi-squared analyses of LRP distribution in response to 1% exogenous sucrose restoration treatments

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12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after sucrose treatment

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Original table

Treatment		gs	stage 0	stage 1	stage 2	stage 3	
Control		0	0	10	73	6	
no bend		76	14	0	0	0	
Dark		58	30	0	0	0	
1% Suc res		0	11	28	25	25	

Table used for chi-squared test

LRP stages	Control	Dark	1% Suc res	
gs to 1	10	88	39	
2 to 3	79	0	50	

Chi-squared test

Rows, columns:	2, 3	df	2	
Chi2:	142.74	p (no assoc.):	<0.000001	
Monte Carlo p :	0.0001			
Fisher´s exact				
p (no assoc.):	<0.000001			

16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose restoration treatment

Original table

Treatment	gs	stage 0	stage 1	stage 2	stage 3	stage 4	stage 5	stage 6
Control	0	0	0	0	21	56	10	1
no bend	79	11	0	0	0	0	0	0
Dark	55	30	4	0	0	0	0	0
1% Suc res	0	11	28	25	25	0	0	0

Table used for chi-squared test

LRP stages	Control	Dark	1% Suc res
gs to 2	0	89	64
3 to 6	88	0	25

Chi-squared test

Rows, columns:	2, 3	df	2
Chi2:	192.43	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact			
p (no assoc.):	<0.000001		

87 < n >89. The cells shaded pink indicate statistical significance ($p<0.05$). df is the degree of freedom. gs represents the ground state stage, as described in chapter 2 of this thesis. 1% Suc res signifies sucrose restoration treatment.

Table 5.7 Kruskal Wallis tests on the distribution of LRPs in response to 1% exogenous sucrose restoration treatments.

12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after sucrose treatment

Kruskal-Wallis test for equal medians

H (chi2):	271.3
Hc (tie corrected):	294.2
p (same):	1.83E-63

Dunn's post hoc test

	No bend	Control	Dark	Restoration
No bend		1.13E-39	0.264	1.02E-33
Control	1.13E-39		1.83E-33	0.2618
Dark	0.264	1.83E-33		5.13E-28
Restoration	1.02E-33	0.2618	5.13E-28	

16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose treatment

Kruskal-Wallis test for equal medians

H (chi2):	292.6
Hc (tie corrected):	311.9
p (same):	2.68E-67

Dunn's post hoc test

	No bend	Control	Dark	Restoration
No bend		3.18E-53	0.1041	1.04E-23
Control	3.18E-53		1.86E-42	9.65E-08
Dark	0.1041	1.86E-42		6.22E-17
Restoration	1.04E-23	9.65E-08	6.22E-17	

87 < n >89*.* The cells shaded pink indicate statistical significance ($p<0.05$). H is the test statistic for the Kruskal-Wallis test. Hc signifies the corrected test statistic in the Kruskal-Wallis test.

5.4.4 Light restores LRP progression in extended darkness-treated plants.

The observation that the developmental progression of LRPs is inhibited under extended darkness and the progression of LRPs under 0% exogenous conditions in the light control as described in the carbon starvation experiments (Figure 5.3), as well as in chapters 3 and 4, raised the question of whether the return of extended darkness-treated plants to the light (16h/light, 8h/dark cycle) conditions without the addition of exogenous sucrose could restore the progression of LRPs in extended darkness-treated plants. To answer this question, extended darkness experiments were conducted using the method and time points for both the extended darkness and control conditions as described earlier.

The mechanics of the light restoration experiments were similar to the sucrose restoration experiments. The experiments consisted of light control, which contained 0% exogenous sucrose, extended darkness control, and return to light (light restoration hereafter) treatments. There were two return-to-light treatments (Figures 5.5A). The first treatment involved the return of 6h extended darkness-treated plants to light conditions at the end of the 6 h of extended darkness. Plants were allowed to grow for an additional 6h after light restoration treatment and the bend sites were analysed as described earlier. A similar approach was used for the other time point (12h extended darkness-treated plants), where 12h extended darkness-treated plants were returned to light conditions and allowed to grow for 6h, the bend sites were analysed as the previous time point.

The difference between the extended darkness and the light controls in the light restoration experiments and the same controls in the extended darkness experiment (described earlier) is that the duration of extended darkness was similar to that described in the section for the sucrose restoration experiments: 12h (18h after gravitropic bending) and 16h (24h after gravitropic bending) for the former and 6h (12h after gravitropic bending) and 12h (18h after gravitropic bending). Similar to the sucrose restoration experiments, the light restoration treatments for 12h extended darkness-treated plants coincided with the bend site analyses of the 6h extended darkness-treated plants (light restored) in principle (Figure 5.4A). Consequently, 12h extended darkness-treated plants were returned to light first, which took about 10 mins per experiment, and then the bend sites of 6h extended darkness-treated plants (light restored) were analysed. Analyses of the data from the bend site revealed statistically significant differences in the distribution of the LRPs among stages under the light restoration, light, and extended darkness control conditions(Table 5.9). The distribution of LRPs among stages revealed that the return of 6h extended darkness-treated plants to light restored the progression of LRPs (Figures 5.5B & 5.5C). Under extended darkness conditions, over 60% of LRPs were found at the ground state while over 35% were found at stage 0 (Figures 5.5B & 5.5C). However, upon return to light conditions, about 65% of LRPs were found at stage 2, about 15% at stage 1, and over 5% were found at stages 0 and the ground state (Figures 5.5B & 5.5C). Although the pattern of LRP distribution under light restoration conditions is not the same as that of the light control, the stages under both conditions were more advanced compared to the extended darkness control (Figures 5.5B, 5.5C, 5.5D & 5.5E).

For example, at 18 hours after gravitropic bending, over 45% of LRPs were found at stages 3 and 4, while over 5% were found at stages 1 under the light control conditions. Relatively, at the same time point, about 65% of LRPs were found at stage 2, about 15% at stage 1, and over 5% were found at stages 0 and the ground state (Figures 5.5B & 5.5C) under light restoration conditions. At 24h after gravitropic bending, 25% of LRPs were found at stage 3, and 75% were found at stage 4 under the light control conditions compared to the extended darkness conditions where over 60% of LRPs were found at the ground state, and over 40% were found at stage 0, while <10% were found at stage 1 (Figures 5.5D & 5.5E). Similarly, under light restoration conditions, over 70% of LRPs were found at stage 3, over 15% were found at stage 4, over 10% were found at stage 2, and about 5% were found at stage 1. Taken together, these data show that the return of extended darkness-treated plants to 16h/light and 8h/dark conditions restores the inhibited LRP progression phenotype.

The rates of progression from these sucrose restoration experiments were calculated as described in the sucrose restoration section. At 18h after gravitropic bending, there were statistically significant differences among all treatments (Table 5.9). The average rates of LRP progression were: mean=0.18 n/h; median and mode=0.17 n/h under light conditions (Table 5.8). Under the light restoration conditions, the mean rate of LRP progression was 0.1446 n/h and the median and the modal rates of LRP progression were 0.1667 n/h compared to a mean rate of LRP progression of 0.02 n/h and modal and median rates of LRP progression of 0.00 n/h (Table 5.8). At 24h after gravitropic bending, the mean rate of LRP progression was 0.18 n/h under the light control condition, and 0.14 n/h under the light restoration condition, while the modal and median rates of LRP progression were 0.17 n/h and 0.13 n/h under the light control condition and light restoration condition respectively (Table 5.8). Statistical tests were also conducted for the distribution of LRPs among stages between treatment conditions and data showed statistically significant differences in the distribution of LRPs among stages under light restoration conditions compared to the extended darkness control at all time points (Table 5.10). There were statistically significant differences in the distribution of LRP among stages under the light control and light restoration conditions (Table 5.10). Taken together these data suggest that the return of extended darkness-treated plants to light restores the progression of their LRPs, but not to control levels.

