

**ENVIRONMENTAL REGULATION OF
PLANT ROOT DEVELOPMENT**

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Abstract

Soil compaction represents a major agronomic challenge, inhibiting root growth and resource capture, causing significant yield losses. Roots employ ethylene to sense soil compaction as its reduced air space causes this gaseous signal to accumulate around root tips (Pandey *et al.*, 2021b). One of the key aims of Chapter 2 is to investigate the underlying hormonal signals and regulatory genes that control compaction resistance in tomato. The aims will be achieved by exploiting prior knowledge gained from the model plants and crops (i.e., *Arabidopsis*, rice, and maize). My results reveal that during soil compaction stress tomato roots employ the volatile hormone signal ethylene to promote root growth inhibition. For example, less ethylene-sensitive tomato lines were observed to exhibit root growth resistance to compaction stress. The aim of my thesis objective is to identify the underlying genes that control root penetration to provide a mechanistic understanding of root-determined processes that influence traits within and beyond the root. This knowledge can be vital for longer-term breeding strategies to deliver novel varieties into commercial practice.

Hormone signals like ethylene have recently been reported to play a critical role in soil compaction responses in crops like rice (Pandey *et al.*, 2021b). Ethylene is likely to employ other downstream signals to mediate these compaction responses like cortical radial cell expansion. Rice roots grown in compacted soil conditions exhibit elevated levels of the signal abscisic acid (ABA). In Chapter 3, I explore the role of ABA during rice root compaction responses. My results reveal that soil compaction inhibits rice root growth by increasing the ethylene response, which then upregulates several abscisic acid (ABA) biosynthesis genes. Testing the functional importance of their upregulation revealed mutations in rice ABA biosynthetic genes like *OsMHZ5* disrupt radial cortical expansion in compacted soil, suggesting ABA acts downstream of ethylene to control this adaptive response.

Water stress regulates several root adaptive responses including compaction (focus of Chapter 3). In Chapter 4, I investigate how water stress controls lateral root outgrowth. Lateral root branching and length play essential roles during adaptation to water stress. To identify potential regulators of this adaptive process, GWAS predictions on a root phenotype dataset generated for a maize diversity panel subjected to water stress were analysed. One promising loci predicted by GWAS encoded a suberin synthesis gene. I tested the impact of a loss-of-function mutant allele in *Arabidopsis*, which exhibited significantly higher lateral root (LR) length compared to wild-type (WT; Col-0). Moreover, mutant roots display a similar longer lateral root phenotype as suberin degradation line *pCASP1::CDEF*, suggesting there is a link between root surface area and suberin-based water-impermeable barrier formation in roots. The spatial relationship between suberin biosynthesis and lateral root development under environmental stress conditions awaits expressing these transgenic lines in a mutant background.

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Publications

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Abbreviations

°C	Degrees Celsius
3D	Three Dimensional
AAO	Absciscic aldehyde oxidase
ABA	Absciscic acid
AC	Ailsa Craig
Ac	Anticlinal division
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ACS	1-aminocyclopropane-1-carboxylic acid synthase
AM	Arbuscular mycorrhizae
AP2	APETALA2
BD	Bulk density
c1	Outer cortex layer
c2	S cortex layer
c3	Inner cortex layer
Cas9	CRISPR-associated protein 9
CASP1	Casparian strip membrane domain protein 1
cDNA	Complementary DNA
CEI	Cortex-endodermis initial
cm	Centimetre
cmx	Central metaxylem
Col	Columella
Cor/co	Cortex

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS	Casparian strip
CTF	Controlled traffic farming
cv	Cultivar
DBD	DNA-binding domain
DNA	Deoxyribonucleic acid
eFP	Electronic Fluorescent Pictograph
Endo/en	Endodermis
ep	Epidermis
<i>epi</i>	<i>Epinastic</i>
ER	Endoplasmic reticulum
ERF	Ethylene response factor
ET	Ethylene
ex	Exodermis
EZ	Elongation zone
FA	Fatty acid
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FC	Fold change
g	Gram
GPAT5	Glycerol-3-phosphate acyltransferase 5
<i>gr</i>	<i>Green-ripe</i>
gRNA	Guide RNA

GSA	Gravitropic set-point angle
GWAS	Genome-Wide Association Study
HC	Hair cell
IL	Introgression line
<i>KCS4</i>	<i>3-Ketoacyl-CoA Synthase 4</i>
kV	Kilovolt
L(l)	Litre
LR	Lateral root
LR2	Second lateral root
LRC	Lateral root cap
LRP	Lateral root primordia
M	Molar mass
M82	<i>Solanum lycopersicum</i> cv. M82
Mb	Megabase
mc	Middle cortex
MCS	Multiseriate cortical sclerenchyma
mg	Milligram
<i>MHZ4</i>	<i>Mao huzi 4</i>
ml	Millilitre
mm	Millimetre
mol	Mole
ms	Millisecond
mx	Metaxylem
NCED	9-cis-epoxycarotenoid dioxygenase
NHC	Non-hair cell

<i>nor</i>	<i>Non-ripening</i>
<i>not</i>	<i>Notabilis</i>
<i>Nr</i>	<i>Never-ripe</i>
P	Pearson
PAR	Photosynthetically active radiation
PBS	Phosphate buffered saline
Pc	Periclinal division
PCC	Phloem companion cells
PCR	Polymerase chain reaction
pe	Pericycle
Penn	<i>Solanum pennellii</i>
PFA	Paraformaldehyde
ppm	Part per million
PPP	Phloem pole pericycle
PR	Primary root
Pro	Procambium
QC	Quiescent centre
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
QTL	Quantitative trait loci
RAM	Root apical meristem
RH	Root hair
<i>rin</i>	<i>Ripening inhibitor</i>
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute

RSA	Root system architecture
s/sec	Second
SAM	S-adenosyl-L-methionine
sc	Sclerenchyma
SDR	Short-chain dehydrogenase/reductase
st	Stele
UHPLC-MS/MS	Ultra-high performance liquid chromatography-electrospray tandem mass spectrometry
UV	Ultraviolet
VFN8	<i>Solanum lycopersicum</i> cv. VFN8
VLCFA	Very-long-chain fatty acid
WT	Wild-type
XPP	Xylem pole pericycle
ZEP	Zeaxanthin epoxidase
μA	Microampere
μl	Microlitre
μm	Micrometre
μmol	Micromole

1. Chapter 1: Background

1.1. The importance of soil compaction for agriculture and global food security

Global food demand is expected to rise by 70% as the world's population approaches 9.2 billion by 2050 (Ray *et al.*, 2013; FAO, 2017). Tilman *et al.*, (2011) claim that between 2005 and 2050, global food consumption will increase by 100–110%. To attain future food security, a "next-generation Green Revolution" is required. To achieve a more sustainable agricultural development, radical new thoughts and practices are required. The next Green Revolution will require a far more comprehensive, systems-based plan that includes the society, economy, and environment at all levels of organization (Nüsslein and Dhankher, 2016). If a major food production disaster is to be avoided to meet the needs of a growing global population, transformative research across the agri-food sector is required. Agriculture in the future will require individualised solutions that account for not only fundamental shifts in existing knowledge and supporting technologies, but also the need to protect the environment and meet societal demands (Dhankher and Foyer, 2018).

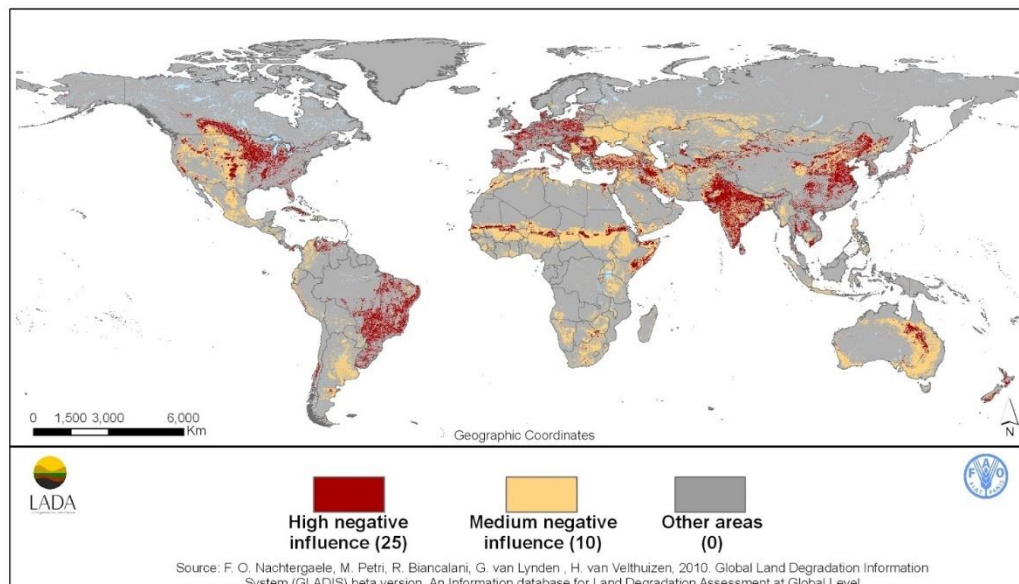


Figure 1.1. Global map of effects of soil compaction (adapted from Nachtergaele *et al.*, 2010).

Root penetration is reduced by soil compaction in both the deeper and surface soil layers. As a result of this, global agricultural yield is being reduced (Correa *et al.*, 2019). Compaction susceptibility in natural soils is a term used to describe the susceptibility of agricultural soils to compaction. As shown in Figure 1.1 and 1.2, Houšková and Montanarella, (2008) and Nachtergaele *et al.*, (2010) showed natural soil susceptibility to compaction, in the case of agricultural soils.

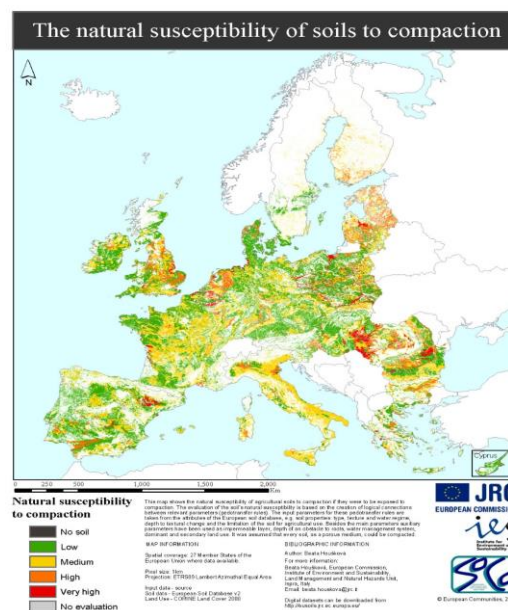


Figure 1.2. Europe map of natural soil susceptibility to compaction in the case of agricultural soils (adapted from Houšková and Montanarella, 2008).

Modern agricultural techniques have increased soil compaction, primarily due to intensification of activities resulting in the use of tillage practices and heavier machinery (Soane *et al.*, 1982; Mangalassery *et al.*, 2015), causing the degradation of ~68 million hectares of land worldwide (Hamza and Anderson, 2005). Compaction reduces soil porosity and increases bulk density, limiting water and nutrient flow and availability (Horn *et al.*, 1995; Hamza and Anderson, 2005). The reduction in soil pore space, particularly in large air-filled pores, also limits gas transmission between the rhizosphere and roots (Fujikawa and Miyazaki, 2005). Pandey *et al.*, (2021b) reported that in compacted soils, entrapped ethylene triggers root growth inhibition.

Roots have been observed to undertake adaptive growth responses, such as an enhanced radial expansion of root tips, to potentially enlarge cracks and penetrate compacted soils (Correa *et al.*, 2019; Pandey *et al.*, 2021b). However, Pandey *et al.*, (2021b) and Vanhees *et al.*, (2021) have reported that in compacted soils, rice and maize roots that are less ethylene-sensitive and remained narrow are better able to penetrate hard soils.

Figure 1.3 illustrates an overview of the impact of soil compaction on crop and soil caused by field trafficking, ground pressure, and heavy machinery.

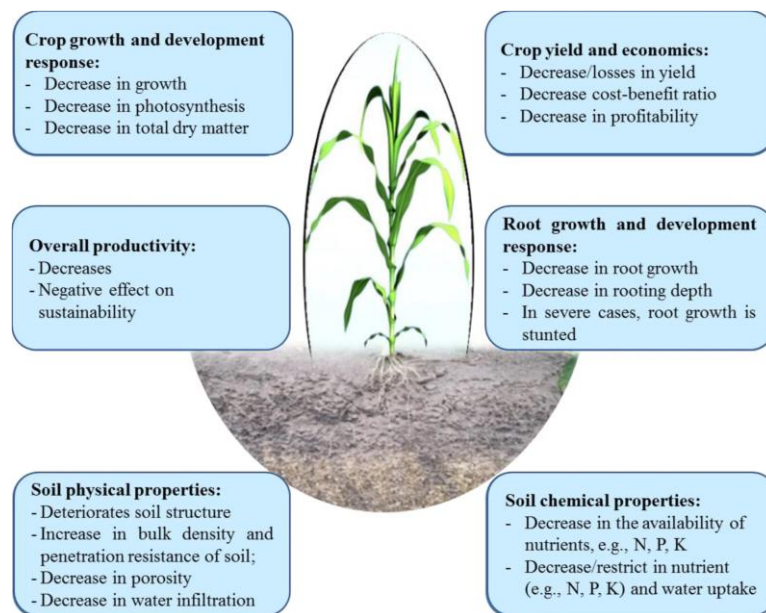


Figure 1.3. An overview of soil compaction's effects on agricultural productivity and soil properties (adapted from Shaheb *et al.*, 2021).

1.2. Root system architecture (RSA)

The term "root system architecture" (RSA) refers to the structure and shape of a root system and is defined as the spatial configuration of a root system in soil (de Dorlodot *et al.*, 2007). The term "architecture" refers to a complex assemblage of subunits' spatial configuration, with the implication that the overall configuration has functional importance (Lynch, 1995).

RSA has significant agronomic importance. The uptake of minerals, nutrients, and water is influenced by the spatial configuration of a crop's root system. For example, root angle has a major impact on foraging for immobile nutrients like phosphate that preferentially accumulates in topsoil (Huang *et al.*, 2018). Forde and Lorenzo, (2001); López-Bucio *et al.*, (2003); Liu *et al.*, (2020) showed that the number and length of lateral roots affect the capture of mobile resources such as water and nitrate. Many root traits are highly responsive to environmental signals such as water and nutrient availability (Huang *et al.*, 2018). Understanding the molecular mechanisms regulating these adaptive responses is important for future crop improvement (Hodge *et al.*, 2009).

Figure 1.4 illustrates an idealised eudicot root system at 2 stages of development, featuring a primary embryonic (also known as allorhizic or taproot) root system. This 'herring bone' arrangement contrasts markedly with the homorhizic or fibrous root systems typical in monocots (Atkinson *et al.*, 2014).

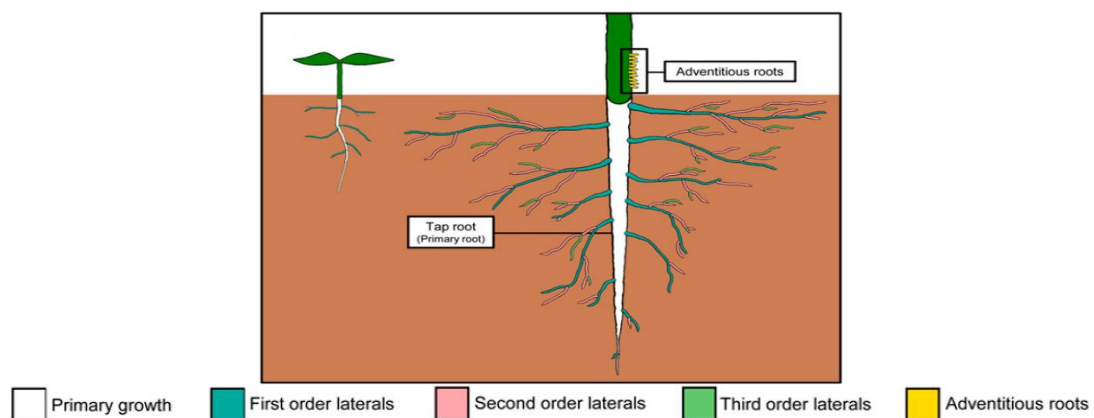


Figure 1.4. Schematic of eudicot root system architecture (adapted from Atkinson *et al.*, 2014).

Figure 1.5 illustrates a real tomato RSA growing in soil utilising non-invasive X-ray microscale computed tomography (CT) (Atkinson *et al.*, 2014). This 3D image reveals the dominant 'taproot' which gives rise to multiple lateral roots that emerge horizontally to forage the surrounding soil.

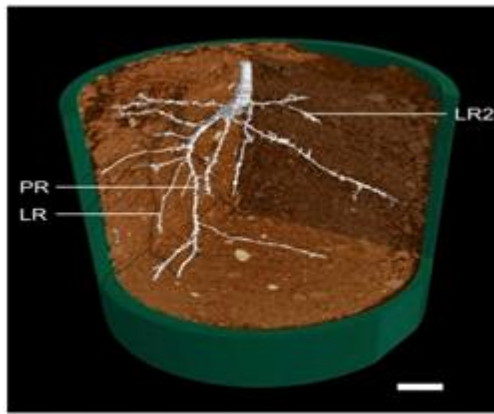


Figure 1.5. An X-ray microCT scan of tomato root system architecture. 12-day-old tomato plant. Scale bar represents 5 mm. Abbreviations: **LR2:** second lateral root; **LR:** lateral root; **PR:** primary root (adapted from Atkinson *et al.*, 2014).

1.2.1. Genetic analysis of tomato root development

Tomato root development has been the focus of several studies to characterise its genetic regulation (Ron *et al.*, 2013; Toal *et al.*, 2018; Zhang *et al.*, 2021). For example, Ron *et al.*, (2013) characterised the root development of domesticated tomato and wild species, *Solanum lycopersicum* (cv. M82) and *Solanum pennellii*. The authors observed major differences in their anatomy and root morphology including radial cell division, stem cell niche structure, numbers of cortical cell layers, gravitropic set-point angle (GSA), and root length. They also identified loci that control different root traits by utilizing an introgression population between *S. pennellii* and cultivated parent M82.

Figure 1.6 displays that IL4-1 has the longest primary root length compared to the cultivated parent M82. The cultivated parent M82 is shown in red, while the inbred accession of *S. pennellii* is shown in green. QTL analysis for root length across the introgression line population was performed by Ron *et al.*, (2013). Figure 1.6 shows that several genotypes displayed a transgressive enhancement in root length compared to *Solanum lycopersicum* (Ron *et al.*, 2013).