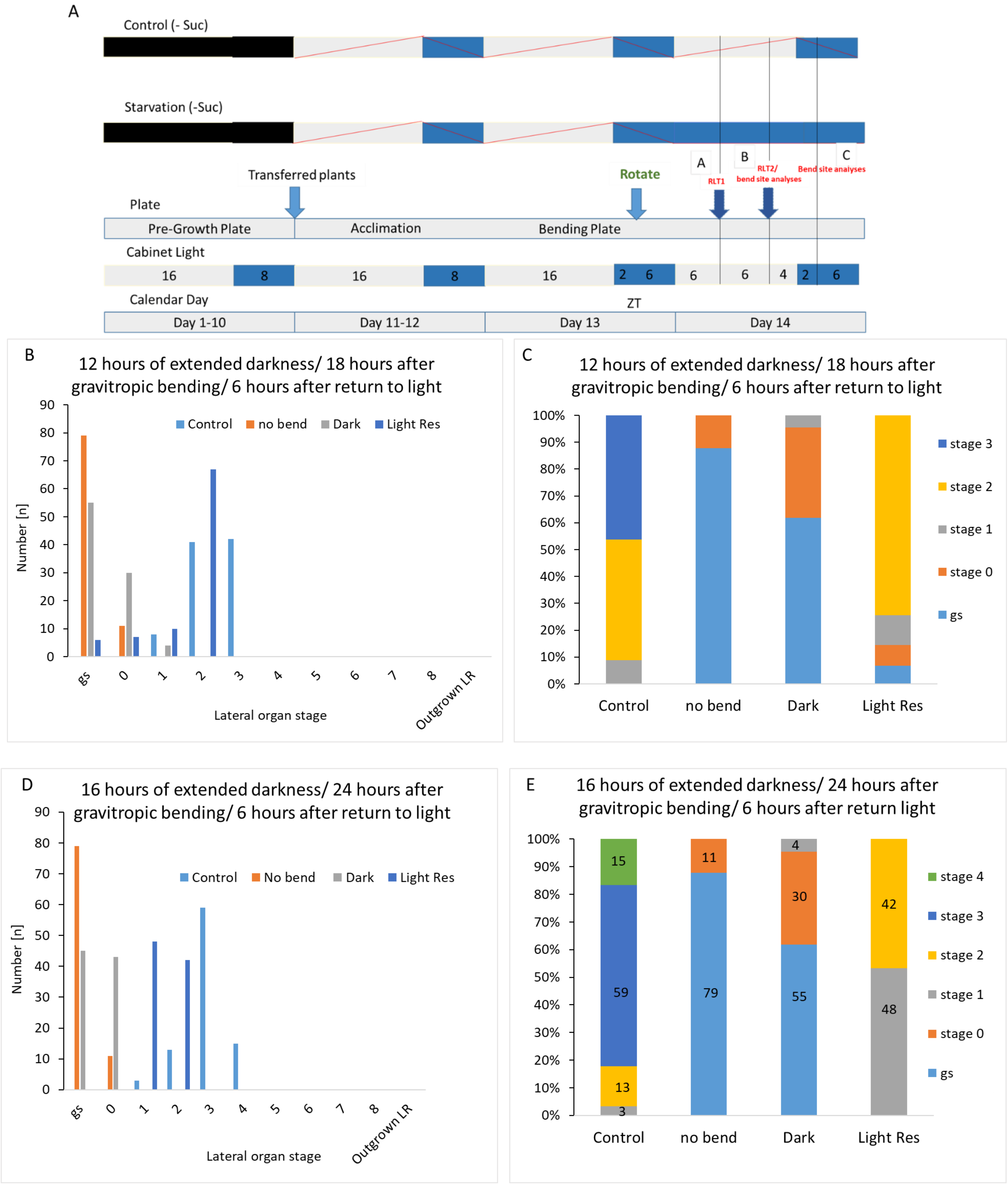
A
Control (- Suc)
Starvation (-Suc)
Transferred plants
Rotate
A
B
C
RLT1
RLT2/ bend site analyses
Bend site analyses
Plate
Pre-Growth Plate
Acclimation
Bending Plate
Cabinet Light
16
8
16
8
16
2
6
6
6
4
2
6
Calendar Day
ZT
Day 1-10
Day 11-12
Day 13
Day 14
B
12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after return to light
Control
no bend
Dark
Light Res
Number [n]
90
80
70
60
50
40
30
20
10
0
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
C
12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after return to light
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Control
no bend
Dark
Light Res
stage 3
stage 2
stage 1
stage 0
gs
D
16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after return to light
Control
No bend
Dark
Light Res
Number [n]
90
80
70
60
50
40
30
20
10
0
gs
0
1
2
3
4
5
6
7
8
Outgrown LR
Lateral organ stage
E
16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after return light
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
15
59
13
3
11
79
4
30
55
42
48
Control
no bend
Dark
Light Res
stage 4
stage 3
stage 2
stage 1
stage 0
gs

Figure 5.5 **Light restores LRP progression in extended darkness-treated plants.**

(A) Schematic of experimental design. **A**, 6 hour-extended darkness-treated plants were returned to normal circadian and allowed to grow for 6 hours. RTL1 signifies the first (**1**) **R**eturn **T**o **L**ight treatment which is referred as light restoration treatments in this chapter. **B**, Bend sites of plants subjected to the treatment described in (A) were analysed as described in section 4.3 of chapter 4; 12 hour-extended darkness-treated returned to normal circadian conditions and allowed to grow for 6 hours. RTL2 signifies the second (**2**) **R**eturn **T**o **L**ight treatment. **C**, Bend sites of plants subjected to the treatment described in (B) were analysed as described in section 4.3 of chapter 4.

(B-C) The distribution of LRPs among stages in response to 1% exogenous sucrose treatment in 6h dark treated plants. n =90. Data were pooled from 3 independent experiments. gs represents the ground state stage, as described in chapter 2 of this thesis. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Light Res signifies light restoration treatment (return to light).

(D-E) The distribution of LRPs among stages in response to 1% exogenous sucrose treatment in 12h dark treated plants. n =90. Data were pooled from 3 independent experiments. gs represents the ground state stage, as described in chapter 2 of this thesis. Numbers in stacked column graphs represent the sample sizes (number of roots) at each stage. Light Res signifies light restoration treatment (return to light).

Table 5.8 Average rates of LRP progression in response to light restoration treatments.

	12 hours of extended darkness/18 hours after gravitropic bending/ 6 hours after return to light			**16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after return to light**		
	per hour [h]			**per hour [h]**		
	Dark	**Control**	**Restoration**	**Dark**	**Control**	**Restoration**
Average	**Rate of LRP progression**					
Mean	0.02	0.18	0.1446	0.02	0.14	0.09
Median	0.00	0.17	0.1667	0.00	0.13	0.08
Modal	0.00	0.17	0.1667	0.00	0.13	0.08

n =90. Rates of LRP progression were calculated by dividing averages of LRP stages at the indicated conditions by the number of hours after gravitropic bending at the indicated time points.

Table 5.9 Chi-squared analyses of LRP distribution in response to light restoration treatments.