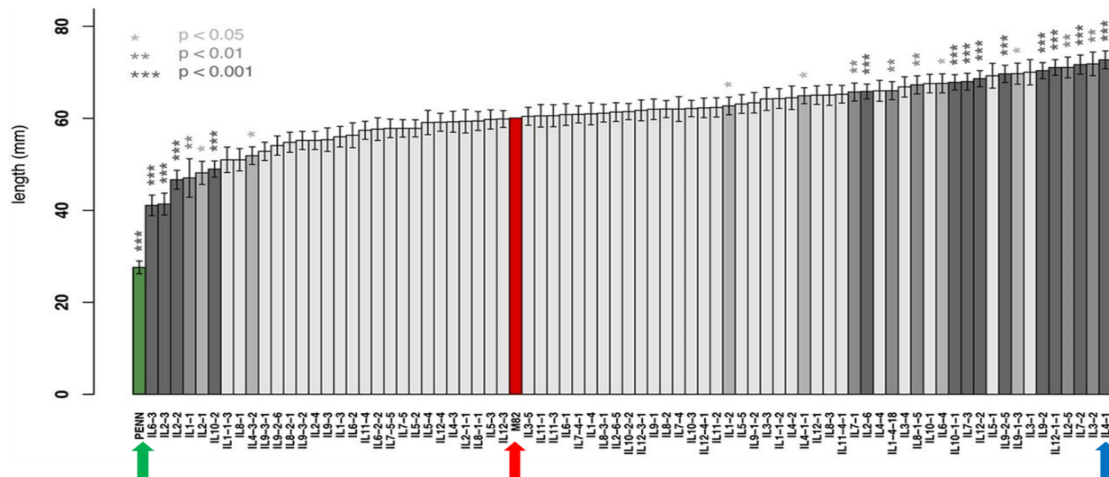


Figure 1.6. Average values of primary root length across the tomato introgression lines. Green, red, and blue arrows indicate *Solanum pennellii*, *Solanum lycopersicum* cv. M82 and IL4-1, respectively (modified from Ron *et al.*, 2013).

Figure 1.7 illustrates differences in tomato ILs root angle. *S. pennellii* has a shallow root system. Many desert plants have shallow root systems to capture water (Schenk and Jackson, 2002; Toal *et al.*, 2018; Kirschner *et al.*, 2021). *S. pennellii* exhibits a very shallow root system just underneath the soil surface, presumably to capture rainwater (Ron *et al.*, 2013). Root angle also plays a significant role in phosphate uptake efficiency (PUE; Miguel *et al.*, 2015). Because of phosphate accumulation by plants in the topsoil, many plants' roots deviate from the vertical to forage for phosphate better (Brown *et al.*, 2013).

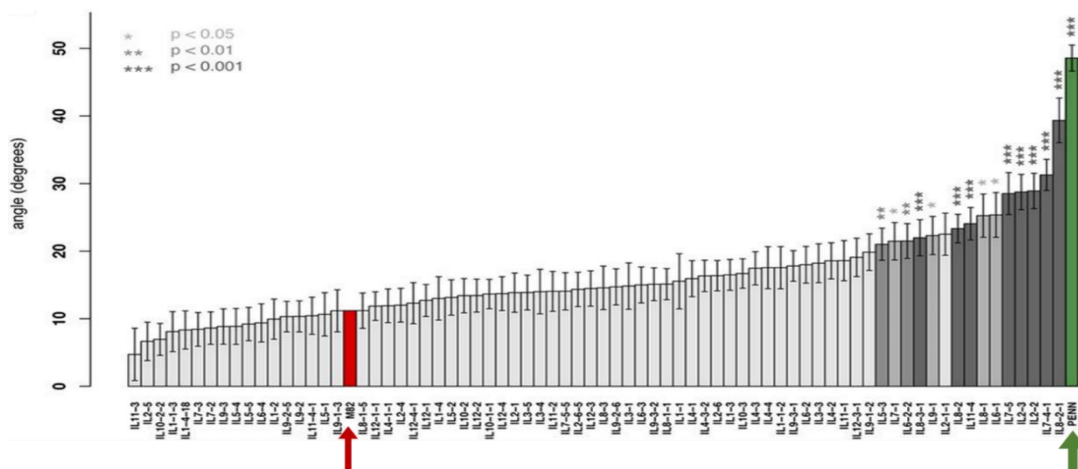


Figure 1.7. Mean root angle values across tomato introgression lines. Red and green arrows indicate *Solanum lycopersicum* cv. M82 and *Solanum pennellii*, respectively (modified from Ron *et al.*, 2013).

1.2.2. Tomato root anatomy

Differences between root systems of wild and cultivated tomato species extend to their anatomy. Ron *et al.*, (2013) have performed root development characterization of *S. pennellii* and cultivated line M82 to reveal differences in how tomato species root morphogenesis. The authors identified important developmental differences between the parental and introgression lines by measuring multiple root traits ranging from root growth to numbers of quiescent centre cells, and cortex cell layers.

Figure 1.8 illustrates the number of quiescent centre cells in *S. pennellii* and cv. M82. Ron *et al.*, (2013) reported that there are two layers with fewer cells in *Solanum pennellii* root, while there are three layers of cells in the cultivated parent M82. For comparison, quiescent centre cell numbers can form between 800-1200 cells in maize roots to ~4 cells in *Arabidopsis* roots (Jiang *et al.*, 2003).

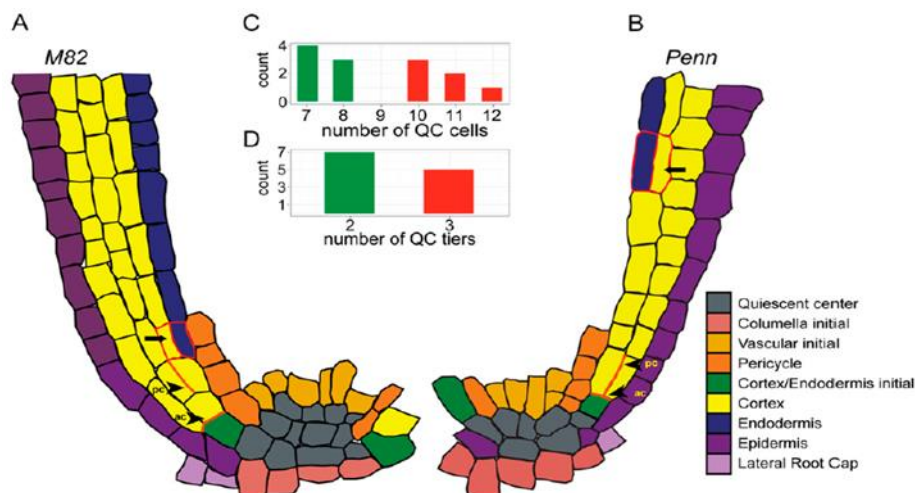


Figure 1.8. The root stem cell niche characterization of *Solanum pennellii* and cv. M82. (A-B) Ac, anticlinal division; pc, periclinal division. (C-D) Quiescent centre cell numbers and layers. Green and red colours indicate *S. pennellii* and *S. lycopersicum* cv. M82, respectively (modified from Ron *et al.*, 2013).

Figure 1.9 illustrates the cellular anatomy and root morphology of *S. pennellii* (Penn) and *S. lycopersicum* cv. M82. Figure 1.9A shows that *S. pennellii* roots are deviating. Ron *et al.*, (2013) performed QTL analysis for root primary length through the introgression line population.

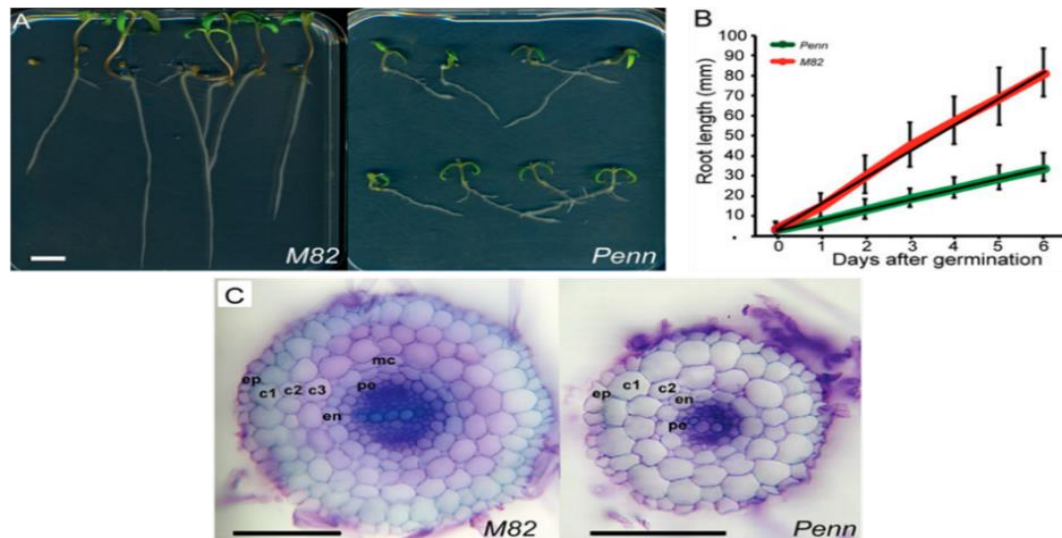


Figure 1.9. Root cell anatomy and morphology of cultivated parent M82 and *S. pennellii*. **(A)** Root length of *S. pennellii* and cv. M82. Scale bar represents 10 mm. **(B)** *S. pennellii* and cv. M82 root length. $n = 73\text{--}79$ (*Solanum pennellii*), $n = 24\text{--}60$ (*S. lycopersicum* cv. M82). **(C)** Root cross-sections of cultivated parent M82 and the inbred accession of *S. pennellii*. Scale bars represent 100 μm . Abbreviations: **pe**: pericycle; **en**: endodermis; **mc**: middle cortex; **c3**: inner cortex layer; **c2**: S cortex layer; **c1**: outer cortex layer; **ep**: epidermis. Error bars = SD (adapted from Ron *et al.*, 2013).

Figure 1.9 reveals differences in root length between M82 and *S. pennellii*. Figure 1.9 displays that the short-rooted wild species had fewer cells in the root meristem than *S. lycopersicum*. Strikingly, *S. pennellii* has no inner cortex layer (Figure 1.9) (Ron *et al.*, 2013). The number of cells for xylem, pericycle, endodermis, and cortex was also increased in M82 compared with *S. pennellii* (Figure 1.9C). Ron *et al.*, (2013) have reported that *S. lycopersicum* and *S. pennellii* have diarch vascular architecture, however *S. pennellii* had dramatically fewer cells than *S. lycopersicum* (Figure 1.10).

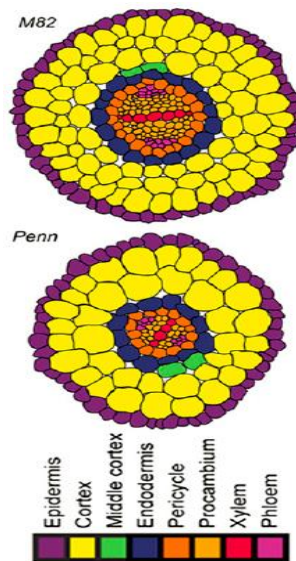


Figure 1.10. Schematic representation of the radial cell patterning in *S. pennellii* and cultivated parent M82. Representative figures from cross-sections in Figure 1.9C (adapted from Ron *et al.*, 2013).

1.2.3. Rice (*Oryza sativa* L.) RSA

The RSA of eudicots species like tomato and *Arabidopsis* differ greatly from monocot species like rice (Hochholdinger and Zimmermann, 2008; Rebouillat *et al.*, 2009) (Figure 1.11). For example, the seedling root systems of many eudicots species are composed of many lateral roots originating from a single primary (tap) root (Figure 1.4). In contrast, cereals such as rice (*Oryza sativa* L.) initially produce several embryo-derived seminal roots (Figure 1.12A), but later develop a dense fibrous root system that consists of many post-embryonic adventitious roots that emerge from the tiller stems (Figure 1.11).

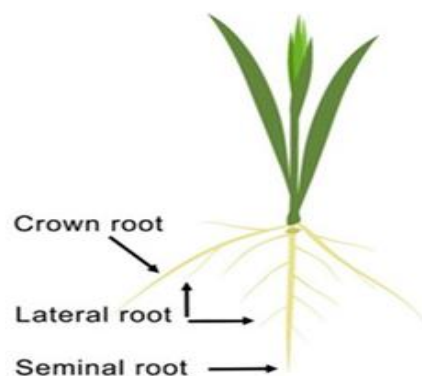


Figure 1.11. Schematic of rice root system architecture (adapted from Seo *et al.*, 2020).

Anatomically, while rice and tomato root radial morphologies appear superficially similar, rice contains more cortical layers (3-8) and extra outer layers (e.g., sclerenchyma; Figure 1.12). These additional layers arise from extra formative divisions that take place in the enlarged stem cell niche at the rice root apex (Figure 1.13).

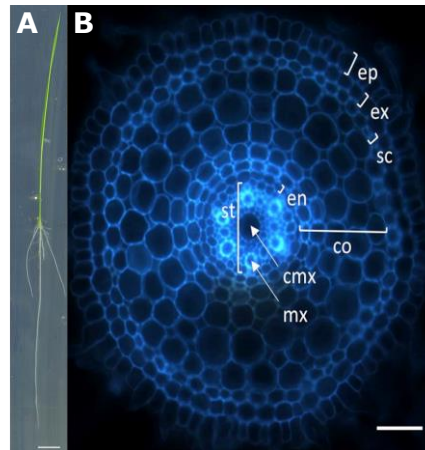


Figure 1.12. The root system architecture and cross-section of rice (*Oryza sativa* cv. Nipponbare). **(A)** Representative image of 6-day-old rice seedling grown on $\frac{1}{2}$ MS media. **(B)** Representative autofluorescence microscope image of root cross-section of cv. Nipponbare. Abbreviations: **mx**: metaxylem; **cmx**: central metaxylem; **st**: stele; **co**: cortex; **sc**: sclerenchyma; **ex**: exodermis; **ep**: epidermis. Scale bars represent 10 mm and 100 μ m in panels **A** and **B**, respectively (adapted from Lartaud *et al.*, 2015).

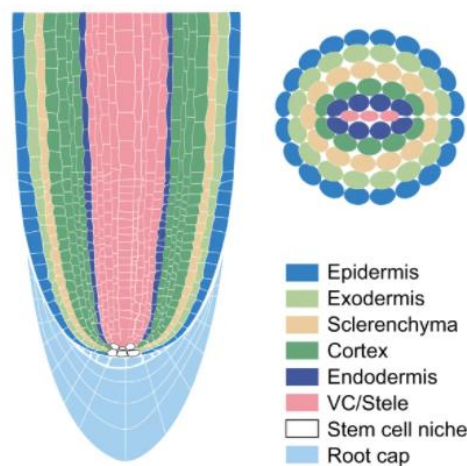


Figure 1.13. The anatomy of a rice radicle is depicted in this diagram. In root apical meristem (RAM), cell files and cellular patterns are drawn schematically. Within the RAM, the endodermis, epidermis, stele, and cap are converged and distinct (adapted from Zhang *et al.*, 2021).

1.2.4. *Arabidopsis thaliana* root anatomy

Arabidopsis represents a simple model from a molecular genetic and morphological perspective (Birnbaum *et al.*, 2003). For instance, *Arabidopsis* contains ~4 quiescent centre (QC) cells (Figure 1.14) (Jiang *et al.*, 2003).

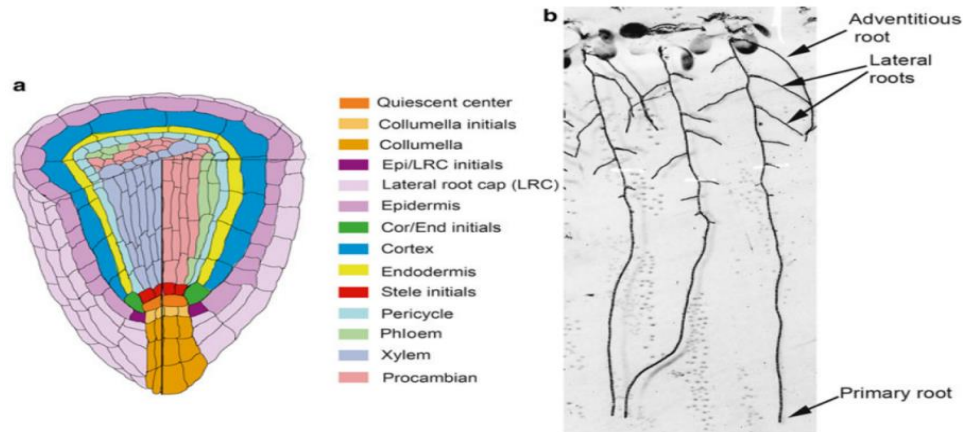


Figure 1.14. The root system architecture of *Arabidopsis thaliana*. (a) The cellular structure of *Arabidopsis* root tip. (b) 10-day-old *Arabidopsis* roots (Duan *et al.*, 2015).

Furthermore, *Arabidopsis* has just one cortex layer, whereas most dicot and monocot species have several cortical layers that are the result of successive divisions of cortex-endodermis initial cells (Figure 1.10 and 1.11). In addition, *Arabidopsis* lacks a suberinized exodermis, an outer layer of the root cortex with a suberinized cell wall to prevent the loss of water and solutes (Ron *et al.*, 2013).

1.2.5. Effects of hormones and abiotic stress signals on root growth and development

During root growth and development, hormone signals play major regulatory roles. For example, auxin and cytokinin regulate numerous processes which include root vascular development, gravitropism, and lateral roots initiation (Gupta *et al.*, 2013). For example, auxin promotes the formation of lateral and adventitious roots in tomatoes. Consistently, Ivanchenko *et al.*, (2006) reported lateral root development is reduced in the auxin-insensitive *diageotropic (dgt)* mutant.

Several groups (Ioio *et al.*, 2008; Müller and Sheen, 2008; Marhavý *et al.*, 2011; Zhang *et al.*, 2011) have reported cytokinin exhibits crosstalk with auxin during root development, often acting in an antagonistic manner, to control lateral root organogenesis, root growth, meristem size, and root stem-cell niche establishment.

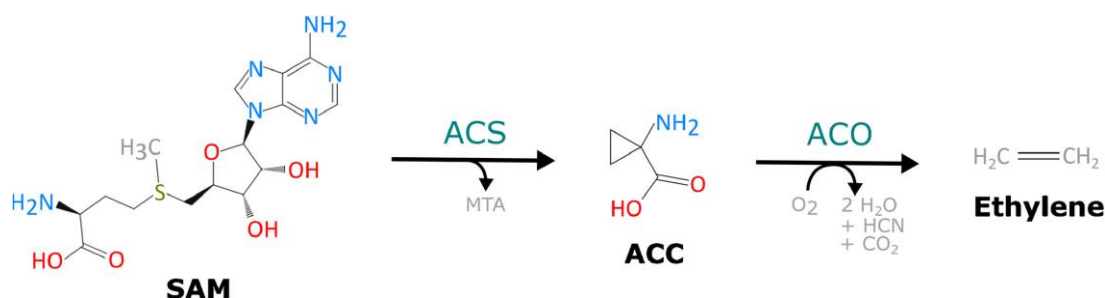


Figure 1.15. Schematic overview of ethylene biosynthesis pathway (adapted from Pattyn *et al.*, 2021).