12 hours of extended darkness/ 18h after gravitropic bending/ 6h after return to light

Original table

Treatment	Gs	stage 0	stage 1	stage 2	stage 3
Control	0	0	8	41	42
Dark	55	30	4	0	0
Light Res	6	7	10	67	0

Table used for Chi-squared tests

	Control	Dark	Restoration
gs to1	8	89	23
2 to 3	83	0	67

Chi-squared test

Rows, columns:	2, 3	df	2
Chi2:	171.1	p (no assoc.):	<0.000001
Monte Carlo p :	0.0001		
Fisher´s exact			
p (no assoc.):	<0.000001		

16 hours of extended darkness/ 24h after gravitropic bending/ 6h after return to light

Original table

Treatment	Gs	stage 0	stage 1	stage 2	stage 3		stage 4
Control	0	0	3	13	59		15

Dark	55	30	4	0	0		0
Light Res	0	0	48	42	0		0

Table used for Chi-squared tests

	Control	Dark	Restoration	
gs to 1	3	89	48	
2 to 4	87	0	42	

Chi-squared test

Rows, columns:	2, 3	df	2	
Chi2:	167.63	p (no assoc.):	<0.000001	
Monte Carlo p :	0.0001			
Fisher´s exact				
p (no assoc.):	<0.000001			

n =90. The cells shaded pink indicate statistical significance (p<0.05). df is the degree of freedom. gs represents the ground state stage, as described in chapter 2 of this thesis.

Table 5.10 Kruskal Wallis tests on the distribution of LRPs among stages in response to light restoration treatments.

12 hours of extended darkness/ 18 hours after gravitropic bending/ 6 hours after sucrose treatment

Kruskal-Wallis test for equal medians

H (chi2):	261
Hc (tie corrected):	2.86E+02
p (same):	9.63E-62

Dunn's post hoc test

	No bend	Control	Dark	Restoration
No bend		5.77E-47	0.08569	1.53E-25
Control	5.77E-47		9.85E-37	1.32E-04
Dark	8.57E-02	9.85E-37		2.50E-18
Restoration	1.53E-25	1.32E-04	2.50E-18	

16 hours of extended darkness/ 24 hours after gravitropic bending/ 6 hours after sucrose treatment

Kruskal-Wallis test for equal medians

H (chi2):	303.3
Hc (tie corrected):	3.22E+02
p (same):	1.87E-69

Dunn's post hoc test

	No bend	Control	Dark	Restoration
No bend		1.33E-53	0.03805	1.96E-29
Control	1.33E-53		5.93E-40	9.55E-06

Dark	0.03805	5.93E-40		1.17E-19
Restoration	1.96E-29	9.55E-06	1.17E-19	

n = 90. The cells shaded blue indicate non-normal distribution (p<0.05). The cells shaded oink indicate statistical significance (p<0.05). H is the test statistic for the Kruskal-Wallis test. Hc signifies the corrected test statistic in the Kruskal-Wallis test.

5.5 Discussion

Some physiological studies on establishing a causal relationship between carbon availability and LR formation (and PR growth) have used variations in either the availability, intensity, and concentrations of factors that stimulate endogenous sucrose synthesis (aside from the supply of exogenous sucrose) (e.g., light intensity and CO_2 concentrations) (Freixes et al. 2002; Lee-Ho et al., 2007; Miotto et al., 2021; Muralidhara et al., 2021). The study presented in this chapter was aimed at understanding whether extended darkness would affect the developmental progression of LRPs. The results from the study showed that an extended darkness treatment of just 6 hours led to a near-even distribution of LRPs at the ground state and stage 0 compared to the control (Figures 5.3B & 5.3C). This phenotype was conserved even after 12 hours of extended darkness treatment (Figures 5.3D & 5.3E).

The distribution of nearly 50% of LRPs at the ground state and stage 0 in response to 6 and 12 hours of extended darkness compared to the about 95% of LRPs at the ground state under no bend (no extended darkness treatment), suggest progression of some LRPs from the ground state to stage 0 (which encompasses founder cells that have undergone the nuclear migration stage) even under extended darkness conditions. This observation demonstrates that some LRPs can undergo the nuclear migration stage under extended darkness conditions, suggesting a partial inhibition of nuclear migration in response to extended darkness treatment. The data in (Figure 5.3) also suggests that nuclear migration is partially inhibited under extended darkness conditions, while we do not know emphatically that sucrose plays any specific role in the nuclear migration process (probably as a signal), the nuclear migration process will definitely be dependent on the energy provision role of sucrose, just like any other developmental process in plants.

Extended darkness treatments have been suggested to cause an adjustment in starch degradation to ensure some carbon availability until the next expected light/day (Graf et al., 2010) (in the case expected light/day comes on early enough or the plants may die when subjected to extended darkness for too long). Because of this, it is likely that there was still some carbon availability during extended darkness of up to 12 hours, however, these levels of carbon in some of the plants studied were only sufficient to facilitate the early processes of LR formation up to the nuclear migration stage. Additionally, the results shown in (Figure 5.3) and all the results from follow-on experiments presented in section 5.4 of this chapter did not study the effects of extended darkness at time points longer than 12 hours, however, based on the observation of LRP distribution among the ground state and the stage 0 in all the time points studied, it is expected that pattern will remain the same over longer periods till the plants eventually die.

The results presented in (Figure 5.3) also suggest that LRPs are unable to progress to stage 1 under extended darkness conditions, potentially due to carbon starvation. Under control conditions, over 90% of LRPs were found at stage 1 and 15% were found at stage 0 at 12 hours after gravitropic bending, compared to the almost 50% of LRPs found at both the ground state and stage 0 at 6 hours of extended darkness/ 12 hours after gravitropic bending (Figure 5.3C). At 18 hours after gravitropic bending, almost 90% of LRPs were found at stage 2, over 15% were found at stage 2 and less than 5% were found at stage 0, however, under extended darkness conditions at the same time point after gravitropic bending (12 hours of extended darkness/ 18 hours after gravitropic bending), nearly 50% of LRPs were found at the ground state and stage 0.

It is also noteworthy that under both 6 and 12 hours of extended darkness conditions, less than 5% of LRPs were found at stage 1, this, therefore, suggests that some of the LRPs in some plants (individual LRPs represent one plant based on the bend assay experimental method as discussed in chapter 4 of this thesis) can progress to stage 1, but this progression to stage1 is too insignificant across a larger population of plants to make biological interpretations from. Additionally, the rates of LRP progression show 0.00 n/h median and modal rates of progression at 6 and 12 hours (12 and 18 hours after gravitropic bending) of extended darkness compared to 0.15 n/h and 0.17 n/h at 12 and 18 hours after gravitropic bending under control conditions (Table 5.2). Taken together, these data suggest that LRPs are unable to transition from stage 0 to stage 1; that is founder cells are unable to undergo the first anticlinal cell division that is needed to initiate LRPs. The study presented in this chapter did not follow up to understand how extended darkness might be affecting LRP initiation due to time constraints.

The study presented in this chapter also set out to determine whether the addition of exogenous sucrose could restore LRP progression in extended darkness-treated plants. The results showed that the addition of 1% exogenous sucrose could restore LRP progression to control levels after 6 hours of extended darkness (Figures 5.4B & 5.4C; Table 5.7). However, at longer extended darkness treatments (12 hours), 1% exogenous sucrose could still restore LRP progression but not to control levels (Figures 5.4D & 5.4E; Table 5.7). There were also no statistically significant differences in the distribution of LRPs among stages between the sucrose restoration treatment to 6 hours of extended darkness-treated plants (18 hours after gravitropic bending) and the control (18 hours after gravitropic bending). Similarly, the rates of LRP progression are similar for both the sucrose restoration treatment 6 hours of extended darkness-treated plants and the control, however, the rate of LRP progression under control conditions at 24 hours after gravitropic bending is higher than the rate of LRP progression for the sucrose restoration to 12 hours extended darkness-treated plants (24 hours after gravitropic bending).