Plant roots also synthesise gaseous hormone signals like ethylene. The ethylene biosynthesis pathway is well-characterised, with only two dedicated enzymatic reactions (Figure 1.15). The initial substrate S-adenosyl-L-methionine (SAM) is converted by ACC synthase (ACS) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979; Boller *et al.*, 1979). The enzyme ACC oxidase (ACO) converts ACC to ethylene in the second step (Hamilton *et al.*, 1990; Ververidis and John, 1991) (Figure 1.15).

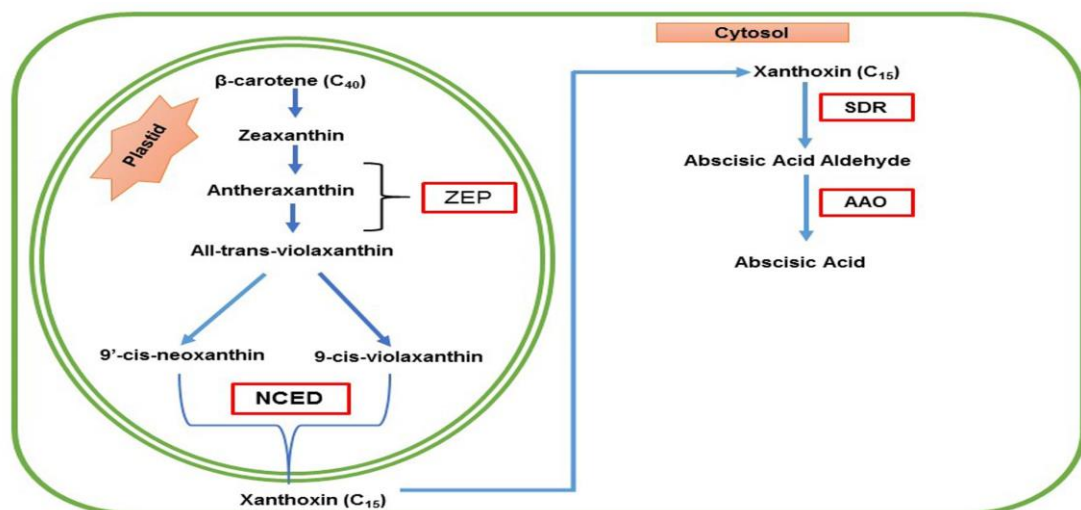


Figure 1.16. Schematic overview of abscisic acid (ABA) biosynthesis pathway (adapted from Sah *et al.*, 2016).

Plant roots also synthesise hormone signals like abscisic acid (ABA) that provides protection against abiotic stresses. The conversion of zeaxanthin and antheraxanthin to all-trans-violaxanthin, catalysed by zeaxanthin epoxidase (ZEP), is the first step in the abscisic acid (ABA) biosynthesis pathway (Figure 1.16). The 9-cis-epoxy carotenoid dioxygenase (NCED) converts all-trans-violaxanthin to 9'-cis-neoxanthin or 9-cis-violaxanthin, which results in xanthoxin. Two enzymatic reactions then convert xanthoxin to ABA; short-chain dehydrogenase/reductase (SDR) converts xanthoxin to an ABA aldehyde, and then the abscisic aldehyde oxidase (AAO) catalyses the oxidation of the abscisic aldehyde to ABA (Sah *et al.*, 2016).

Hormone regulated root developmental mechanisms are often the target for environmental signals in soil. For example, asymmetric water availability in soil is sensed by roots using the lateral root hydropatterning response (Bao *et al.*, 2014), where the auxin signalling machinery is modified by SUMO-mediated post-translational modification (Orosa-Puente *et al.*, 2018). Recent results in tomato roots suggest that auxin also plays an important role during adaptation to water stress, acting downstream of the key signal abscisic acid (ABA) (Figure 1.17) (Zhang *et al.*, 2021).

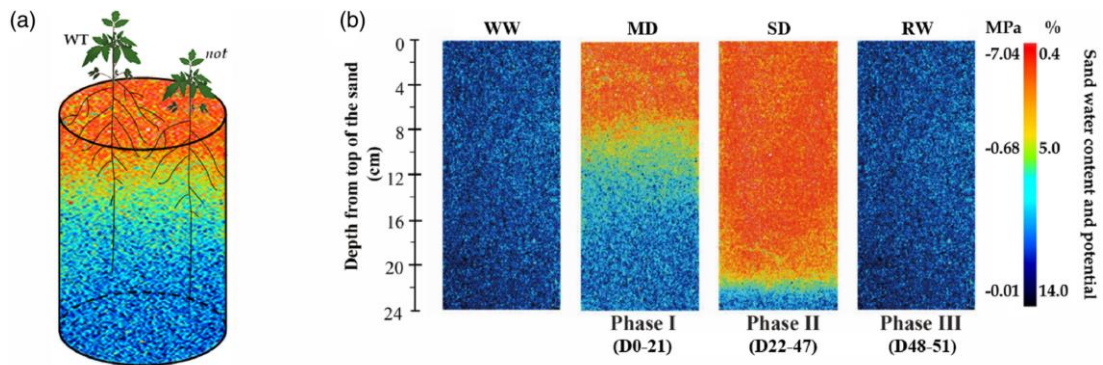


Figure 1.17. Plant growth of ABA-biosynthesis mutant *notabilis* (*not*) and wild-type (WT; *Solanum lycopersicum* L. cv. Lukullus) in three progressive stages of drought recovery and stress. (a) To minimise differences in growth conditions, both genotypes are grown in the same pot. (b) Water profile in the pot during the phases of re-watering, drought, and drying. Blue and red colours indicate high water content and low water content in the sand, respectively (adapted from Zhang *et al.*, 2021).

Hormones are also important for triggering adaptive growth responses to soil physical properties such as compaction. For example, as soil bulk density increases, root branching is reduced in tomato wild-type (WT; cv. Ailsa Craig). Nevertheless, as shown in Figure 1.18, cv. Ailsa Craig produced more lateral roots than its ABA-deficient mutant *notabilis* (*not*) (Tracy *et al.*, 2015), highlighting the positive role of ABA in stress adaptation.

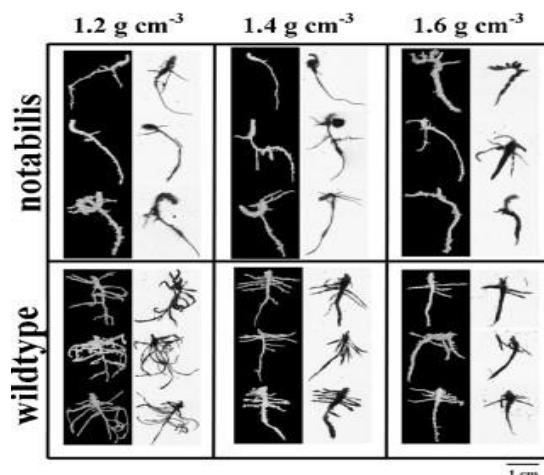


Figure 1.18. Examples of individual root systems that have been segmented. ABA-biosynthesis mutant *notabilis* (*not*) and wild-type (WT; cv. Ailsa Craig). Scale bar represents 10 mm (Tracy *et al.*, 2015).

The characteristic reduction in root elongation during a soil compaction response (Figure 1.19A versus B) has recently been demonstrated to be controlled by the gaseous signal ethylene (Pandey *et al.*, 2021b). As soil becomes more compacted, fewer interconnected pores cause ethylene to accumulate around the root tip, triggering growth inhibition. However, ethylene response mutants remain able to penetrate compacted soil (1.6 g cm⁻³ BD; Figure 1.19), revealing that this gaseous signal (rather than soil physical impedance) causes growth cessation.

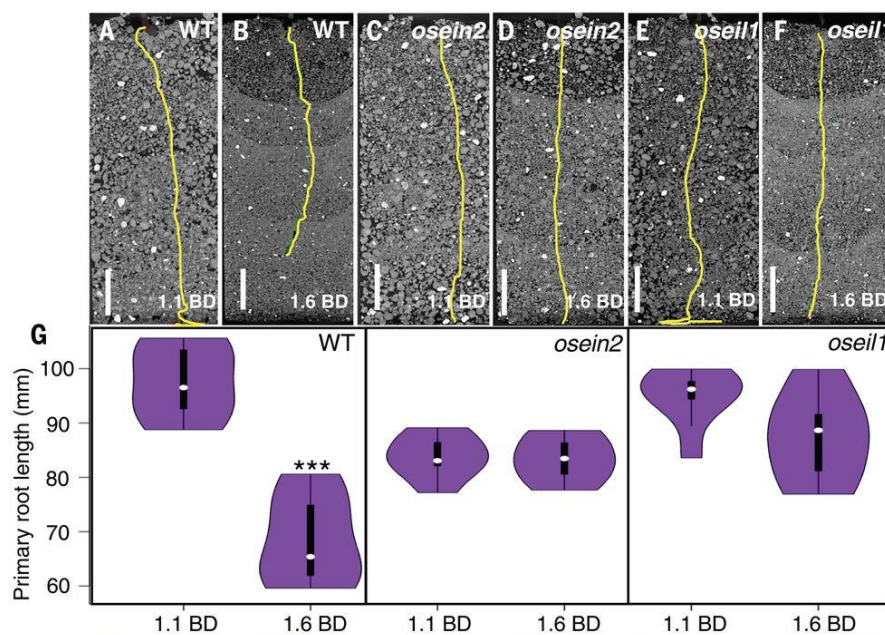


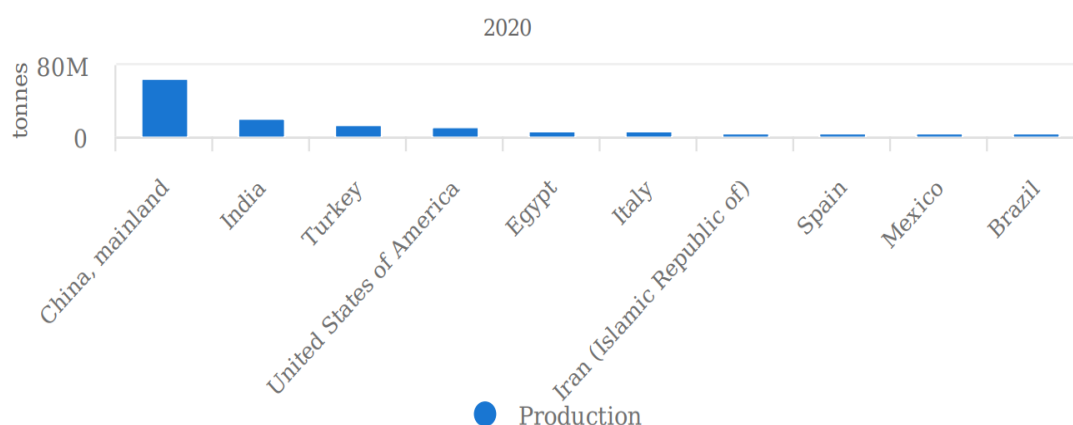
Figure 1.19. Rice root growth resistance to compacted soil is conferred via disrupting the ethylene response. Representative CT (Computed Tomography) images of primary root length in (A, B) wild-type (WT; cv. Nipponbare) as compared with (C, D) *osein2*, and (E, F) *oseil1* in non-compacted (1.1 g cm⁻³ BD) and compacted (1.6 g cm⁻³ BD) soil, respectively. (G) Violin plots display the root penetration ability of wild-type (WT; cv. Nipponbare), *osein2* and *oseil1* rice seedlings in compacted soil (1.6 g cm⁻³ BD) versus non-compacted (1.1 g cm⁻³ BD) soil. *** represents p -value ≤ 0.0001 , calculated by Student's t -test. Scale bars represent 10 mm in panels A-F (adapted from Pandey *et al.*, 2021b).

1.3. Global importance of tomato

Tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Mill.) is the most intensively studied of 3000-4000 Solanaceae species, which are classified into ~90 genera (Knapp *et al.*, 2004; Gebhardt, 2016) and is one of the 17 core species in the tomato clade (Lippman *et al.*, 2007). It originated in western South America and was domesticated in Mexico. Spanish Conquistador Hernán Cortés took back tomato seeds to Spain in the 16th century (Picó and Nuez, 2000).

Between 2000 and 2019, global vegetable production increased by ~65%. In 2019, the four main vegetable crops accounted for ~39% of the total: tomatoes with ~16%, followed by onions (~9%), gherkins and cucumbers (~8%), and cabbages (~6%) (FAO, 2021). It is one of the most consumed vegetables around the globe, with a global production of approximately 186 million tonnes in 2020 (FAOSTAT, 2020).

It is a warm-season crop and cannot tolerate low temperatures. Figure 1.20 displays that the top four biggest tomato producers are China, India, Turkey, and the USA (FAOSTAT, 2020).



Source: FAOSTAT (Feb 06, 2022)

Figure 1.20. Production quantities of tomatoes of top 10 producers in 2020 (FAOSTAT, 2020 [accessed 06 February 2022]).

In the human diet, tomatoes are an essential source of phytochemicals, vitamins; A, C, and E, and minerals; K⁺, Ca⁺⁺, and Fe⁺⁺ (Seymour *et al.*, 2013). Scientists have sequenced the tomato genome (The Tomato Genome Consortium, 2012). Its diploid genome of 950 Mb is arranged as 12 chromosomes. The tomato (*S. lycopersicum*) genome is 7 times bigger than *Arabidopsis thaliana* (~135 Mb) and has approximately the same number of genes (35,000; Kaul *et al.*, 2000; Barone *et al.*, 2008; The Tomato Genome Consortium, 2012).

Many studies of tomatoes have focused on fruit development, flowers and, leaves (Mao *et al.*, 2000; Chitwood *et al.*, 2013; Uluisik *et al.*, 2016), In contrast, tomato roots have not been studied at the same level of detail (Santisree *et al.*, 2011; Tracy *et al.*, 2012; Ron *et al.*, 2013; Kumari *et al.*, 2021). Roots are important for anchorage, mineral, and water uptake (Gupta *et al.*, 2013). Tomato rootstocks are also in widespread use because they have a positive effect on yield by stimulating scion vigour. Rootstocks are employed to produce fresh market tomatoes in Southern Europe and almost all protected tomatoes in Northern Europe. The global market value for rootstocks is ~US\$100 million/year for tomatoes alone (UKRI Gateway, 2014).

Tomato has been used as a model organism for eudicots due to its well-developed genetic and genomic resources (Mueller *et al.*, 2005). *Solanum lycopersicum* cv. M82 is sensitive to abiotic stresses, including chilling and drought stresses. Temperature extremes, salinity, and drought, which are abiotic stresses, may lead to subsequent oxidative damage, increased levels of reactive oxygen species (ROS), loss of protein activity, disorganization of membranes, and turgor loss. Wild plant species often exhibit tolerance to abiotic stresses, while domesticated crops are generally sensitive (Arms *et al.*, 2017).

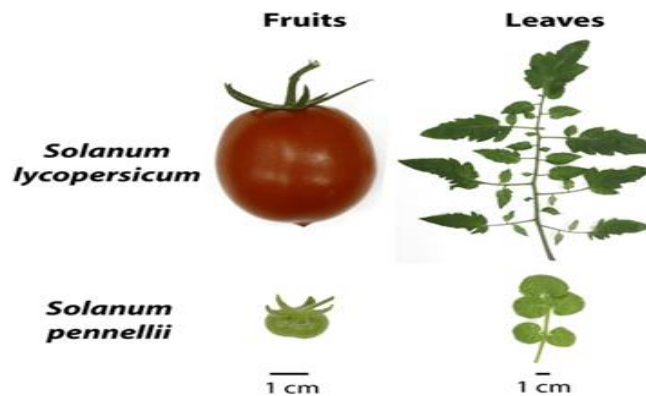


Figure 1.21. Phenotypic differences between wild tomato *S. pennellii* and cultivated tomato *S. lycopersicum* cv. M82 (adapted from Chitwood *et al.*, 2013).

Figure 1.21 displays the phenotypic diversity exhibited by the leaves and fruit of the modern tomato cultivar *S. lycopersicum* cv. M82 and a wild species *S. pennellii*. The desert species *S. pennellii* is a distant relative of modern tomato (Lippman *et al.*, 2007). In this project, the small, green-fruited desert species *S. pennellii* and modern tomato cultivar *S. lycopersicum* cv. M82 and their derived introgression line (IL) population will be used (Eshed and Zamir, 1995). *Solanum pennellii* displays pathogen resistance, and salt and drought tolerance compared to cultivar M82. *S. pennellii* is found in arid, rocky soil and coastal deserts (Chitwood *et al.*, 2013). Hence, the introgression lines (ILs) derived from these diverse parents are likely to exhibit great phenotypic variation which can be exploited to pinpoint key loci regulating tomato growth and development.

1.4. Genetic resources in tomato

To address whether the newly identified hormone-regulated mechanisms for root compaction responses are targets for natural variation in crops like tomato requires access to collections of landraces, wild species and/or mapping populations. For example, Eshed and Zamir (1994) have developed a permanent mapping population designed for quantitative trait loci (QTL) analysis by utilising whole-genome marker analysis. This resource comprises *Solanum lycopersicum* cv. M82 which has single introgressed genomic regions from *Solanum pennellii*. This congenic resource is composed of 76 introgression lines (ILs) of tomato plants harbouring chromosome segments from *S. pennellii*.

S. pennellii was crossed as the male parent with *S. lycopersicum*, and then the first generation was backcrossed to the *S. lycopersicum* parent. Homozygous lines were derived via two rounds of self-fertilization for each of 76 lines (Figure 1.22) (Giovannoni, 2006).

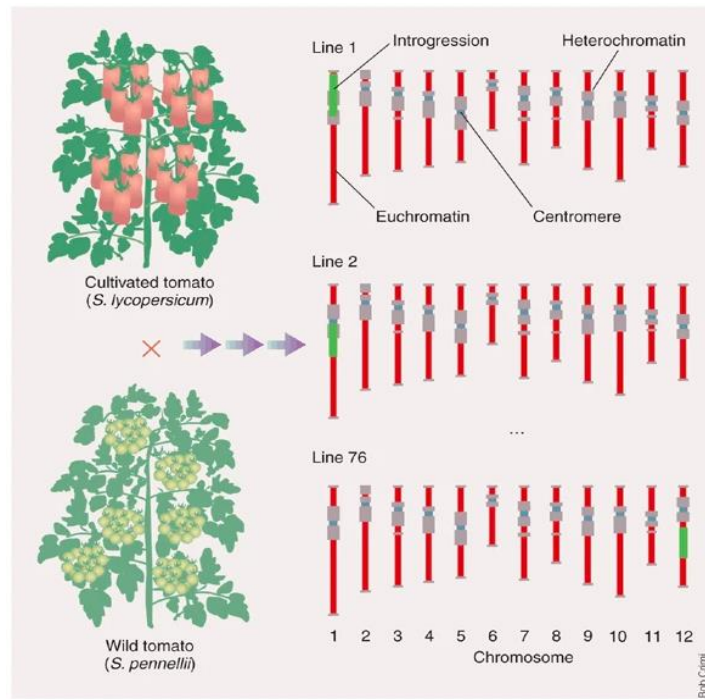


Figure 1.22. Introgression lines are being developed in order to map and define QTLs for crop improvement. Green segments are introgressed *S. pennellii* segments. Reds are representing *S. lycopersicum* chromosomes (adapted from Giovannoni, 2006).

Through consecutive marker-assisted selection and introgression backcrossing, introgression lines were created to construct a collection of recurrent parent lines with single introgressed segments (Eshed and Zamir, 1994). The genome of the wild-type tomato (*S. pennellii*) was sequenced and published by Bolger *et al.*, (2014). It has a genome size of 942 Mb and contains 44,966 protein-coding genes and 32,273 high-confidence genes (Bolger *et al.*, 2014). Because of its distinctive morphology and exceptional stress tolerance, *S. pennellii* is a crucial genetic donor for the cultivated tomato *S. lycopersicum* (Rick and Tanksley, 1981).

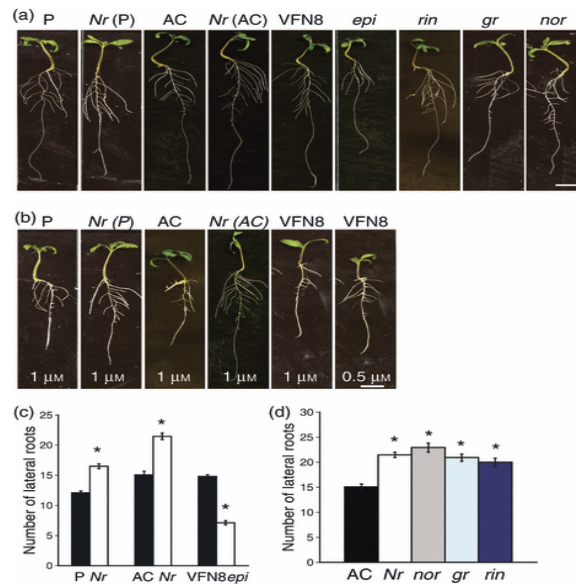


Figure 1.24. In tomato (*Solanum lycopersicum* L.), the lateral root formation is affected via mutations that change ethylene synthesis and signalling. 8-day-old tomato seedlings. **(a)** Seedlings' root phenotypes on control or **(b)** 1 or 0.5 μ M ACC, as specified. **(c)** The mean of lateral roots number. **(d)** In each genotype, the mean of an emerged lateral number. Abbreviations: **P**: Pearson (wild-type); **Nr**: *Never-ripe* mutant; **AC**: Ailsa Craig (wild-type); **VFN8**: *Solanum lycopersicum* cv. VFN8; **epi**: *epinastic* mutant; **nor**: *non-ripening*; **gr**: *green-ripe*; **rin**: *ripening-inhibitor* (adapted from Negi *et al.*, 2010).

Negi *et al.*, (2010) analysed mutants insufficient in fruit ripening and ethylene signalling to examine if ethylene regulates root formation. Figure 1.24a displays a comparison of lateral root formation and primary root elongation of *Never-ripe* mutant (*Nr*) in the background of wild-type lines, which are Ailsa Craig (AC) and Pearson (P) and other fruit-ripening mutants, which are *rin*: *ripening-inhibitor*, *nor*: *non-ripening*, and *gr*: *green-ripe*. Figure 1.24d illustrates the significant increase in lateral roots of all three mutants (*rin*, *nor*, and *gr*). As shown in Figure 1.24a, the increased root branching of *rin*, *nor*, and *gr* is unrelated to increases in the growth of leaf or hypocotyl. Because of the role of *ripening inhibitor*, *green-ripe*, and *Never-ripe* mutants in regulating ethylene signalling or synthesis, ethylene appears to be negative to lateral root development. Negi *et al.*, (2010) have analysed that increased ethylene levels restrict lateral root formation. Figure 1.24b shows decreased emerged lateral root numbers and primary root elongation.

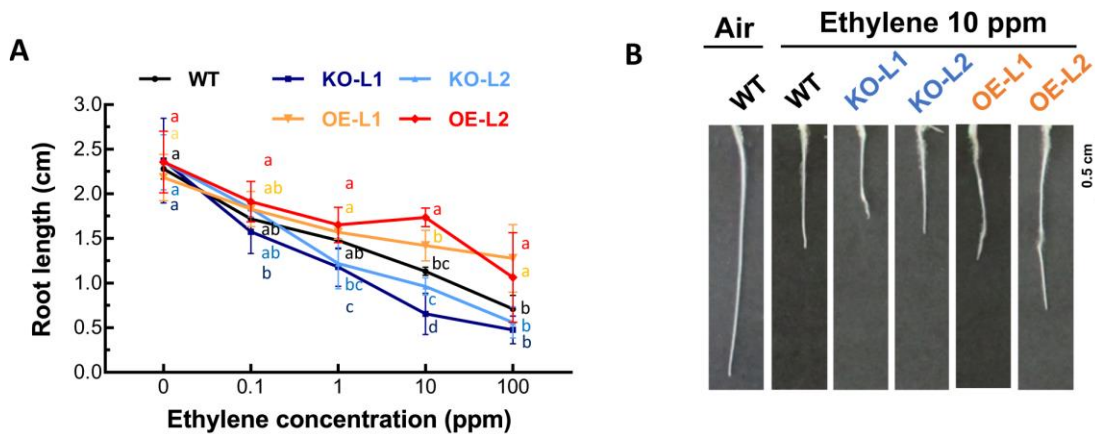


Figure 1.25. The effects of SIETR7 mutants on etiolated seedling responses to ethylene. (**A-B**) In *OE-L2*, *OE-L1*, *KO-L2*, *KO-L1*, and wild-type (WT; cv. Micro-Tom) lines, seedling root length variations as a function of ethylene concentration. Six-day-old tomato seedlings were grown in the dark at 25°C. Scale bar represents 5 mm. To compare tomato lines at each ethylene concentration, Tukey's HSD tests were used ($p < 0.05$), and significant differences were underlined by various letters (adapted from Chen *et al.*, 2020).

Chen *et al.*, (2020) investigated the effects of exogenous ethylene on etiolated seedlings to evaluate the effects of ethylene on these mutants (Figure 1.25). Ethylene inhibited the growth of the wild-type (WT; cv. Micro-Tom) roots, as seen in Figure 1.25, which is consistent with the findings of others (Ma *et al.*, 2014). When compared to wild-type (WT; cv. Micro-Tom), neither the KO nor the OE lines showed significant differences in root growth inhibition. Chen *et al.*, (2020) showed that only *KO-L1* was more responsive to 1 and 10 ppm ethylene (C_2H_4), while *OE-L2* was less responsive than *Solanum lycopersicum* cv. Micro-Tom at 10 and 100 ppm ethylene (C_2H_4).

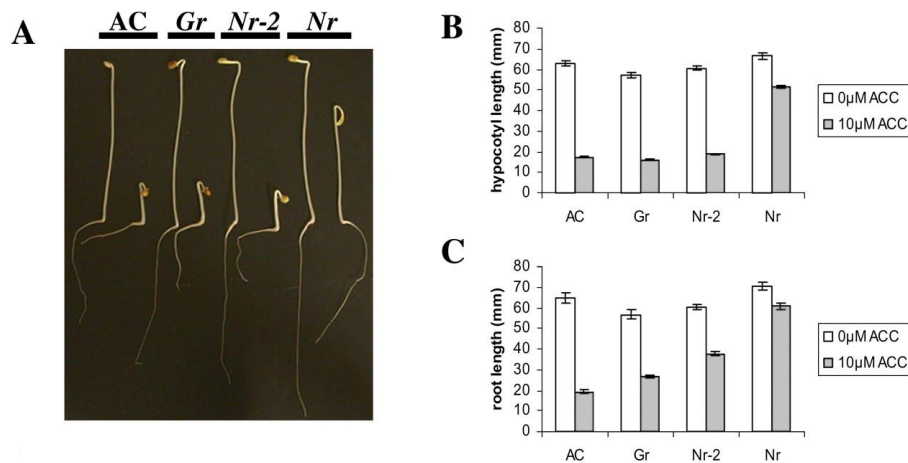


Figure 1.26. Tomato seedling triple response of wild-type (WT; cv. Ailsa Craig [AC]), *Never-ripe 2* (*Nr-2*), *Green-ripe* (*Gr*), and *Never-ripe* (*Nr*). **(A)** Representative image of *Nr*, *Nr-2*, *Gr*, and wild-type (WT; cv. Ailsa Craig) seedlings. The effects of ethylene on the root **(C)** and hypocotyl **(B)** lengths were measured. Seedlings of wild-type (WT; cv. Ailsa Craig) and mutant were sterilised and cultivated in the presence (+) or absence (-) of 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, and incubated for 7 days at 25°C in the dark. At least 21 seedlings were examined independently (adapted from Barry *et al.*, 2005).

The triple response phenotype is characterised by root and hypocotyl shortening and thickening, as well as increased curvature of the apical hook when tomato seedlings are treated with 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor. *Never-ripe* (*Nr*) roots were less sensitive than wild-type (WT; cv. Ailsa Craig) roots (Figure 1.26) (Barry *et al.*, 2005).

1.5. Thesis aims and objectives

➤ Chapter 2

Soil compaction represents a major agronomic challenge, inhibiting root growth and resource capture, causing significant yield losses. Roots employ ethylene to sense soil compaction as its reduced air space causes this gaseous signal to accumulate around root tips (Pandey *et al.*, 2021b). The resulting ethylene response triggers root growth inhibition, in part by promoting cortical cell radial expansion. Based on this recent knowledge and discovery of new signalling paradigm where diffusion of a gaseous hormone ethylene plays critical roles to sense soil compaction, I aim to investigate:

- 1- How dicots plants like tomato respond to compaction and understand the role of underlying hormone signals and molecular mechanism regulating compaction resistance in tomato?
- 2- Whether the ethylene sensitivity-based assay also confers the similar compaction resistance in tomato roots?
- 3- How root anatomical responses are regulated by ethylene and compaction in tomato roots?
- 4- What are the underlying genes which control the soil compaction/ethylene responses in tomato roots?

➤ Chapter 3

Radial expansion of root cortical cells and epidermal cells reduction are considered as the prime responses of roots undergoing compaction. Understanding the mechanism of these root responses in compacted soil was a challenge until now. In this chapter I aim to investigate:

- 1- Whether ethylene alone confers the root responses (radial swelling and epidermal cell reduction), or it requires downstream signals?
- 2- How downstream signals cross-talks to ethylene to regulate swelling responses of root tips in compacted soil conditions?
- 3- What are the consequences of downstream signals conferring soil compaction responses?

➤ Chapter 4

Water stress impacts several root adaptive responses including compaction (focus of Chapter 3). As compacted have less water (accessible) availability than noncompacted soil. Water stress affect the soil exploration capacity of the plants by modulating root system architecture. It is unclear how water stress affects the root architecture in compaction. Therefore, my aim is to investigate:

- 1- The potential regulators of water stress responses using maize GWAS based study.
- 2- Functional characterization of the candidate genes.
- 3- How will the gene affect root system architecture?

2. Chapter 2: Ethylene regulates tomato root responses in compacted soil

2.1. Introduction

Soil compaction causes inhibition of root penetration, reducing nutrients and water uptake (Lynch *et al.*, 2021). Soil compaction is a major challenge facing modern agriculture. Breeding crops to better withstand compacted soil offers a genetic solution (Hamza and Anderson, 2005). Understanding the plant regulatory signals causing alterations to root growth in response to soil compaction will be critical to finding a 'genetic solution'.

Until recently, roots were considered unable to penetrate compacted soils due to the mechanical force required (Passioura, 2002; Correa *et al.*, 2019). However, Pandey *et al.*, (2021b) recently reported roots sense soil compaction employing the gaseous hormone ethylene. Compacted soil restricts diffusion of ethylene away from root tips, causing inhibition of root elongation and promoting radial swelling (Pandey *et al.*, 2021b). Strikingly, roots of mutants insensitive to ethylene in *Arabidopsis* and rice can penetrate compacted soil (Pandey *et al.*, 2021b). Unlike wild-type roots which exhibited inhibition of cell elongation and promotion of cortical cell radial expansion, roots of ethylene-response mutants remained narrow and able to penetrate compacted soil (Pandey *et al.*, 2021b). This conclusion is in agreement with Vanhees *et al.*, (2020) who noted that root thickening was not associated with deeper rooting in compacted soils; rather, thinner roots of older nodes increased rooting depth in maize.

Ethylene, which decreases root elongation and promotes radial thickening, appears to represent important signal-regulating root responses to compacted soil conditions (Pandey *et al.*, 2021b). This new knowledge offers novel opportunities to breed crop varieties less sensitive to ethylene which are potentially better able to grow in compacted soil. Nevertheless, this elegant solution to select less ethylene and compaction resistant varieties needs to be demonstrated to translate from models like *Arabidopsis* and rice to other crops like tomato. While this plant hormone has long been known to play a function in climacteric fruit ripening, ethylene's role during tomato root compaction responses is not known. This chapter investigates the role of ethylene during root compaction responses in tomato and explores whether natural genetic variation exists for this important agronomic trait.

2.2. Materials and methods

2.2.1. Plant materials

The population of 76 introgression lines (ILs) used in this study was derived from a cross between the wild tomato *Solanum pennellii* (accession LA0716) and *Solanum lycopersicum* cv. M82 (accession LA3475) (Eshed and Zamir, 1995). Tomato introgression lines were obtained from the University of California, Davis, Tomato Genetics Resource Center (TGRC; <https://tgrc.ucdavis.edu/>). Tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant was kindly shared by Prof. Graham B. Seymour from the University of Nottingham. The genetic background of tomato *Never-ripe* mutant is in the wild-type (WT; cv. Ailsa Craig [AC]).

2.2.2. Plant growth conditions

2.2.2.1. Glasshouse conditions for tomato plants

Tomato seeds of *S. lycopersicum* cv. M82, *S. pennellii* and 76 introgression lines (ILs) were surface sterilised for 5 minutes with 20% bleach (diluted from 19% CaOCl₂ stock), then washed five times with sterilised water and then were sown in 9 cm pots of Levington M2 soil mix (Scotts Professional, Ipswich, UK), and after that one-month-old tomato plants were placed in 18 cm pots of soil mix. These tomato plants were grown in glasshouse maintained at 22°C during the day and 18°C at night till maturity.

2.2.2.2. Seedling growth methods

Tomato seeds (cv. M82, *S. pennellii*, cv. Ailsa Craig, ethylene-insensitive *Never-ripe* (*Nr*) mutant, and 76 introgression lines) were surface sterilised for 5 minutes with 20% bleach (diluted from 19% CaOCl₂ stock), then washed five times with sterilised water. Tomato seeds were stratified in the dark for 48-hour at 4°C before transferring to a tomato growth chamber (maintained at 21°C, 12-hour photoperiod, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 70% relative humidity), sterilised seeds were germinated on moist sterilised filter paper in the dark for 3 days.

2.2.3. Ethylene treatment

Pre-germinated tomato seedlings (cv. M82, *S. pennellii*, cv. Ailsa Craig, *Never-ripe* mutant, and 76 introgression lines) (see 2.2.2.2) were transferred on a 10" x 15" moist (with sterilised water) germination paper (catalogue no. PG1015; Hoffman Manufacturing, Inc, Oregon, USA) and rolled. Then, 4-day-old equally germinated tomato seedlings were transferred into a gas-tight jar GasPak Plus™ System (BBL-71040 Anaerobic System Envelopes with Palladium Catalyst; Becton Dickinson, Cockeysville, Maryland, USA) which was injected with a defined dose of ethylene (10 or 30 ppm) from ethylene (C₂H₄) gas cylinder (N.03-grade; minimum purity 99.9%) which obtained from BOC Ltd (Guildford, Surrey, UK) (Figure 2.1). And they were grown for 3 days in gas-tight jars at 21°C with a 12-hour photoperiod, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, and 70% relative humidity. After 3 days of ethylene treatment, these seedlings were imaged using D5100 (Nikon DSLR camera) for quantifying their root lengths. For anatomical analysis of root tips exposed with ethylene, root tips (up to the elongation zone) were harvested and placed in 4% paraformaldehyde (PFA; CAS no. 30525-89-4; Merck) for 1-hour. Subsequently, root tips were washed two times in 1x PBS buffer and thereafter cleared in ClearSee solution for 15 days. Cleared root tips were then treated with 1% Calcofluor dye (Calcofluor White Stain; Sigma) and imaged using a confocal microscope (Leica, TCS-SP5) as described previously (Pandey *et al.*, 2021a).

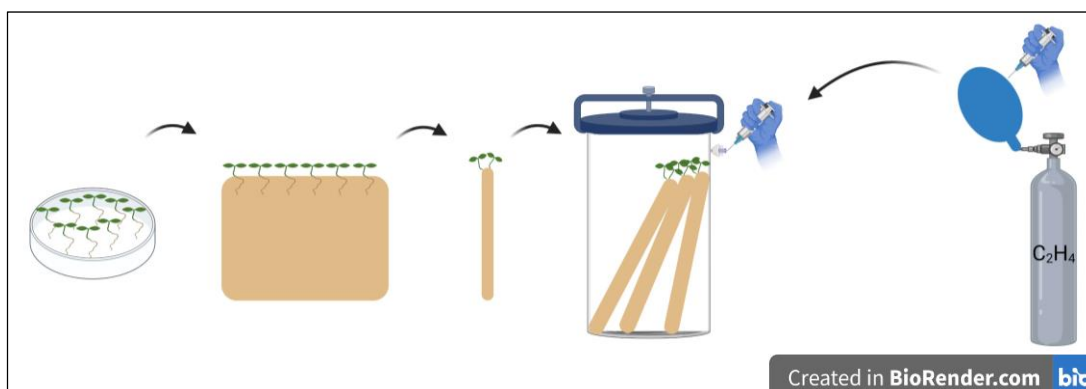


Figure 2.1. Schematic of ethylene-based (C_2H_4) root growth bioassay where germinated tomato seedlings at equivalent stage of growth were transferred to a moist paper roll, then placed in a gas-tight jar which was injected with a defined dose of ethylene (10 or 30 ppm). This figure was created using BioRender.com.

2.2.4. Radial and longitudinal sectioning, and confocal imaging for ethylene treatment

Pre-germinated tomato seedlings cv. M82 (LA3475), *S. pennellii* (LA0716), IL2-1-1 (LA4036), IL2-6-5 (LA4042), and IL3-1 (LA4043) were treated with 10 ppm ethylene gas as described previously (Pandey *et al.*, 2021b). After 3 days of ethylene treatment, root tips were harvested and subsequently, ten root tips from control and ethylene treatment were cleared in ClearSee solution for 15-20 days for confocal imaging of longitudinal sections. Root tip samples (apical 1.5 cm) were embedded in 3.5% melted agarose and 100 μ m thick transverse sections were cut from the elongation zone using a Leica vibratome (model: VT1000s; Leica Microsystems, UK), stained with 1% Calcofluor white dye for one minute on a glass slide, and subsequently imaged using a Leica TCS-SP5 confocal microscope using 10% diode laser excited at 404 nm wavelength to obtain cross-sectional images.