There were higher rates of LRP progression under the sucrose restoration conditions compared to the extended darkness control at both time points studied. For example, the modal and media rates of LRP progression were 0.1667 n/h and 0.00 n/h for the sucrose restoration treatment and the extended darkness control, respectively at 18 hours after gravitropic bending (Table 5.5). As the similar pattern was reported for 24 hours after gravitropic bending (Table 5.5). A statistically significant difference in the distribution of LRPs was also reported for the two conditions at the same time points discussed earlier (Table 5.7). The data showed that 1% exogenous sucrose can restore LRP progression in extended darkness-treated plants and it is sufficient to promote the developmental progression of LRPs independent of light availability.

As expected, the return of extended darkness-treated plants to light conditions restored LRP progression under all the time points studied (Figure 5.5; Tables 5.8 & 5.10). Exogenous sucrose supply is reported to not be sufficient to override the requirement of light for root growth and development (Miotto et al., 2021). Although the experimental methods used by Miotto et al (2021) are different from the one presented in this chapter, that is, Miotto et al (2021) conducted the experiment cited in this section on 4dag seedlings, while the experiments presented in this chapter were conducted on 10 dag seedlings, and their plants were grown in the dark longer than those presented in this chapter—the pattern of reduced PR growths is expected under the experimental conditions in the study presented in this chapter.

Preliminary data from an experiment where exogenous was supplied to 10 dag seedlings at transfer and grown in complete darkness for 39 hours (Figures 5.6A & 5.6B) showed reduced PR growth under 0% exogenous conditions, however, PR growth under 1% exogenous sucrose conditions was similar to the 1% exogenous sucrose conditions in the control (Figure 5.6B). This preliminary data, together with the observation under sucrose restoration conditions presented in this chapter suggest that probably the supply of exogenous sucrose can restore root growth and in the context of this thesis the developmental progression of LRPs when plants are grown in complete darkness conditions for shorter periods. This phenomenon might be possible due to a likely adjustment of starch degradation to produce sucrose to sustain growth until an expected light comes on (Graf et al., 2010) as explained earlier.

Dark treatments inhibit mitotic activity in the RAM as reported by the *CYCB1;1:GUS* reporter line (Sassi et al., 2012; Miotto et al. 2021). The *CYCB1;1:GUS* reporter line (Ferreira et al. 1994) has been widely used to study the occurrence of the first asymmetric cell divisions in LR formation and PR growth in Arabidopsis (Beeckman et al., 2001; Casimiro et al., 2001; Himanen et al., 2002; Sassi et al., 2012; Miotto et al., 2021;). Therefore, the *CYCB1;1:GUS* reporter line could be used to study the occurrence of the first asymmetric cell division under extended darkness conditions at the bend sites using the experimental methods discussed in this thesis. This experiment could not be performed for this thesis due to time constraints.

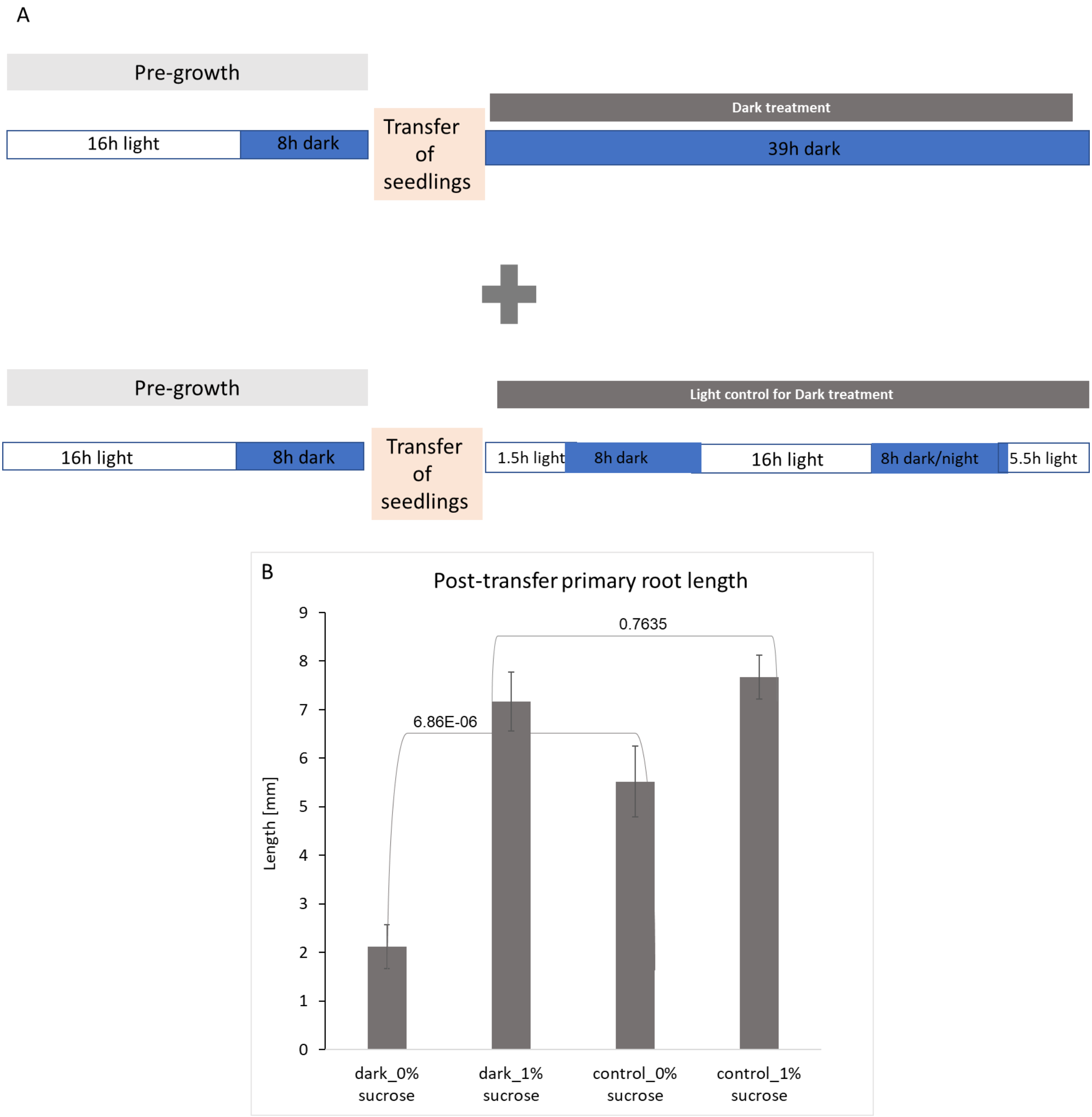

Figure 5.6 **Effect of extended darkness on PR growth**

(A) Schematic of experimental design

(B) Post-transfer PR growth in response to extended darkness and exogenous sucrose supply.. n =32. Error bars are standard error of the mean .Numbers on error are p values from a Turkey Kramer's post hoc test (p<0.05) after one-way anova tests following a normality test (Table 5.11).