2.2.5. Soil materials

Loamy sand soil of the Newport series (FAO Brown Soil) (83.2% sand, 12.1% clay, and 4.7% silt; pH = 6.35; organic matter 2.93%) was used in all soil experiments. The soil was collected at a farm near the University of Nottingham in Bunny, Nottinghamshire, England (52° 52' N 1° 07' W), sieved to less than 2 mm, and the water content was calculated by drying the soil at 65°C until a certain weight was reached.

2.2.6. Preparation of soil columns

The methods for the preparation of soil columns were performed as in the previous description (Pandey, *et al.*, 2021a). 99.82 g and 122.50 g of sieved (<2 mm) air-dried soil was added to the 3D-printed columns, respectively, to reach soil bulk densities of 1.1 g cm^{-3} and 1.5 g cm^{-3} . The columns were placed in a tray filled with sterile water to a depth of 3 cm and left at room temperature overnight to allow the soil to saturate from the bottom of the column upwards. After 8-12 hours, the soil's surface is checked to see if it is fully saturated, and the columns are removed from the tray of water and allowed to drain freely for 48-hour before recording their mass. The mass of the column is equal to the notional field capacity moisture of the soil. A 1 cm space was filled with 1.1 g cm^{-3} bulk density soil (9.98 g) at the top of the compacted zone (1.5 g cm^{-3} BD) to help the pre-germinated seedlings establish before hitting the compacted soil layer.

2.2.7. Soil compaction experiment

The methods for root compaction assays were performed as in the previous description (Pandey *et al.*, 2021a). After radicle emergence (4-6 mm in length), seedlings were gently placed in topsoil (upper 1 cm non-compacted) in a 3D-printed column (33 mm diameter x 100 mm height) for compaction experiments as described below. The pre-germinated seeds were transferred to the top of the soil packed at different compaction levels. For non-compacted soil, entire column was packed with 1.1 g cm^{-3} bulk density soil. For compaction, top 1 cm soil was also prepared at 1.1 g cm^{-3} bulk density soil and remaining 9 cm of column was packed with 1.5 g cm^{-3} bulk density soil. The radicles of germinated seedlings were gently pushed in non-compacted soil in both types of columns. These seedlings were grown in a controlled growth chamber (maintained at 21°C , 12-hour photoperiod and $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$ PAR with 70% relative humidity) for 7 days.

After seven days of compaction treatment, root tips were removed carefully by washing the soil medium to keep the root tips intact for confocal imaging and subsequent root anatomical imaging. The root length was measured via ImageJ (<https://imagej.nih.gov/ij/>) (Schindelin *et al.*, 2012).

2.2.8. Longitudinal sectioning and confocal imaging for soil compaction experiment

Tomato roots were removed from soil mesocosms and immediately washed with sterilised water in three consecutive water-filled dishes using fine brush strokes to remove soil particles from root tips. Washing and clearing of the root tips were performed as described (Pandey *et al.*, 2021a). After 7 days of compaction experiment, root tips were harvested and subsequently, ten root tips from non-compacted and compacted were cleared in ClearSee solution for 15-20 days for imaging of longitudinal sections with a Leica TCS-SP5 confocal microscope using 10% diode laser excited at 404 nm wavelength.

2.2.9. Propidium iodide (PI) staining and cell length analysis

Seeds were germinated in the dark at 21°C for 4 days, and germinated seedlings were grown in compacted and non-compacted soil for 7 days. Primary roots were harvested, cleaned with sterilised water, immersed in 1 μ M PI for 3 min, and imaged using Leica Laser Scan Microscope (TCS-SP5) at an excitation wavelength of 561 nm and emission wavelengths of 600–650 nm. Confocal images were analysed via ImageJ (<https://imagej.nih.gov/ij/>) (Schindelin *et al.*, 2012). Cell length analysis was performed as described previously (Pandey *et al.*, 2021a).

2.2.10. X-ray Computed Tomography (CT) imaging

Equally germinated seedlings of cv. M82, *S. pennellii*, IL2-1-1, IL3-1, cv. Ailsa Craig and *Never-ripe (Nr)* were grown in 3D-printed columns (33 mm diameter x 100 mm height) filled with sandy loam soil at bulk densities of 1.1 g cm⁻³ and 1.5 g cm⁻³ as non-compacted and compacted soil conditions, respectively. These plants were grown in a controlled growth chamber maintained at 21°C, 12-hour photoperiod and 300 μ mol m⁻² s⁻¹ PAR with 70% relative humidity.

Seven-day-old seedlings were scanned using Phoenix v|tome|x M 240 kV X-ray μ CT system (Waygate Technologies (a Baker Hughes business); Wunstorf, Germany) at the Hounsfield Facility (University of Nottingham, Sutton Bonington Campus, UK). The scans consisted of the collection of 2520 projection images in FAST mode (continuous rotation), with an X-ray tube energy and a current of 140 kV and 200 μ A, respectively.

The detector exposure time was 131 ms, and the voxel resolution was 57 microns. The scan time was 5 minutes. Reconstruction of these 2520 images was performed using GE Datos rec software. A polyline measurement tool was used to trace the length of primary roots grown in $\pm$ compacted soil conditions in VGStudio MAX software V2.2 (Volume Graphics GmbH, Germany).

2.3. Results

2.3.1. Optimising a high throughput ethylene (C₂H₄) bioassay for tomato root growth

Ethylene sensitivity of root growth has been shown to be closely linked with compaction responsiveness in rice, *Arabidopsis*, and maize (Pandey, *et al.*, 2021b; Vanhees *et al.*, 2021). To determine whether tomato roots also exhibit this regulatory relationship, tomato roots from the cv. M82 x *S. pennellii* mapping populations were assessed for variation in their sensitivities towards ethylene. To do this, initially, a high throughput root growth bioassay was optimised, using the *S. pennellii* introgression lines (ILs) population.

To determine the optimum dose for the analysis of ethylene sensitivity of tomato IL root growth, the population's parental line *Solanum lycopersicum* cv. M82 (accession LA3475) was treated with 10 and 30 ppm of ethylene for 3 days. After 3 days, primary root length was measured via ImageJ (Schindelin *et al.*, 2012). Roots of M82 show a dose-dependent response to ethylene (C₂H₄); ~63% root growth inhibition at 30 ppm versus ~52% root growth inhibition at 10 ppm treatment. Based on this result, 10 ppm ethylene was used in all further tomato root growth analyses.

2.3.2. Tomato introgression lines (ILs) root growth varies in their sensitivity towards ethylene (C₂H₄)

The high throughput root growth bioassay was optimised using cv. M82 (Figure 2.1) was next used to screen the *S. pennellii* introgression line (IL) population. A heatmap was generated based on their primary root lengths either under control (0 ppm ethylene) and after ethylene (C₂H₄) (10 ppm for 3 days) treatment of each IL compared to cv. M82, cv. Ailsa Craig and the *Never-ripe* ethylene-insensitive mutant (Figure 2.2). Root growth for the majority of tomato ILs was ethylene-sensitive. However, root growth of the *Never-ripe* mutant and two ILs (IL2-1-1 and IL3-1) were much less ethylene-sensitive (Figure 2.2).

There is a clear impact of ethylene treatment for seedling growth of both wild-type species (cv. M82 and *S. pennellii*) and the majority of tomato introgression lines (ILs). For example, the majority of tomato ILs exhibited triple response (e.g., long root hairs and wide hypocotyls in addition to the short root length). Note that *S. pennellii* was excluded from the heatmap (Figure 2.2), despite displaying ethylene related phenotypes (e.g. increased root hairs, root thickening, and reduced primary root length). since its growth rate is very slow compared to tomato ILs and WT (cv. M82).

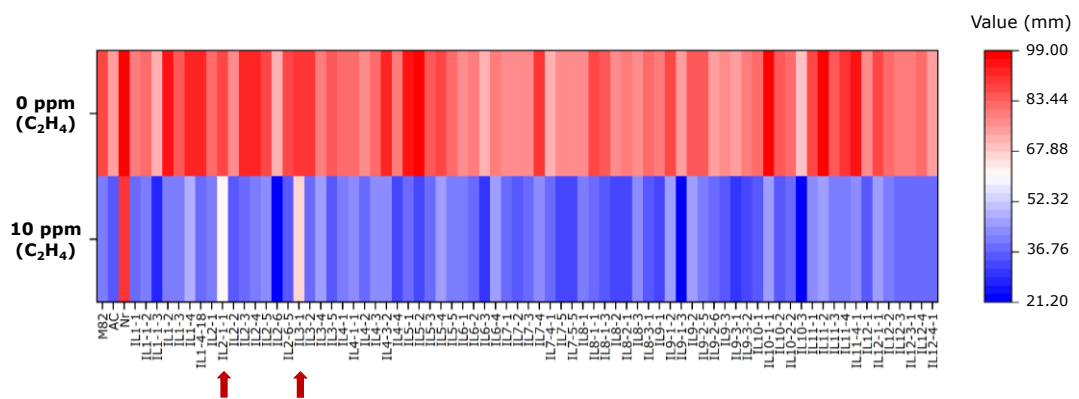


Figure 2.2. Heatmap showing the primary root length of control (0 ppm ethylene) and after ethylene (C₂H₄) (10 ppm for 3 days) treatment of each IL compared to cv. M82, cv. Ailsa Craig (AC), and tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant. Colours from blue to red indicate the primary root length from low to high. Red arrows indicate less ethylene-sensitive tomato ILs (IL2-1-1 and IL3-1). *n* = 12, two biological replicates were done for each IL, wild-type lines (cv. M82 and cv. Ailsa Craig), and *Nr* mutant. This figure was generated using Origin(Pro) Version 2021b (OriginLab Corporation; Northampton, MA, USA).

2.3.3. Less ethylene-sensitive tomato introgression lines (ILs) do not show a typical triple response

To determine whether the reduced ethylene sensitivity for root growth of the IL2-1-1 and IL3-1 lines extended beyond roots to other organs, I examined their growth responses in roots and other seedling tissues. Neljubow, (1901) originally reported that ethylene altered growth of multiple organs in etiolated pea (*Pisum sativum*) seedlings. This growth effect became known as the "triple response" which included the radial expansion of hypocotyls combined with reduced shoot and primary root length. The "triple response", a visually scored ethylene response, has been shown to be effective in identifying mutants and less ethylene-sensitive lines with altered ethylene response, perception, and synthesis (Kieber and Ecker, 1993; Lanahan *et al.*, 1994).

I initially examined the impact of both ILs on root growth following ethylene treatment using the high throughput ethylene (C₂H₄) bioassay screen (see 2.2.3; Figure 2.2). The ethylene bioassay revealed that growth of IL2-1-1, IL3-1, and *Never-ripe* (*Nr*) roots are much less inhibited (~30%, ~26%, and only ~9% reduction, respectively) compared to wild-type (WT; cv. M82) and highly ethylene-sensitive introgression line IL2-6-5 (~52% and ~63% reduction, respectively) (Figure 2.3). The ethylene-induced radial expansion of hypocotyls combined with reduced shoot length was also reduced in IL2-1-1, IL3-1, and *Nr* compared to cv. M82 and IL2-6-5 (Figure 2.3). Hence, genetic variation in IL2-1-1 and IL3-1 appears to disrupt ethylene-sensitivity in multiple tomato seedling tissues.

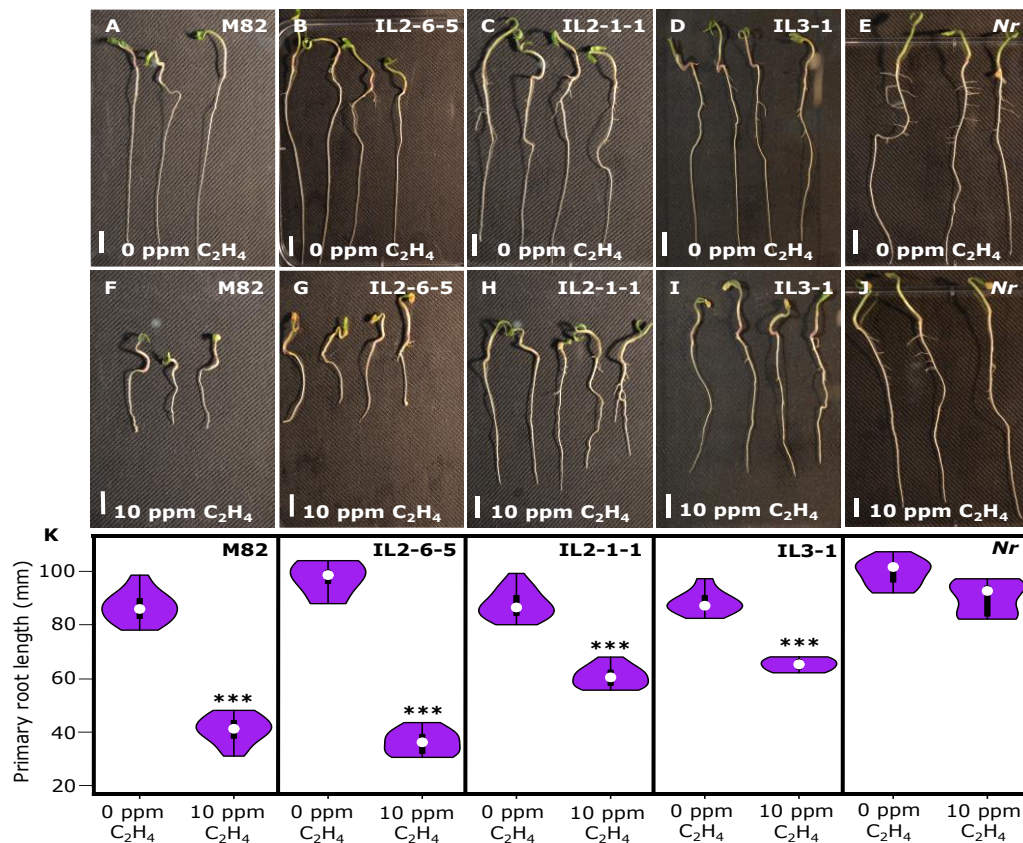


Figure 2.3. Seedlings of less ethylene-sensitive tomato introgression lines (ILs) IL2-1-1 and IL3-1 did not exhibit a classical 'triple response' compared with wild-type (WT; cv. M82) and highly ethylene-sensitive tomato introgression line IL2-6-5. Representative images of primary root length in (A, F) wild-type (WT; cv. M82) as compared with (B, G) IL2-6-5, (C, H) IL2-1-1, (D, I) IL3-1, and (E, J) tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant as control (0 ppm ethylene) and after ethylene (C₂H₄) (10 ppm for 3 days) treatment, respectively. Violin plots represent the primary root length of control (0 ppm C₂H₄) versus ethylene (C₂H₄) treated (10 ppm for 3 days) for wild-type (cv. M82), highly ethylene-sensitive tomato introgression line IL2-6-5 and less ethylene-sensitive tomato ILs (IL2-1-1 and IL3-1), and ethylene-insensitive mutant *Never-ripe* (*Nr*) seedlings. (K) Violin plots were generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). *** represents *p*-value of ≤ 0.0001, calculated by Student's *t*-test. *n* = 12; all graphs are the average of two repeats. Scale bars represent 10 mm in panels A-J.

2.3.4. Ethylene treatment triggers root growth inhibition and radial expansion in sensitive lines

To understand the cellular basis of ethylene responses on tomato root growth, the anatomies of primary roots treated with ethylene for selected tomato lines were examined. Roots were sectioned, then imaged on a confocal microscope (see 2.2.4). Lines (cv. M82 and *S. pennellii*) whose growth was sensitive to ethylene exhibited a reduction in root cell length and an increase in radial cell expansion (Figure 2.4 and 2.5).

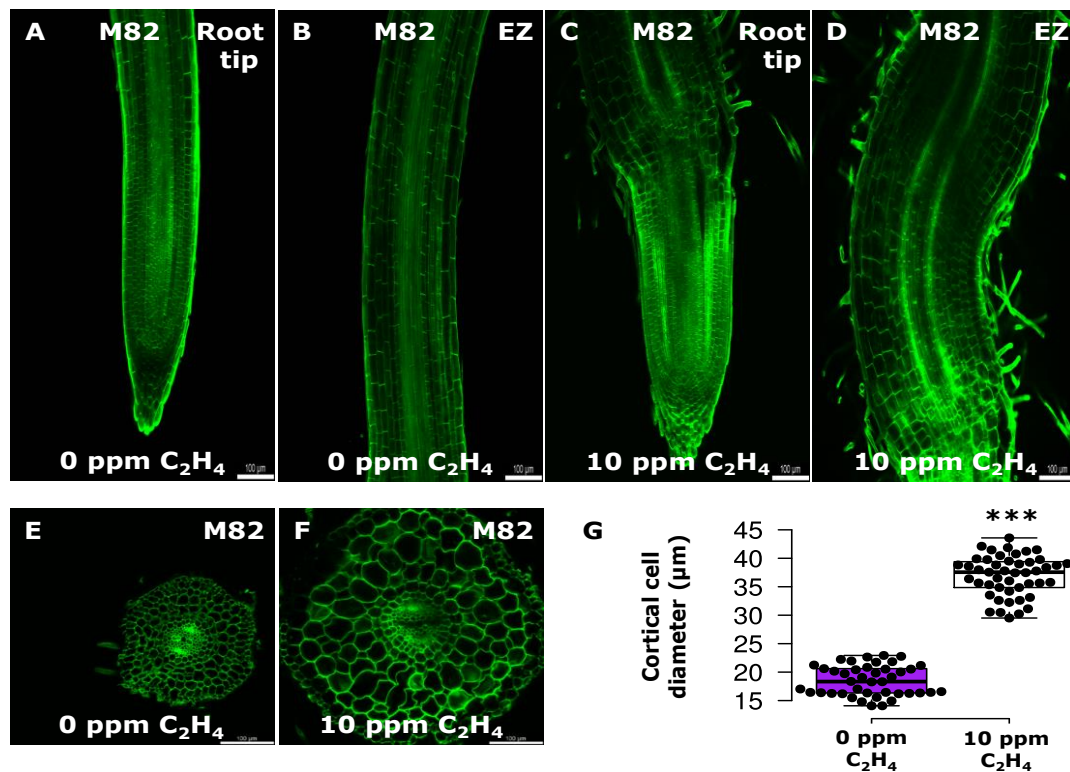


Figure 2.4. Ethylene promotes the radial expansion of wild-type (WT; cv. M82) root cortical cells. (**A** and **B**) Representative confocal images of WT (cv. M82) as a control in the root tip and elongation zone (EZ). (**C** and **D**) Representative images of WT (cv. M82) after ethylene (C₂H₄) (10 ppm for 3 days) treatment showing a radial expansion of cortical cells in the root tip and elongation zone (EZ). (**E**) Representative image of WT (cv. M82) as a control in the root tip. (**F**) Representative image of WT (cv. M82) after ethylene (10 ppm for 3 days) treatment showing radial expansion (diameter) of cortical cells in the root tip. (**G**) Quantitative box plot showing the radial expansion (diameter) of control and ethylene-treated root tips of wild-type (WT; cv. M82). Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). Control and ethylene treated root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. All the experiments were repeated two times independently. At least 6 primary roots were examined independently. $n = 42$ and 47 , respectively. *** represents p -value ≤ 0.001 , calculated by Student's t -test. Scale bars represent 100 μ m in panels **A-F**.

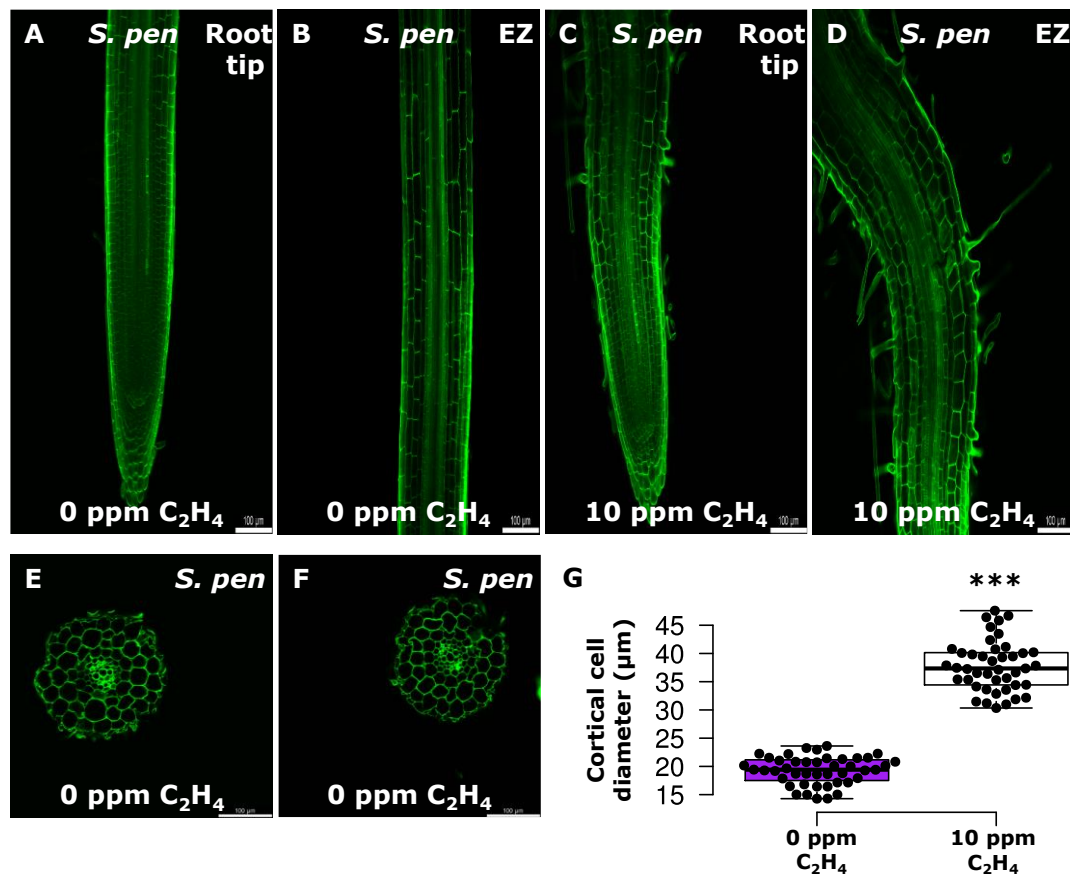


Figure 2.5. Ethylene promotes the radial expansion of the desert wild-species *Solanum pennellii* root cortical cells. **(A-B)** Representative confocal images of the desert wild-species *Solanum pennellii* as a control in the root tip and elongation zone (EZ). **(C-D)** Representative images of *S. pennellii* after ethylene (C₂H₄) (10 ppm for 3 days) treatment showing a radial expansion of cortical cells in the root tip and elongation zone (EZ). **(E-F)** Representative images of *S. pennellii* as a control in the root tip. **(G)** Quantitative box plot showing the radial expansion (diameter) of control and ethylene-treated root tips of *S. pennellii*. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). Control and ethylene treated root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. All the experiments were repeated two times independently. At least 6 primary roots were examined independently. $n = 44$ and 43 , respectively. *** represents p -value ≤ 0.001 , calculated by Student's t -test. Scale bars represent 100 μm in panels **A-F**.

2.3.5. Less ethylene-sensitive tomato introgression lines exhibit higher root penetration in compacted soil

The impact of root ethylene sensitivity on the ability of tomato introgression lines to penetrate compacted soil was studied. From the ethylene bioassay screen (see 2.2.3), two less ethylene-sensitive tomato ILs (IL2-1-1 and IL3-1) were selected to study their root growth responses in compacted soils. Pre-germinated tomato seeds were transferred to 1.1 g cm⁻³ BD and 1.5 g cm⁻³ BD 3D-printed soil columns, and their root lengths were measured after 7 days of growth. The soil compaction assay revealed that IL2-1-1 and IL3-1 root growth is much less inhibited (~31% and ~28% reduction, respectively) compared to the wild-type (WT; cv. M82) (~55% reduction) and highly ethylene-sensitive introgression line IL2-6-5 (~49% reduction) (Figure 2.6). Hence, cortical cell swelling in response to soil compaction does not appear to aid root penetration capacity. Instead, this ethylene-dependent root swelling response appears to reduce root growth when exposed to compacted soil.

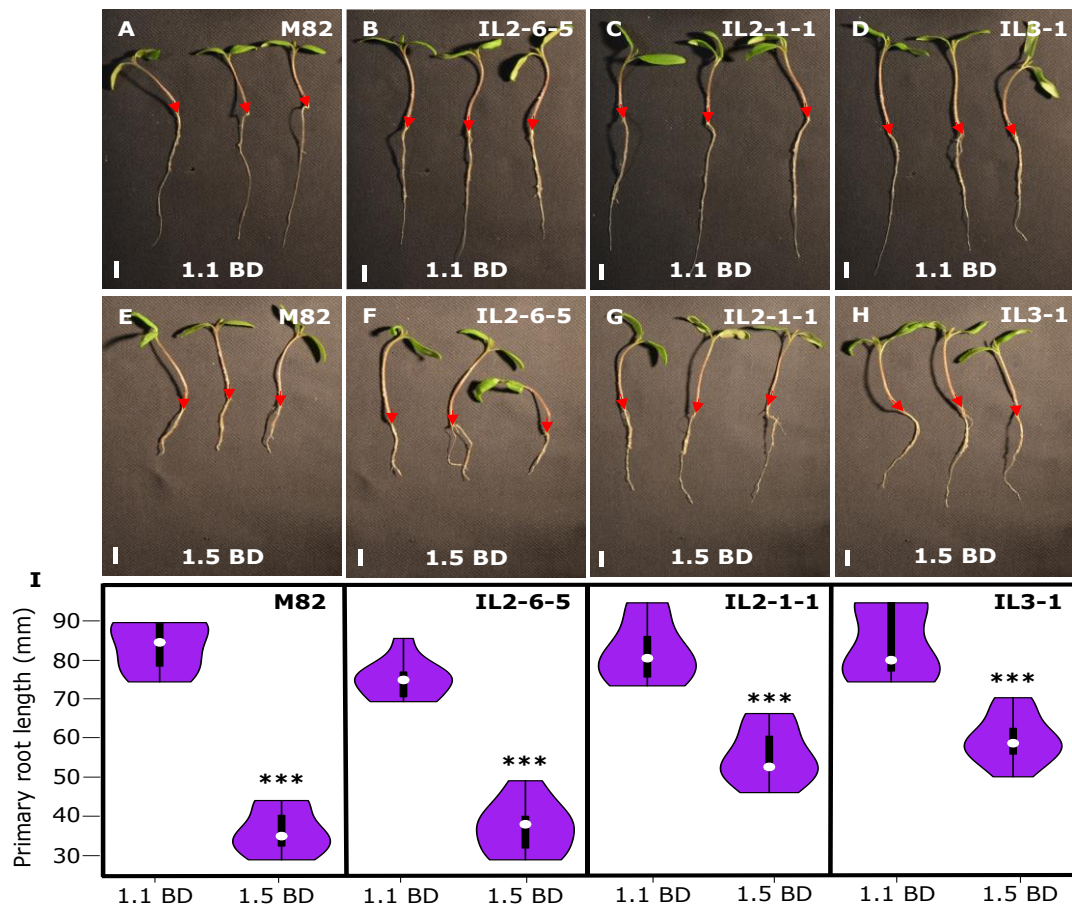


Figure 2.6. Less ethylene-sensitive tomato introgression lines IL2-1-1 and IL3-1 have better root penetration ability than wild-type (cv. M82) and highly ethylene-sensitive tomato introgression line IL2-6-5. Representative CT (Computed Tomography) images of primary root length in (A, E) wild-type (WT; cv. M82) as compared with (B, F) highly ethylene-sensitive tomato introgression line IL2-6-5, (C, G) IL2-1-1 and (D, H) IL3-1 in non-compacted [1.1 g cm^{-3} bulk density (BD)] and compacted soil [1.5 g cm^{-3} bulk density (BD)], respectively. Red arrows indicate the location of the root-shoot junction to reveal the hypocotyl length. (I) Violin plots represent the primary root length of cv. M82, IL2-6-5, IL2-1-1 and IL3-1 tomato seedlings in non-compacted (1.1 g cm^{-3} BD) versus compacted (1.5 g cm^{-3} BD) conditions. For CT imaging, 3 replicates were imaged in 1.1 g cm^{-3} BD and 1.5 g cm^{-3} BD soils. The CT experiment was independently repeated twice. Violin plots were generated using BoxPlotR (shiny.chemgrid.org/boxplotr/) (Spitzer *et al.*, 2014). $n = 12$; all graphs are the average of two repeats. *** represents p -value ≤ 0.0001 , calculated by Student's t -test. Scale bars represent 10 mm in panels A-H.

Root penetration capabilities of several additional tomato lines (cv. M82, *S. pennellii*, IL2-1-1, IL2-6-5, IL3-1, cv. Ailsa Craig and tomato ethylene-insensitive mutant *Never-ripe*) were examined to test this theory independently. X-ray computed tomography (CT) was employed to non-invasively observe how tomato root growth is changed in response to soil compaction. When cultivated in compacted soil, microCT imaging of less ethylene-sensitive tomato introgression lines revealed both these lines have greater root penetrating abilities than WT (Figure 2.7). IL2-1-1 and IL3-1 roots growth are much less inhibited (~31% and ~28% reduction, respectively) compared to the wild-type (WT; cv. M82) (~55% reduction) and *S. pennellii* (~60% reduction) (Figure 2.7). Hence, cortical cell swelling in response to soil compaction does not appear to aid root penetration capacity. Instead, this ethylene-dependent root swelling response appears to reduce root growth when exposed to compacted soil.

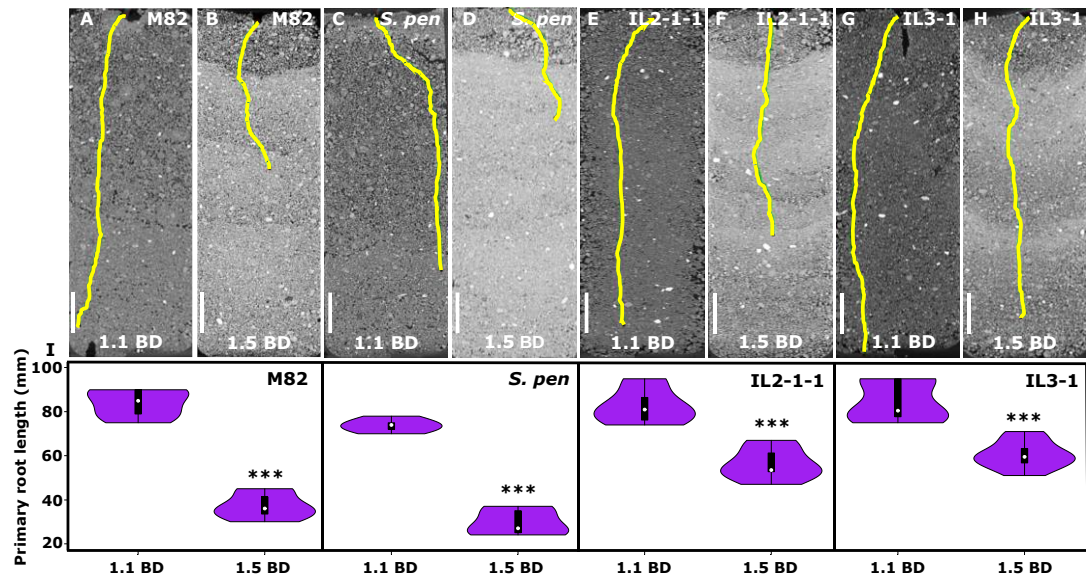


Figure 2.7. Less ethylene-sensitive tomato introgression lines IL2-1-1 and IL3-1 have better root penetration ability than wild-type (WT; cv. M82) and its wild relative *S. pennellii* in compacted soil. Representative CT (Computed Tomography) images of primary root length in (A, B) wild-type (WT; cv. M82) as compared with (C, D) *S. pennellii*, (E, F) IL2-1-1 and (G, H) IL3-1 in non-compacted [1.1 g cm^{-3} bulk density (BD)] and compacted soil (1.5 g cm^{-3} BD), respectively. Violin plots represent the primary root length of cv. M82, *Solanum pennellii*, IL2-1-1 and IL3-1 tomato seedlings in non-compacted (1.1 g cm^{-3} BD) versus compacted (1.5 g cm^{-3} BD) conditions. (I) The upper 1 cm is non-compacted soil (1.1 g cm^{-3} BD). For CT imaging, 3 replicates were imaged in 1.1 g cm^{-3} BD and 1.5 g cm^{-3} BD soils. $n = 3$; all graphs are the average of two repeats. *** represents p -value ≤ 0.0001 , calculated by Student's t -test. The CT experiment was independently repeated twice. Violin plots were generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). Scale bars represent 10 mm in panels A-H.

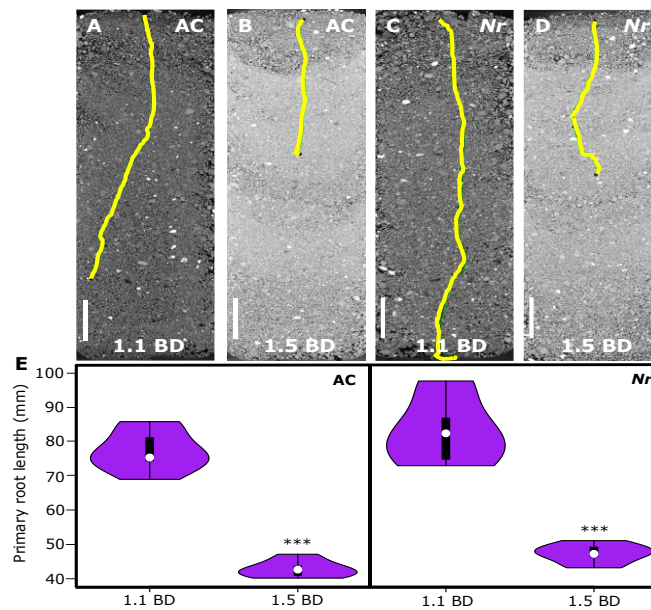


Figure 2.8. Tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant is also highly ethylene-sensitive compared with wild-type (WT; cv. Ailsa Craig [AC]) in compacted soil. Representative CT (Computed Tomography) images of primary root length in (**A, B**) wild-type (WT; cv. Ailsa Craig) as compared with (**C, D**) tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant in non-compacted [1.1 g cm^{-3} bulk density (BD)] and compacted soil (1.5 g cm^{-3} BD), respectively. Violin plots represent the primary root length of cv. Ailsa Craig and *Never-ripe* tomato seedlings in non-compacted (1.1 g cm^{-3} BD) versus compacted (1.5 g cm^{-3} BD) conditions. (**E**) The upper 1 cm is non-compacted soil (1.1 g cm^{-3} BD). For CT imaging, 3 replicates were imaged in 1.1 g cm^{-3} BD and 1.5 g cm^{-3} BD soils. The CT experiment was independently repeated twice. Violin plots were generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014).*** represents p -value ≤ 0.0001 , calculated by Student's t -test. Scale bars represent 10 mm in panels **A-D**.

The reduced ethylene response in introgression lines IL2-1-1 and IL3-1 appears to provide resistance to their root growth in the compacted soil. To explore the role of ethylene further, ethylene-sensitive wild-type (WT; cv. Ailsa Craig [AC]) and ethylene-insensitive *Nr* lines were grown in non-compacted (1.1 g cm^{-3} BD) or compacted (1.5 g cm^{-3} BD) soil. CT imaging in compacted soil conditions revealed, rather surprisingly, that tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant is highly sensitive ($\sim 43\%$ reduction) to compaction and is comparable to wild-type (WT; cv. Ailsa Craig [AC]) ($\sim 45\%$ reduction) (Figure 2.8). This surprising result suggests led us to explore the role of ethylene on root compaction responses further.

2.3.6. Soil compaction promotes the radial expansion of cortical cells in ethylene-sensitive tomato lines

The phenotypes of different tomato lines were studied to see if ethylene sensitivity is correlated with cortical cell radial expansion under compacted soil conditions. Wild-type (WT; cv. M82) was grown in non-compacted ($1.1 \text{ g cm}^{-3} \text{ BD}$) or compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil, then cleared for confocal imaging (see 2.2.8). When cultivated in compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil conditions, cv. M82 roots exhibited a significant increase in root diameter (Figure 2.9). Confocal imaging revealed radial expansion of wild-type (WT; cv. M82) primary root cortical cells after compaction treatment.