Light is not only needed for sucrose synthesis but also for auxin transport (Sassi et al., 2012; Sassi et al., 2013). In light-grown seedlings, shoot-derived auxin (auxin synthesised in the young leaf primordia) are known to promote LR emergence (Bhalerao et al., 2002). The phenotype of dark-grown seedlings is known to look like that of light-grown seedlings with the shoot apex decapitated or polar auxin transport (PAT) inhibited by *N*-1-naphthylphthalamic acid (NPA) applied at the hypocotyl/root junction (Reed et al., 1998; Grieneisen et al., 2007). This, therefore, suggests that light promotes PAT from the shoot to the root and that the transport of auxin may be inhibited under dark conditions. *PIN1* gene expression in hypocotyls is reduced under dark conditions and is induced upon return to light (Sassi et al.. 2012). This reduction in *PIN1* gene expression could lead to decreased availability of auxin in the root which could lead to a reduction in the number of LRPs at advanced stages (≥ stage 5), (Bhalerao et al., 2002), which is effectively a reduction in the developmental progression of LRPs.

Auxin transport in the root is also influenced by light availability, it has been demonstrated that dark treatments promote vacuolar targeting of PIN2 for degradation in the RAM (as opposed to the expected plasma membrane localisation), leading to reduced PIN2 protein levels in the plasma membrane (Laxmi et al., 2008). This response is proposed to alter local auxin transport in the root apex, thereby reducing the size of the RAM leading to reduced mitotic activity as explained earlier (Laxmi et al., 2008; Sassi et al., 2012; Miotto et al., 2021). Taken together, these observations suggest that auxin transport may be inhibited under extended darkness conditions discussed in this chapter, which may be blocking the occurrence of the first asymmetric cell divisions required to initiate LRPs (progression from stage 0 to stage 1). Expression levels of *PIN1* and *PIN2* genes must be studied using the experimental methods and under the conditions discussed in this chapter to understand the dynamics of auxin transport in response to extended darkness treatments at the time points studied in this chapter.

Sucrose and its hexose (glucose) have been reported to induce the expression of auxin biosynthetic and transport genes (Lilley et al., 2012; Sairanen et al., 2012). The addition of up to 5% (from 1 to 5%) of glucose led to increases in IAA contents in the roots of 10-day-old wild-type Col-0 seedlings (Sairanen et al., 2012). Increases in the transcript levels of *YUCCA8*, *YUCCA9*, *CYP79B2*, and *CYP79B* which are members of the auxin biosynthesis gene families were observed when whole seedlings were pre-treated for 4 hours in liquid medium and then treated for 15 h in medium containing 0, 1, or 5% glucose (Sairanen et al., 2012). Additionally, the supply of exogenous sucrose to whole plants increased IAA levels in the root compared to the shoot, suggesting a role of sucrose promoting auxin biosynthesis in the root (Lilley et al., 2012). Increases in the transcript levels of components of the auxin signalling pathway such as *IAA3*, *IAA17*, *SMALL AUXIN UPREGULATED15* (*SAUR15* , *IAA1*, the auxin transporter PIN7 and the auxin biosynthetic *YUCCA8* were reported in response to exogenous sucrose supply (Lilley et al., 2012).

These responses were identified to be both Phytochrome Interactor Factor (PIF), the master regulator of photomorphogenesis dependent and independent (Lilley et al., 2012). Taken together, these observations suggest that sucrose promotes auxin biosynthesis, transport, and signalling. It is, therefore, not surprising that the supply of exogenous to extended darkness-treated plants restored the LRP progression phenotype. This could have been possible potentially due to the induction of auxin biosynthesis, transport, and/or signalling which would have been inhibited under extended darkness conditions as discussed earlier. The induction of auxin biosynthesis, transport, and/or signalling would then activate the cell cycle, leading to the resumption of the asymmetric cell division needed to initiate LRPs.

5.6 Conclusion

The results from the study presented in this chapter have shown that some of the LRPs can undergo the nuclear migration process, but are unable to undergo LRP initiation. Suggesting an extended darkness-induced partial inhibition of nuclear migration and complete inhibition of LRP initiation. Additionally, the supply of exogenous sucrose to extended darkness-treated plants restored their LRP progression phenotype, and so did the return of extended darkness-treated plants to light. The restoration of LRP progression in response to exposure to light and exogenous sucrose could be due to their roles in auxin biosynthesis and transport. Sucrose- and/or light-induced auxin biosynthesis and transport would make auxin available to control the cell division that underpins LRP initiation.

Experiments testing the occurrence of the first asymmetric cell division required to initiate LRPs, auxin levels, and the expression of key auxin biosynthetic and transporter genes at bend sites under extended darkness conditions and in response to exogenous sucrose and light restoration should be conducted to understand the auxin biosynthesis and transport dynamics and their relationship with LR initiation must be conducted.

Chapter 6 General discussion and conclusions.

6.1 Summary of study

Most of the efforts to determine the role of sucrose on LR formation have been focused on measuring the lengths of LRs, quantifying LR numbers and calculating LR densities (Freixes et al., 2002; Lee-Ho et al. 2007). Those studies that have scored LRPs have not categorised them into the various developmental stages (Macgregor et al., 2008). In the absence of data on LRP distribution among the various developmental stages in response to sucrose supply potential checkpoints of regulation by sucrose to be determined. Therefore, the study presented in this thesis set out to understand how sucrose promotes LR formation by focusing on determining the effect of sucrose on the distribution of LRPs among the various developmental stages, thereby determining a checkpoint of regulation by sucrose.

The first data chapter (chapter 3) of this thesis describes the development of a filter paper/ Parafilm strip method that allows the direct supply of exogenous sucrose to the shoot without the contact of the root with sucrose via the media. The development of this method was necessary because the conventional method of growing Arabidopsis on plates involves the supply of exogenous sucrose to both shoot and root, suggesting uptake of sucrose by both shoot and root. Under soil conditions, roots do not take up sucrose, instead, sucrose is transported from the shoot to the root to promote its growth and development. Consequently, translation of insights from plate-based experiments to soil conditions may not yield reproducible results or biological insights.

The study also explored the effects of various methods of supplying exogenous sucrose: (i) directly to the shoot, (ii) directly to the root via the media, and (iii) to both shoot and root on LR numbers and densities, and LRP numbers. The results (Figures 3.4, 3.5, 3.6 & 3.7) revealed that supply of exogenous sucrose directly to the shoot is necessary to observe a statistically significant progression of LRPs from early stages (≤ stage 3) to later stages (> stage 3). A significant number of LRPs were concentrated at the early stages in both the presence and absence of exogenous under both conditions where exogenous sucrose was supplied: (i) directly to the root via the media, and (ii) to both shoot and root. This observation suggested that, under *in vitro* conditions, the uptake of exogenous sucrose by the shoot, and its concomitant transport alongside endogenous sucrose to the root promotes the developmental progression of LRPs. These observations answered the questions asked in the chapter about whether there were differences in the responses of LR parameters (e.g., LR, LR density, LRP, LRP density) to exogenous sucrose supplied via the three approaches discussed earlier.

Follow-up analyses of early-stage LRPs revealed that a significant number of LRPs were concentrated at stage 1 in the absence of exogenous sucrose compared to the significant concentration of LRPs at stages >3 in the presence of exogenous sucrose. This indicated a role of sucrose on the progression of LRPs from stage 1 to later stages. Taken together, these observations suggested a potential role of sucrose transport, possibly the transport of a molecule or a signal (likely auxin) from the shoot to the root that promotes the developmental progression of LRPs. Sucrose transporters such as AtSWEET11, AtSWEET12, and SUC2 could be studied to elucidate the role of sucrose in promoting the developmental progression of LRPs in Arabidopsis.