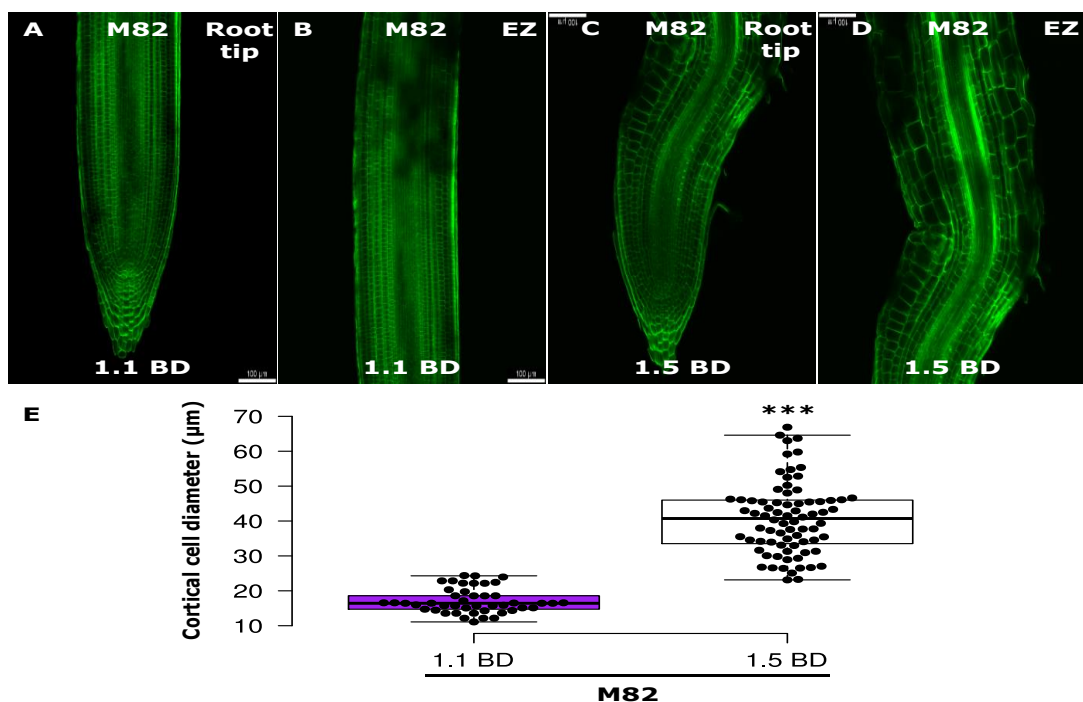


Figure 2.9. Tomato cv. M82 (LA3475) primary root cortical cells undergo radial expansion when growing in compacted soil. Representative confocal images of wild-type (WT; cv. M82) as a control in the root tip and elongation zone (EZ) (**A** and **B**, respectively) in non-compacted ($1.1 \text{ g cm}^{-3} \text{ BD}$). Representative confocal images of wild-type (cv. M82) in the root tip and elongation zone (EZ) (**C** and **D**, respectively) in compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil. (**E**) Quantitative box plot showing the radial expansion (diameter) of root cortical cells in primary root tips of WT (cv. M82). All the experiments were repeated two times independently. At least 6 primary roots were examined independently. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). $n = 45$ and 76 , respectively. Non-compacted and compacted root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. *** represents p -value ≤ 0.001 , calculated by Student's t -test. Scale bars represent $100 \mu\text{m}$ in panels **A-D**.

Less ethylene-sensitive introgression line (IL2-1-1) was grown in non-compacted (1.1 g cm^{-3} BD) or compacted (1.5 g cm^{-3} BD) soil before being cleared for confocal imaging (see 2.2.8). In comparison to wild-type roots, IL2-1-1 roots did not exhibit a significant increase in root diameter when grown in compacted (1.5 g cm^{-3} BD) soil conditions (Figure 2.10).

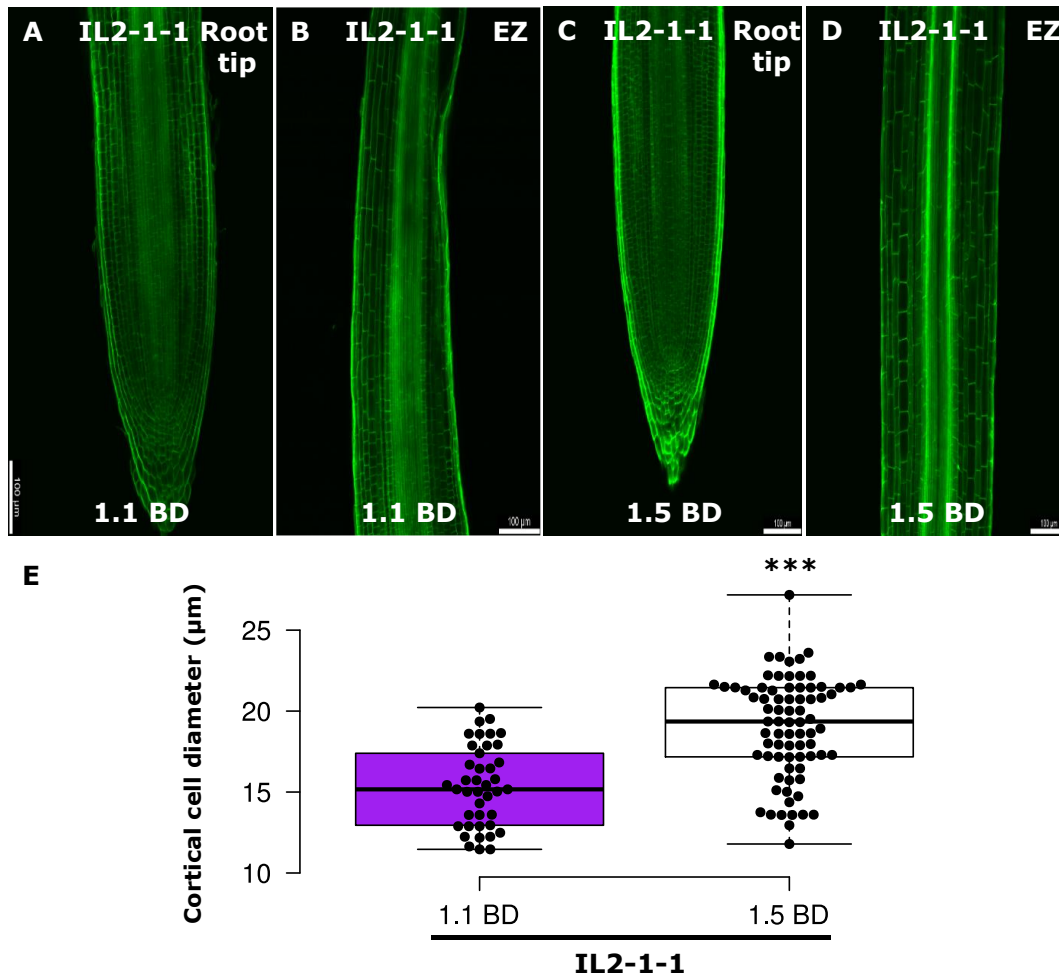


Figure 2.10. Less ethylene-sensitive tomato introgression line IL2-1-1 (LA4036) does not show increased root cortical cell diameter when grown in compacted soil. Representative confocal images of IL2-1-1 as a control in the root tip and elongation zone (EZ) (**A** and **B**, respectively) in non-compacted (1.1 g cm^{-3} BD). Representative confocal images of IL2-1-1 in the root tip and elongation zone (EZ) (**C** and **D**, respectively) in compacted (1.5 g cm^{-3} BD) soil. (**E**) Quantitative box plot showing the radial expansion (diameter) of root cortical cells in primary root tips of IL2-1-1. All the experiments were repeated two times independently. At least 6 primary roots were examined independently. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). $n = 41$ and 77 , respectively. Non-compacted and compacted root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. *** represents p -value ≤ 0.001 , calculated by Student's t -test. Scale bars represent $100 \mu\text{m}$ in panels **A-D**.

Less ethylene-sensitive tomato introgression line (IL3-1) was grown in non-compacted ($1.1 \text{ g cm}^{-3} \text{ BD}$) or compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil before being cleared for confocal imaging (see 2.2.8). In comparison to wild-type roots, IL3-1 roots did not exhibit a significant increase in root diameter when grown in compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil conditions (Figure 2.11).

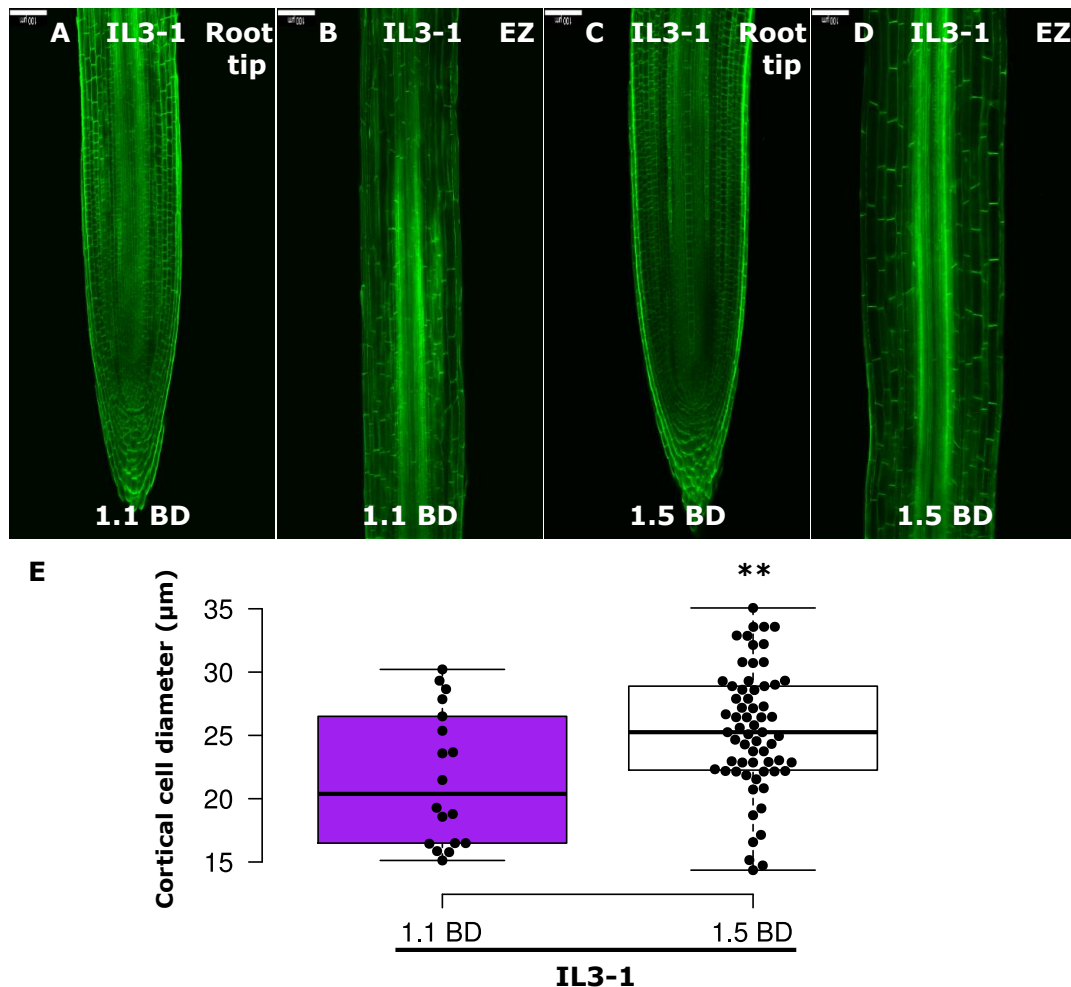


Figure 2.11. Less ethylene-sensitive tomato introgression line IL3-1 (LA4043) does not show increased root cortical cell diameter when grown in compacted soil. Representative confocal images of IL3-1 as a control in the root tip and elongation zone (EZ) (**A** and **B**, respectively) in non-compacted ($1.1 \text{ g cm}^{-3} \text{ BD}$). Representative confocal images of IL3-1 in the root tip and elongation zone (EZ) (**C** and **D**, respectively) in compacted ($1.5 \text{ g cm}^{-3} \text{ BD}$) soil. (**E**) Quantitative box plot showing the radial expansion (diameter) of root cortical cells in primary root tips of IL3-1. All the experiments were repeated two times independently. At least 6 primary roots were examined independently. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). $n = 18$ and 64 , respectively. Non-compacted and compacted root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. ** represents p -value ≤ 0.01 , calculated by Student's t -test. Scale bars represent $100 \mu\text{m}$ in panels **A-D**.

Highly ethylene-sensitive tomato introgression line IL2-6-5 was grown in non-compacted (1.1 g cm^{-3} BD) or compacted (1.5 g cm^{-3} BD) soil before being cleared for confocal imaging (see 2.2.8). In comparison to wild-type roots, IL2-6-5 roots exhibited a significant increase in root diameter when grown in compacted (1.5 g cm^{-3} BD) soil conditions (Figure 2.12).

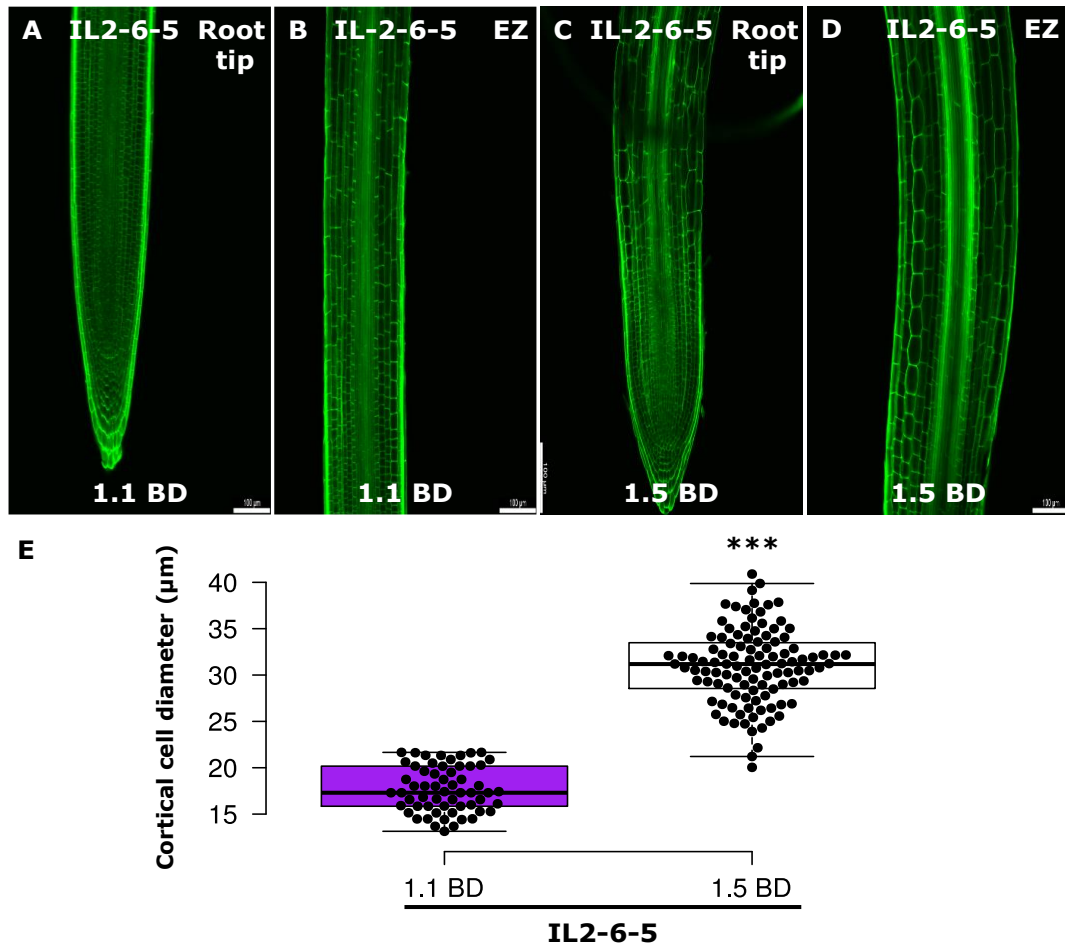


Figure 2.12. Highly ethylene-sensitive tomato introgression line IL2-6-5 (LA4042) shows increased root cortical cell diameter when grown in compacted soil. Representative confocal images of IL2-6-5 as a control in the root tip and elongation zone (EZ) (**A** and **B**, respectively) in non-compacted (1.1 g cm^{-3} BD). Representative confocal images of IL2-6-5 in the root tip and elongation zone (EZ) (**C** and **D**, respectively) in compacted (1.5 g cm^{-3} BD) soil. (**E**) Quantitative box plot showing the radial expansion (diameter) of root cortical cells in primary root tips of IL2-6-5. All the experiments were repeated two times independently. At least 6 primary roots were examined independently. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). $n = 59$ and 107 , respectively. Non-compacted and compacted root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. *** represents p -value ≤ 0.001 , calculated by Student's t -test between genotypes in compacted compared with non-compacted conditions. Scale bars represent $100 \mu\text{m}$ in panels **A-D**.

2.3.7. Genome-enabled identification of candidate loci controlling ethylene-insensitivity in IL2-1-1 and IL3-1

Understanding the genetic regulation of root penetration in compacted soil could have significant implications for marker-assisted selection (or future gene-editing efforts) to create novel compaction resistant tomato varieties. To aid this goal, I attempted to pinpoint the putative ethylene-response regulatory gene(s) that control compacted soil penetration in both tomato introgression lines (IL2-1-1 and IL3-1). This objective was greatly assisted by the genomic segments introgressed within each IL line having been fine mapped and the genome sequences being available for these regions from the wild-relative *Solanum pennellii* (Bolger *et al.*, 2014).

To identify ethylene-related candidate genes, the genetic markers that characterise the endpoints of each IL's overlapping region were used to identify the genomic sequence in the tomato genome assembly provided at the Sol Genomics Network (SGN, assembly ITAG4.1; <https://solgenomics.net/>) (Fernandez-Pozo *et al.*, 2015).

In the case of IL2-1-1, the introgressed region contained 337 genes, two of which were classified as putative ethylene-related genes. Figure 2.13 illustrates that (Soly02g022920 and Soly02g030210) have been identified as encoding an ethylene-responsive nuclear protein and an AP2-like ethylene-responsive transcription factor candidate, respectively (Table 2.1). These genes also have Arabidopsis homologs (Table 2.2).



Figure 2.13. Schematic view of Solyc02g022920 and Solyc02g030210 which encode an ethylene-responsive nuclear protein and an AP2-like ethylene-responsive transcription factor, respectively. Name of genes and locations of IL bins were adapted from (Chitwood *et al.*, 2013) and using Sol Genomics Network (SGN, assembly ITAG4.1; <https://solgenomics.net/>) (Fernandez-Pozo *et al.*, 2015).

In the case of IL3-1, the introgressed region contained 191 genes, five of which were classified as putative ethylene-related genes. Figure 2.14 illustrates the five genes (Solyc03g005500, Solyc03g005510, Solyc03g005520, Solyc03g005600, and Solyc03g006320) have been predicted to encode an ethylene-responsive transcription factor and a S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (Table 2.1). These genes also have Arabidopsis homologs, several of which have ethylene-related functions (Table 2.2).



Figure 2.14. Schematic view of Solyc03g005500, Solyc03g005510, Solyc03g005520, Solyc03g005600, and Solyc03g006320 which encode an ethylene-responsive transcription factor and a S-adenosyl-L-methionine-dependent methyltransferases superfamily protein, respectively. Name of genes and locations of IL bins were adapted from (Chitwood *et al.*, 2013) and using Sol Genomics Network (SGN, assembly ITAG4.1; <https://solgenomics.net/>) (Fernandez-Pozo *et al.*, 2015).

Table 2.1. In the introgressed regions, 7 ethylene-related gene models have been identified. The SOL name of the genes, chromosome numbers, start and stop positions, and descriptions were retrieved from Sol Genomics Network, SGN (<https://solgenomics.net/>) (Fernandez-Pozo *et al.*, 2015).