Crosses of *atsweet11atsweet12* double mutant and *suc2* single mutant with the *pGATA23:NLS-GFP* and the *GATA23:H2B:3xmCherry/pUBI: 3xGFP: PIP1, 4* reporter lines, respectively were generated. However, they could not be successfully used to understand how a defect in sucrose transport as mediated by *AtSWEET11*, *AtSWEET12,* and *SUC2* would affect the developmental progression of LRPs for the following reasons: (i) a wild type allele was found in the PCR results using the atsweet11 gene specific primer and TDNA. And there was not enough time left to troubleshoot the problem, (ii) the *suc2-1* homozygote does not produce any viable seeds, the heterozygote would require a regular genotyping at every experiment to determine heterozygosity which would lead to a never ending cycle that would be both time and resource-intensive.

The second data chapter (chapter 4) of this thesis was aimed at understanding the temporal dynamics of the developmental progression in response to exogenous sucrose supply (via the shoot) this was necessary to understand whether there is a particular checkpoint at which exogenous sucrose would control the developmental progression of LRPs . To understand this, LRP formation was first synchronised by adapting the filter paper/Parafilm strip experimental system described in chapter 3 to the gravitropic bending assay (Ditengou et al., 2008; Lucas et al., 2008; Richter et al., 2009). This was to allow for time course experiments to be conducted to quantify the developmental progression of LRPs from the nuclear migration stage (which signals the initiation of LRPs) to the emergence of LRPs and outgrowth of LRs.

The data (Figure 4.5C & 4.5D) showed that an LRP is induced at the gravity-induced curvature (bend site) as early as 6h after gravitropic bending, and also corroborated the results in chapter 3 that the supply of exogenous sucrose indeed promotes the developmental progression of LRPs. A checkpoint of regulation by sucrose was, however, not determined potential because the plants used in this study did harbour mutations in sucrose synthesis, transport or metabolism, endogenous sucrose is expected to be made available to roots in both the presence and absence of exogenous sucrose. Therefore, carbon starvation experiments via dark treatments would provide a tool for dissecting the role of sucrose under limited or reduced endogenous sucrose levels.

The dynamics of LRP progression in response to exogenous sucrose was, however, determined by calculating rates of progression of LRPs. The data in (Figure 4.8) revealed that the supply of exogenous sucrose promotes the developmental progression of LRPs by increasing the speed of progression and that this speed of progression remains steady across developmental times. This observation, therefore, suggests that sucrose merely increases the speed of LRP developmental progression and may not be driving the development of LRPs itself. This is likely that sucrose fuels or promotes some aspect of the biology of a regulator of LRP development (e.g., auxin) either as a resource or a signal or both, and this factor (likely auxin) is the engine that drives LRP development ‘apparatus’. Auxin regulates the asymmetric cell division that underpins LRP development, sucrose could promote some aspect of auxin’s biology, and auxin would drive LRP development.

Follow up series of experiments in chapter 5 of this thesis on the effect of extended darkness, which is an unexpected darkness. Under the light/dark cycle, which plants depend on for their growth, development, and physiology (e.g., 16h/light, 8h/dark regime), plants are expecting light at the end of the dark period—beginning of dawn. However, when expected light is replaced with darkness, that is referred to as unexpected darkness or extended darkness.

The effect of extended darkness on LRP progression revealed that extended darkness treatments led to the partial and complete inhibition of nuclear migration and LRP initiation (Figure 5.3), respectively and these LRP phenotypes were conserved across the two extended darkness treatments studied (6h and 12h). The data in (Figures 5.4 & 5.5) showed that the availability of a carbon source either directly by the supply of exogenous sucrose or indirectly by the growth of Arabidopsis plants under expected light/dark cycles promoted the transition from the nuclear migration stage to LRP initiation and morphogenesis stages at just 6h after the supply of exogenous sucrose or return of extended darkness-treated plants to expected light/dark conditions. The data in (Figure 5.4) also revealed that the supply of exogenous sucrose was sufficient to restore the extended darkness-induced LRP phenotype. Taken together, these observations suggest that nuclear migration (stage 0) and LRP initiation (stage 1) are checkpoints at which sucrose controls LRP formation.

6.2 Interpretation of study

6.2.1 Phloem-based long-distance transport from shoot to root may be involved in promoting the developmental progression of LRPs.

The fact that the pattern of distribution of LRPs between the early and later stages under conditions where exogenous sucrose was supplied to both shoot and root was similar to the distribution of LRPs under conditions where exogenous sucrose was supplied only to root, and not to that under conditions where exogenous sucrose was supplied only to the shoot suggests that under *in vitro* conditions where both the shoot and root are in contact with sucrose, exogenous sucrose is preferentially taken up by the root, instead of the shoot. The root has been reported to serve as a carbon source under conditions where both the shoot and the root are in contact with sucrose via the media (Papaioannou et al., 2018).

In a study by Papaioannou, (2018), where exogenous sucrose was supplied to both the root and the shoot *in vitro*, AtSWEET1*1* and AtSWEET12 were expressed in both the shoot and the root. However, there were comparatively higher expression levels of AtSWEET11 and AtSWEET12 in the root than in the shoot in Col-0 plants. Relatively, higher expression levels of AtSWEET11 were observed in the shoot relative to the root under *ex-vitro* conditions (Papaioannou et al., 2018). This study suggests that, under *in vitro* conditions when both the shoot and the root are in contact with sucrose, the root preferentially, takes up sucrose from the media, and transports it to the shoot, and that the directionality of sucrose transport by AtSWEET11 and AtSWEET12 is reversed under such conditions along the sucrose concentration gradient.

The observation of the significantly higher numbers of later stage LRPs in response to exogenous sucrose supply under conditions where exogenous sucrose was supplied to only shoot compared to the supply of exogenous sucrose to only root suggests a requirement of the transport of a shoot-derived factor from the shoot to the root to promote the developmental progression of LRPs. And this factor is likely to be auxin.

According to the Münch hypothesis, sucrose is the driver of phloem transport and shoot-derived auxin is transported through the phloem from the shoot to the root (Münch 1930; Ljung et al. 2001; Marchant et al. 2002; Knoblauch and Peters 2017).

Two pathways are known for the long-distance transport of auxin from the shoot to the root through the phloem: (i) Rapid transportation via bulk flow alongside other assimilates through the mature phloem from the shoot to the root, and (ii) polar auxin transport (Swarup et al., 2001; Vanneste and Friml, 2009). AUX1 is known to load shoot-derived auxin into the mature phloem (Marchant et al. ,2002). In the root, AUX1 unloads auxin from the mature phloem, then transports it acropetally through the protophloem to the root meristem (Swarup et al.,2001). The fact that both sucrose and auxin are transported through via bulk flow (Münch 1930; Knoblauch and Peters, 2017;) and are unloaded at the protophloem (Swarup et al., 2001; Ross-Elliott et al., 2017) coupled with their involvement in LRP formation may suggest a link between the two molecules in regulating LRP formation.

In the study presented in chapter 3, the concentration of LRPs at early stages in the absence of exogenous sucrose under both conditions where the root was in contact with exogenous sucrose, but the shoot was not, suggests the phloem transport of auxin or some other phloem transported factor could be involved in the developmental progression of LRPs. This prediction was arrived at due to the observation of significantly high numbers of early-stage LRPs in the absence of exogenous sucrose under conditions where exogenous sucrose was supplied directly to only shoot, compared to the concentration of LRPs at early stages in the presence of exogenous sucrose under the same conditions. Although phloem auxin transport dynamics in relation to the developmental progression of LRPs were not investigated in the study presented in this thesis, the fact that phloem transport is driven by sucrose makes the aforementioned prediction a testable hypothesis.

In the root, the unloading of auxin from the mature phloem and its subsequent acropetal transport to the PR tip through the protophloem could be mediated by sucrose via a potential regulation of AUX1 activity or its transcription. While there is no currently known evidence of the effect of sucrose on the transcription of AUX1 or its activity, there is evidence for the effect of sucrose on the transcription of the genes that encode other auxin transporters, *PIN1*, *PIN2,* and *PIN3* (Zhang et al., 2020). It is, therefore, possible that sucrose may regulate the transcription of AUX1 too, this is unknown.

AUX1 is also involved in the basipetal transport of auxin from the PR tip to XPP cells, as they are expressed in XPP cells before LRP initiation (Swarup et al., 2001; Marchant et al., 2002). This is evidenced by reduced initiation of LRPs, and a concomitant reduction in the numbers of emerged LRs in the *aux1* mutant (Marchant et al., 2002). Based on the potential regulatory effect of sucrose on AUX1 mentioned earlier, the AUX1-mediated basipetal transport of auxin from the PR tip into XPP cells could also be regulated by sucrose. In the XPP, auxin would then modulate LRP initiation.

6.2.2 LRP initiation may be the checkpoint at which sucrose promotes LRP formation.

The conclusions from the extended darkness experiments in section 5.4 of chapter 5 revealed that the progression from the nuclear migration stage to the LRP initiation stage is blocked under both shorter (6h) and longer (12h) periods of extended darkness. This indicates an inhibition of LRP initiation. And not only that the data also suggested that the nuclear migration process is partially inhibited (Figure 5.3).

Sucrose availability was sufficient to completely restore these phenotypes independent of light availability. This altogether suggests that sucrose availability promotes nuclear migration and the first anticlinal cell division in the LR founder cells which is crucial for LRP initiation. The observation and predictions from section 4.4.3 of chapter 4 of this thesis, that while the supply of exogenous sucrose accelerates the developmental progression of LRPs, compared to the absence of sucrose, the rates of progression remains constant across developmental times. Suggesting that sucrose may not be the direct driver of the aforementioned processes. Instead, it may mediate the homeostasis of the 'chief' regulator of LRP development—auxin, as auxin drives the whole process of LR formation—from pre-patterning—to initiation—to meristem activation—to emergence (De Smet et al., 2007; De Rybel et al., 2010; Péret et al., 2012; Xuan et al,. 2015; Goh et al., 2016).

The initiation of LRPs as underpinned by anticlinal cell division is regulated by auxin biosynthesis, transport, and signalling (Santos Teixeira and Ten Tusscher 2019). Auxin is made available to the founder cells by auxin influx and efflux transporters *AUX1* and *PIN3*, respectively. Auxin availability activates ARF7 and *ARF17* which induce the expression of their direct downstream targets LATERAL ORGAN BOUNDARIES- DOMAIN 16 (LBD16), LBD18 and LBD 33. LBD16, LBD18 and *LBD 33* mediate the nuclear migration and asymmetric divisions, respectively (Okushima et al., 2007; Berckmans et al., 2011; Goh et al., 2012).

Before nuclear migration and asymmetric divisions occur, the founder cells swell which requires endodermal volume loss to allow for the imminent pericycle radial growth; the endodermis volume loss occurs downstream of the auxin signalling module (IAA14/SLR4) involved that mediates LRP initiation (Vermeer et al., 2014; Marhavý et al., 2016; Santos Teixeira and Ten Tusscher, 2019).

Sucrose may also promote LRP initiation (aside from the AUX1-mediated regulation of LRP initiation discussed in section 6.2.1) via a potential effect on local auxin biosynthesis in the root. Sucrose regulates the transcription of biosynthesis-related genes (e.g., *YUCCA2*, *YUUCA6,* and *YUCCA8*) (Lilley et al. 2012; Sairanen et al. 2012; Zhang et al. 2020). YUCCA 6 and YUCCA8 are expressed in the root and are involved in local auxin biosynthesis in the root (Chen et al., 2014). Making it likely for sucrose to regulate local auxin biosynthesis in the root. Sucrose and/or its hexoses (e.g., glucose) in the root may promote local auxin biosynthesis at the PR tip (Müller et al., 1998). Auxin biosynthesis may occur at the PR tip because LRPs do not have the autonomy to synthesise auxin independently of the PR until they become LRs and begin elongation (Müller et al., 1998). Auxin would then be transported basipetally from the PR tip to founder cells at the xylem pole pericycle (XPP) by *PIN3*—*PIN3* activity may be promoted by the availability of sucrose and/or its hexose, glucose (Zhang et al., 2020).

The availability of auxin in XPP cells would lead to the degradation of IAA14/SLR, which would lead to the de-repression of ARF7;19 (Fukaki et al., 2002; Fukaki et al., 2005; Okushima et al. 2007). ARF7;19 would then induce the transcription of LBD 16, LBD18, and LBD 33, which would then activate the transcription of an unknown gene, which would promote nuclear migration (Berckmans et al. 2011), as the downstream target of the LBDs involved in nuclear migration remains unknown (Santos Teixeira and Ten Tusscher, 2019). LBD 16, LBD18, and LBD 33 would also activate the cell cycle by regulating the activities of E2FA (Berckmans et al., 2011) and CYCB1;1 (Vanneste et al., 2005), thereby regulating the formative cell division that initiates an LRP (Berckmans et al., 2011; Goh et al., 2012). In the presence of a sucrose-induced auxin, the IAA12/BDL-ARF5/MP module would also be activated to induce LRP initiation (Figure 6.1B) (De Smet et al., 2010), although the downstream target of the IAA12/BDL-ARF5/MP pair is unknown (Santos Teixeira and Ten Tusscher, 2019).

Sucrose-induced upregulation of PIN3 expression (Zhang et al., 2020) would also lead to auxin flux between the endodermis and the pericycle which would lead to the division of LRFCs, thereby initiating LRPs (Marhavý et al., 2013). The accumulation of auxin in the endodermis would also promote endodermal volume loss which is crucial for the activation of the cell cycle for LRP initiation to occur (Vermeer et al., 2014; Marhavý et al., 2016). Under gravitropic bending conditions, *in vitro*, sucrose is likely to promote the transport of auxin from the PR tip to the elongation zone, it could do this by promoting the relocalisation of PIN1 (Ditengou et al., 2008), thereby causing a differential in accumulation of auxin at the lower side of the elongation zone, leading to the formation of a bend site.

6.3 Limitations of study

In the study presented in this thesis, LRPs were scored using fluorescence reporter lines, without clearing the roots as discussed in detail in chapter 1. Scoring LRPs without fixing the roots means that the plant samples cannot be stored overnight. This, therefore, means that LRPs must be scored quickly compared to fixed roots. Additionally, since LRP development is susceptible to environmental changes (e.g., light), while some root samples are being scored, the other plants on a plate awaiting to be scored may continue growing. This suggests that the LRP stage recorded at the end of analyses, may be more advanced than it was immediately after the experiments were ended. Also, there is no way for samples to be stored and imaged later, as that would kill the plants or lead to physiological changes that may create artifacts in the LRP distribution data.

The scoring of LRPs from several root samples may lead to an under-or over-scoring of certain stages, this may be due to fatigue or simply human error. Additionally, in chapter 4, a mathematical artifact due to the calculation of the rates of LRP progression was discussed and summarised in section 6.1 of this chapter, to avoid this artifact, rates of LRP progressions should only be calculated for time points earlier than 24 hours. For later time points where LRPs would have emerged, the lengths of LRs in response to exogenous sucrose or any other desired treatment must be used.

In this study, sucrose levels and/or its hexoses were not measured under the extended darkness conditions. Therefore, it could not be emphatically concluded that the extended darkness treatments caused carbon starvation. The partial inhibition of nuclear migration under extended darkness conditions suggests some levels of sugar in the root that would have fuelled not only nuclear migration but also the preceding processes (e.g., LRFC identity specification). It is, therefore, recommended for sucrose, glucose, and fructose levels at the bend sites, in the PR, and in whole seedlings to be measured under extended darkness conditions.

This would help to understand the dynamics of the carbon status of the plants and whether carbon availability would control LR formation processes earlier than the nuclear migration stage. Earlier studies had shown that the 1% exogenous sucrose and high light intensity do not affect the earliest process in LR formation, priming, and pre-branch site formation (Moreno-Risueno et al. 2010), however, we do not know whether exogenous sucrose and factors that increase endogenous sucrose levels (e.g., high light intensity and elevated CO_2 levels) would have any effect on the LRFC identity specification, which has been already discussed in chapter 1 as the subsequent process to priming and pre-branch site formation.

6.4 Outstanding questions and proposed experiments

Chapter 3 of this thesis led to the conclusion of the potential role of sucrose transport in promoting LRP developmental progression. This, therefore, raises the question of whether mutants of the sucrose transporters AtSWEET11;12 and AtSUC2, could be used to study how defects in sucrose transport could affect the developmental progression of LRPs, especially, the progression from the nuclear migration stage to the LRP initiation stage. This could be done by making crosses with the known LRP markers, for example, *pGATA23::NLS-GFP* (used in this study). The resulting hybrid line could be used to study the response of LRP distribution to exogenous sucrose supply and extended darkness under the same conditions discussed in this thesis. As already discussed in this chapter and in chapter 3, the crosses for the aforementioned hybrids were already made and hybrids lines generated. However, troubleshooting could be done to identify and solve the problem that led to the identification of a wild type allele in the *atsweet11* mutant.

It is understood that defects in the transport abilities of these sucrose transporters could affect growth and development at the whole plant level, therefore, an Inducible gene expression system such as one based on the XVE factor which is constructed from the bacterial transcriptional repressor LexA as a DNA-binding domain, the transcription-activating domain of the herpes simplex virus transcription factor VP16, and the regulatory region of the human oestrogen receptor could be used (Zuo et al., 2000). The XVE inducible gene expression system is not known to cause any XVE independent gene expression, which makes it ideal for inducing or knocking down gene expression and to study the knock-on effect growth and development or a physiological process (Zuo et al., 2000). The XVE gene expression system is also dose- responsive allowing a quantitative control of gene expression upon induction (Zuo et al., 2000). The placement of XVE under the control of a tissue-specific promoter of choice (e.g., one that is expressed in the XPP) tissue would lead to a quantitative fine-tuning of spatial and temporal gene expression of the gene of interest (e.g., *AtSUC2* and/or *AtSWEET11;12*) (Machin et al., 2019).

The XVE gene expression system is estradiol inducible, therefore, in the presence of estradiol, the XVE factor would enter the nucleus and bind to LexA operator sequences (LexAop) placed upstream of a promoter (that is expressed in the XPP), allowing transcription of the *AtSUC2* or the *AtSWEET11;12 gene*s. The reporter lines generated from this inducible gene expression system could then be used for physiological experiments under extended darkness conditions to understand how a conditional knockdown of *AtSUC2* or the *AtSWEET11;12 gene*s would affect nuclear migration and LRP initiation. Based on the results presented in this thesis, it is expected that a reduction in the activity of these transporters would lead to a partial inhibition of nuclear migration, and a complete inhibition of LRP initiation. it is also expected that the supply of exogenous sucrose would restore the LRP phenotype (Chen et al., 2012).

The prediction that sucrose could be controlling nuclear migration and LRP initiation by being upstream of either auxin biosynthesis and/or transport raises the experimental aim to understand auxin biosynthesis and transport, dynamics in response to sucrose, both under the extended darkness and control conditions. If sucrose indeed promotes LRP initiation by being upstream of auxin transport, then hybrid lines could be generated by crossing the mutants of auxin-transport related genes such as AUX1, PIN3, and LAX3 with pGATA23::NLS-GFP. The resultant hybrid lines could be used for physiological experiments under both the extended darkness and control conditions . The use of the resultant hybrid lines would lead to the initiation of LRPs in the presence of exogenous sucrose. However, in the absence of exogenous sucrose and under extended darkness conditions, LRPs would not be initiated. The data collected from such an experiment would reveal that concentration of LRPs at the ground state, with some LRPs at stage 0 (nuclear migration stage), but not at stage 1, which is the LRP initiation stage just as was shown in chapter 5 of this thesis.

Similarly, mutants of the root-derived auxin-biosynthetic genes such as the yuc3;5;7;8;9, known as the yuc quintuple (yucQ) mutant (Chen et al., 2014) could be used in physiological experiments under the experimental conditions from this thesis, to understand whether sucrose indeed acts upstream of auxin biosynthesis to control LRP initiation. If sucrose regulates LRP progression by mediating local auxin biosynthesis in the root, then LRPs should still be initiated in these mutants in the presence of exogenous. However, no LRPs would be initiated in the absence of exogenous sucrose in these mutants.

Mutants of auxin response and signalling genes such as TIR1/AFB3, IAA14/SLR, and IAA12/BDL could be used in similar experiments to understand whether there is a tripartite relationship among sucrose availability, auxin signalling, and LRP initiation. Additionally, the measurement of *DR5::GUS* activity in response to extended darkness in the presence or absence of exogenous sucrose would provide insight into auxin response dynamics under the experimental conditions discussed in this thesis.

To understand whether sucrose could directly regulate LRP initiation by inducing the transcription of cell cycle-related genes involved in the asymmetric divisions required for LRP initiation, the transcript levels of CYCB1;1 and E2FA should be measured under the extended darkness and control conditions in the presence and absence of exogenous. This could be done by harvesting bend sites under the same time points studied in chapter 5 of this thesis, isolating RNAs, and performing quantitative PCR on the samples to determine the mRNA levels in response to the aforementioned conditions. Translational analyses via western blotting or a more sophisticated Mass spectrometry (MS) (Greenbaum et al., 2003) could also be done to know the abundance of the CYCB1;1 and E2FA proteins in response to the aforementioned experimental conditions. The aforementioned proposed experiment, notwithstanding, the data shown in chapter 4 shows that direct regulation of LRP initiation is not likely, as its effect on the speed of LRP progression remained constant across developmental times.

6.5 Conclusions

1. The main conclusion from the study presented in this thesis is that sucrose promotes the migration of the nuclei of two adjacent founder cells toward their common cell wall, thereby signalling the initiation of LRPs. Sucrose also promotes the initiation of LRPs, particularly, sucrose may be required for the asymmetric cell divisions that are a prerequisite for LRP initiation.

2. The study presented in this thesis has also shown that under *in vitro* conditions, sucrose must be supplied directly to the shoot to observe its effect on the developmental progression of LRPs, suggesting a role of a factor transported alongside sucrose through the phloem from shoot to root to promote nuclear migration and LRP initiation. This factor based on the knowledge from the literature on the regulation of nuclear migration and LRP initiation is likely to be auxin.

3. The filter paper/Parafilm strip experimental system developed for the execution of the experiments discussed in this thesis provides a simple and cost-effective tool for developmental and physiological studies aimed at establishing a causal relationship between shoot-derived resources and root growth and development in Arabidopsis.

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