Name	Chr.	Start position	Stop position	Description
Solyc02g022920	Ch2	15128281	15129522	Ethylene-responsive nuclear protein (AHRD V1 ***- Q38MV1_SOLLC)
Solyc02g030210	Ch2	16023195	16026741	AP2-like ethylene-responsive transcription factor At2g41710 (AHRD V1 ***- AP2L4_ARATH); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding
Solyc03g005500	Ch3	383586	383975	Ethylene-responsive transcription factor 14 (AHRD V1 **** ERF97_ARATH); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding
Solyc03g005510	Ch3	387846	388373	Ethylene-responsive transcription factor 1A (AHRD V1 *** A9P6A4_MEDTR); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding
Solyc03g005520	Ch3	397152	397787	Ethylene responsive transcription factor 1a (AHRD V1 *** C0J9I9_9ROSA); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding
Solyc03g005600	Ch3	438634	441628	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (AHRD V3.3 *** A0A2U1MED4_ARTAN)
Solyc03g006320	Ch3	924278	924928	Ethylene-responsive transcription factor 4 (AHRD V1 *- B6THY5_MAIZE); contains Interpro domain(s) IPR001471 Pathogenesis-related transcriptional factor and ERF, DNA-binding

Table 2.2. The SOL name of the genes and Arabidopsis homologues. Homologous genes for Arabidopsis were retrieved from the Ensemble Plants Database (<https://plants.ensembl.org/>) (Howe *et al.*, 2020).

SOL name of the genes	Arabidopsis homologues
Solyc02g022920	AT4G20880
	AT5G44350
Solyc02g030210	AT2G41710
Solyc03g005500	AT1G04370 (ERF14)
	AT3G23220 (ERF095)
Solyc03g005510	-
Solyc03g005520	AT3G23240 (ERF1B)
Solyc03g005600	AT1G04430
	AT3G23300
	AT4G14360
Solyc03g006320	AT1G03800 (ERF10)
	AT1G28370 (ERF11)

To test if there are any changes in the protein-coding parts in the candidate genes in the introgressed region, M82 and *S. pennellii* amino acid sequences were aligned. Amino acid sequences were aligned using Clustal Omega as implemented in Geneious Prime software (<https://www.geneious.com/>) (version 2022.0.1; Biomatters Ltd.).

Solanum pennellii alleles of Sopen02g006550, Sopen03g001520, Sopen03g001530, and Sopen03g002330 have amino acid deletions when compared to *Solanum lycopersicum* cv. M82 alleles, which would confer altered gene function in lines encoding these introgressed regions. None of these amino acid deletions are in the DNA-binding domain (DBD) (Figure 2.15-2.17).



Figure 2.15. Amino acid sequence alignment between (A) Solyc02g022920 and Sopen02g006550, and (B) Solyc02g030210 and Sopen02g009530 which encode an ethylene-responsive nuclear protein and an AP2-like ethylene-responsive transcription factor, respectively. Below the alignment, a red arrow indicates the position of the AP2/ERF domain-containing as using SMART (<http://smart.embl-heidelberg.de>) (Letunic et al., 2021). Amino acid sequences were aligned using Clustal Omega as implemented in Geneious Prime software (<http://www.geneious.com>) (version 2022.0.1; Biomatters Ltd.).



Figure 2.16. Amino acid sequence alignment between (A) Solyc03g005500 and Sopen03g001520, (B) Solyc03g005510 and Sopen03g001530, and (C) Solyc03g005520 and Sopen03g001540 which encode an ethylene-responsive transcription factor. Below the alignment, a red arrow indicates the position of the AP2/ERF domain-containing as using SMART (<http://smart.embl-heidelberg.de>) (Letunic *et al.*, 2021). Amino acid sequences were aligned using Clustal Omega as implemented in Geneious Prime software (<http://www.geneious.com>) (version 2022.0.1; Biomatters Ltd.).

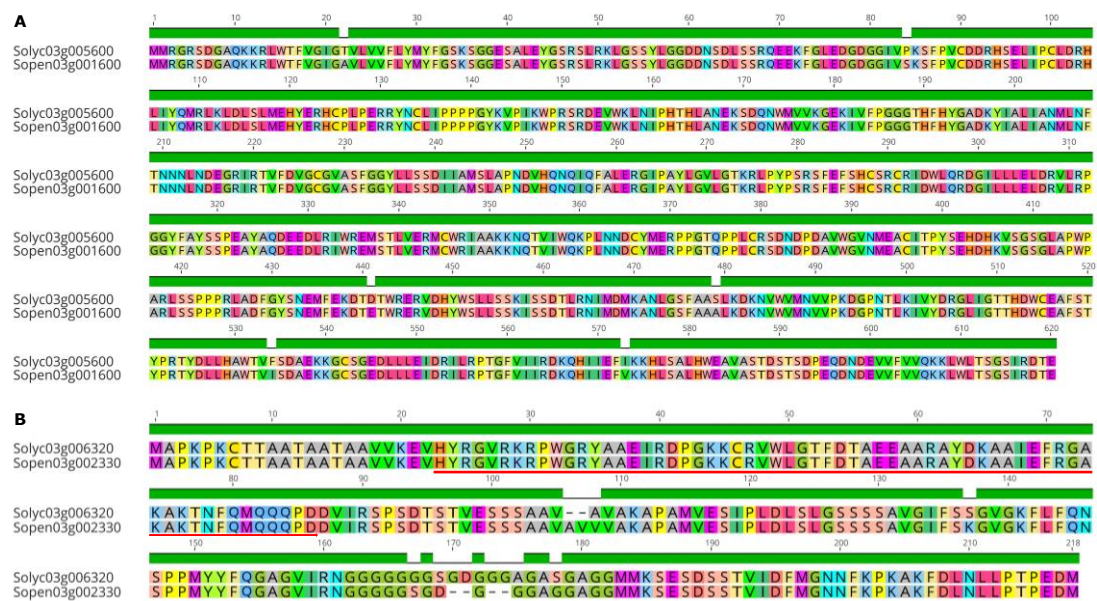


Figure 2.17. Amino acid sequence alignment between (A) Solyc03g005600 and Sopen03g001600, and (B) Solyc03g006320 and Sopen03g002330 which encode a S-adenosyl-L-methionine-dependent methyltransferases superfamily protein and an ethylene-responsive transcription factor, respectively. Below the alignment, a red arrow indicates the position of the AP2/ERF domain-containing as using SMART (<http://smart.embl-heidelberg.de>) (Letunic *et al.*, 2021). Amino acid sequences were aligned using Clustal Omega as implemented in Geneious Prime software (<http://www.geneious.com>) (version 2022.0.1; Biomatters Ltd.).

2.4. Discussion

Classically, root tip swelling was thought to help penetration of compacted soil (Correa *et al.*, 2019). However, recent experiments have shown that *Arabidopsis*, rice, and maize ethylene-insensitive lines and landraces, which disrupt root tip swelling in compacted soil, have increased penetrating abilities (Pandey *et al.*, 2021b; Vanhees *et al.*, 2021). In this study, less ethylene-sensitive tomato introgression lines were discovered that inhibit root tip swelling in compacted soil and exhibit better penetration abilities. This result, when combined with those obtained from other plant species, reveals a clear consensus that roots which remain thinner can penetrate compacted soil easier. In contrast, roots that swell in compacted soil are less able to penetrate. Root tip swelling in compacted soil appears to slow root growth, allowing plant resources to be redirected to other parts of the plant's root system that may be experiencing less-compacted soil conditions (Lynch *et al.*, 2021).

Reduced ethylene responsiveness appears to be a good diagnostic for root-soil compaction resistance in every plant species studied to date. Consistent with this promising design principle, mutating components of the primary ethylene response pathway in *Arabidopsis* and rice confers ethylene and compaction resistance phenotypes. However, in tomato, mutating a primary ethylene signalling component *Never-ripe* (which encodes an ethylene receptor) does not confer compaction resistance. X-ray microCT imaging in compacted soil conditions revealed, rather surprisingly, that tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant is sensitive to compaction stress (~43% reduction in root growth) which is comparable to wild-type (WT; cv. Ailsa Craig [AC]) (~45% reduction) (Figure 2.8). This surprising result suggests led us to explore the role of ethylene on root compaction responses further. Similar observations about *Nr* have been reported by other authors (Clark *et al.*, 1999; Santisree *et al.*, 2011).

Why is the tomato *Never-ripe* mutant not able to penetrate compacted soil? Several possible explanations exist. The *Never-ripe* mutant is reported to be agravitopic (Santisree *et al.*, 2011) which could explain why it is less able to penetrate in soil compaction conditions compared to wild-type (Correa *et al.*, 2019). CT imaging revealed that the *Never-ripe* mutant root appears gravitropic under non-compacted conditions, but its root growth became less gravitropic at higher compaction levels (Figure 2.8). Differences in root exudates and microbiome composition have also been reported for *Never-ripe* (Fu *et al.*, 2021) which could impact the mutant's root penetration ability through compacted soil and hormone accumulation (Oleghe *et al.*, 2017; Korenblum *et al.*, 2020).

Fu *et al.* (2021) showed that tomato ethylene-insensitive *Never-ripe* (*Nr*) mutant lines exhibit considerably different functional and taxonomic profiles of the rhizosphere bacterial community than *Solanum lycopersicum* cv. Micro-Tom. Several of these alterations are closely linked to differences in root exudation metabolites between the cv. Micro-Tom and *Never-ripe* lines. Hence, ethylene signalling mediated by *Never-ripe* can have a direct impact on the function and structure of the bacterial communities in the rhizosphere of tomato plants through detectable changes in root exudates (Fu *et al.*, 2021) which may individually or collectively impact tomato *Nr* roots ability to penetrate compacted soil. These root phenotypic and microbiome differences may explain the contrasting behaviour of ethylene mutants regarding compaction resistance between different species.

Differences also exist in the regulatory relationship between key hormone response pathways in these species that may explain the contrasting impact of ethylene mutants on their root compaction responses. In tomato, the ethylene and ABA signalling pathways are organised differently than in rice and maize, where ABA acts upstream of ethylene in tomato, whereas this is reversed in the cereal models. Tomato roots are more sensitive to compaction stress when ABA synthesis is mutated (Tracy *et al.*, 2015), whereas rice roots are less sensitive (Huang *et al.*, 2022).

Tracy *et al.*, (2015) demonstrated ABA-biosynthesis tomato mutant *notabilis* (*not*) has a reduced ability to penetrate compacted soil. *Notabilis* (*not*) is an ABA-biosynthesis tomato mutant caused by a null mutation in the gene *NCED1*, which encodes a *9-cis-epoxycarotenoid dioxygenase* (Burbidge *et al.*, 1999; Zhang *et al.*, 2021).

Thus, ABA (acting upstream of ethylene) normally negatively regulates root compaction responses such as growth inhibition in tomato. In contrast, ABA biosynthesis mutants in rice exhibit less growth inhibition and radial swelling in compacted soil. Hence, ABA (acting downstream of ethylene) normally positively regulates root compaction responses in rice. In summary, important differences exist in the regulatory relationship between key hormone response pathways in tomato and cereal species that may explain the contrasting impact of ethylene mutants on their root compaction responses.

Despite the results obtained using the *Nr* mutant, I observed that reduced ethylene sensitivity proved an effective way to select tomato introgression lines whose roots exhibited reduced sensitivity towards soil compaction.

I identified two less ethylene-sensitive tomato introgression lines (IL2-1-1 and IL3-1) that are also compaction-resistant. Neither of these tomato ILs (IL2-1-1 and IL3-1) encode primary ethylene signalling components within their introgressed genomic regions. However, these introgressed genomic regions do encode ethylene synthesis and/or downstream signalling components. For example, in the case of IL2-1-1 two genes (Soly02g022920 and Soly02g030210) have been predicted to encode an ethylene-responsive nuclear protein and an AP2-like ethylene-responsive transcription factor, respectively. It is possible that mutating genes downstream of the primary ethylene response pathway will be more effective at conferring soil compaction resistance, particularly if they modify responses to multiple signals like ABA and ethylene.

To pinpoint which of the ethylene-related gene candidates encoded in IL2-1-1 or IL3-1 introgressed regions contained missense or nonsense changes, I compared *S. pennellii* and two other wild-type species (*Solanum pimpinellifolium* and *Solanum chilense*) genome sequences to find out if *S. pennellii* was responsible for these changes. Homologues of most candidate genes do not have amino acid deletions in species such as *Solanum pimpinellifolium* and *Solanum chilense*.

However, *Solanum pennellii* alleles of Sopen02g006550, Sopen03g001520, Sopen03g001530, and Sopen03g002330 have amino acid deletions when compared to *Solanum lycopersicum* cv. M82 alleles (Figure 2.15-2.17), which would confer altered gene function in lines encoding these introgressed regions. Despite these genes representing promising candidates conferring compaction resistance, genetic studies are required to validate this. For example, gene editing could be employed to mutant individual candidate genes within the introgressed regions (assuming the loss-of-function allele exhibits a phenotype) to pinpoint the causative gene(s). Once identified, a gene-editing (and/or backcrossing) based approach could be used to modify elite tomato lines to confer root compaction resistance.

3. Chapter 3: Ethylene inhibits rice root elongation in compacted soil via ABA-mediated radial expansion of cortical cells¹

3.1. Introduction

Soil compaction represents a major challenge facing modern agriculture (Hamza and Anderson, 2005), reducing crop yields by ~25%, and when combined with drought by up to 75%, as roots are challenged to penetrate hard soils (Arvidsson, 1999; Hoque and Kobata, 2000; Gregory *et al.*, 2007).

Soil compaction results in inhibition of root penetration and reduced uptake of nutrients and water. Efforts to mitigate the impact of soil compaction on crop productivity include reducing tillage, controlled traffic farming (CTF), and sub-soil management (Gregory *et al.*, 2007; Jones *et al.*, 2012). However, these approaches can be time-consuming, costly to implement and ineffective for deeper soil profiles.

Breeding crops to better withstand compacted soil offers a genetic solution to improve root growth during mechanical impedance (Jones *et al.*, 2012). Recently, maize roots having small cells with thick walls in outer cortical layers (termed multiseriate cortical sclerenchyma [MCS]) were reported to improve rooting depth in compacted soil in field and greenhouse trials (Schneider *et al.*, 2021). In comparison to non-MCS genotypes, the MCS anatomical trait was associated with greater root tip bending force, tensile strength, and root lignin concentration.

¹ Huang, G., **Kilic, A.**, Karady, M., Zhang, J., Mehra, P., Song, X., Sturrock, C.J., Zhu, W., Qin, H., Hartman, S., Schneider, H.M., Bhosale, R., Dodd, I.C., Sharp, R.E., Huang, R., Mooney, S.J., Liang, W., Bennett, M.J.*, Zhang, D.*, and Pandey, B.K.* (2022). Ethylene inhibits rice root elongation in compacted soil via ABA and auxin mediated mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*. (**Under revision**).

Conventional thinking considers that roots are unable to penetrate compacted soils due to the mechanical force required (Passioura, 2002; Correa *et al.*, 2019). However, Pandey *et al.*, (2021b) recently discovered that plant roots employ the gaseous hormone ethylene to sense soil compaction. Their experiments revealed that compacted soil restricts the diffusion of ethylene out of and away from roots, triggering inhibition of root elongation while also promoting radial swelling (Pandey *et al.*, 2021b). Remarkably, roots of *Arabidopsis* and rice mutants that are insensitive to ethylene are able to better penetrate compacted soil (Pandey *et al.*, 2021b). Ethylene-response mutant roots remain narrow and able to penetrate compacted soil, whereas wild-type roots exhibit inhibition of cell elongation and promotion of cortical cell radial expansion (Pandey *et al.*, 2021b). How ethylene triggers these root growth changes is currently unclear.

Here, a novel role for the signal abscisic acid (ABA) and its biosynthesis pathway as a regulatory target of the ethylene-response pathway during rice root growth responses to soil compaction is revealed. Exogenous treatment with ABA promotes radial expansion of root cortical cells, mimicking soil compaction response. Furthermore, ABA acts downstream of ethylene and auxin signalling during a soil compaction response was demonstrated. Integrating ethylene, auxin, and ABA responses in a sequential regulatory fashion during a soil compaction response appears to support ABA-mediated radial cell expansion. In conclusion, ethylene employs ABA as a downstream signal to modify rice root growth, causing root tips to swell and disrupt their ability to penetrate compacted soil.

3.2. Materials and methods

3.2.1. Soil materials

Loamy sand soil of the Newport series (FAO Brown Soil) (83.2% sand, 12.1% clay, and 4.7% silt; pH = 6.35; organic matter 2.93%) was used in all soil experiments. Soil was collected at a farm near the University of Nottingham in Bunny, Nottinghamshire, England (52° 52' N 1° 07' W), sieved to less than 2 mm, and the water content was calculated by drying the soil at 65°C until a certain weight was reached.

3.2.2. Plant materials and growth conditions

Rice seeds (Nipponbare, Dongjin, *osaba1*, *osaba2*, *mhz4*, *mhz5-1*, *oseil1*, *osein2*, *osaux1-1* and *osaux1-3*) were surface sterilised for 10 minutes with 25% bleach (diluted from 19% CaOCl₂ stock), then washed five times with sterilised water. The genetic backgrounds of rice *osaba1*, *osaba2*, *osmh4*, *osmh5-1*, *oseil1* and *osein2* T-DNA insertional mutants are in the Nipponbare background and the genetic background of *osaux1-1* and *osaux1-3* is Dongjin. In a rice growth chamber (12-hour photoperiod at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR light intensity, 70% relative humidity), sterilised seeds were germinated on a moist sterilised filter paper in the dark for 2 days at 28°C. After primary root emergence (4-6 mm in length), seedlings were gently placed in topsoil packed at different compaction levels (1.1 g cm⁻³ and 1.6 g cm⁻³ BD for non-compacted and compacted soil conditions, respectively; the upper 1 cm contained non-compacted soil in both treatments) in a 3D printed column (33 mm diameter x 100 mm height), as previously described (Pandey *et al.*, 2021a, b).

3.2.3. Preparation of soil columns

The methods for the preparation of soil columns were performed as in the previous description (Pandey *et al.*, 2021a). Rice seeds were surface sterilised for 10 minutes with 25% bleach (diluted from 19% CaOCl₂ stock), then washed five times with sterilised water. In a rice growth chamber (maintained at 28°C, 12-hour photoperiod, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 70% relative humidity), sterilised seeds were germinated on moist sterilised filter paper in the dark for 2 days. 99.82 g and 130.67 g of sieved (<2 mm) air-dried soil were added to the 3D-printed columns, respectively; to reach soil bulk densities of 1.1 g cm⁻³ and 1.6 g cm⁻³.

The columns were placed in a tray filled with sterile water to a depth of 3 cm and left at room temperature overnight to allow the soil to saturate from the bottom of the column upwards. After 8-12 hours, the soil's surface is checked to see if it is fully saturated, and the columns are removed from the tray of water and allowed to drain freely for 48-hour before recording their mass. The mass of the column is equal to the notional field capacity moisture of the soil.

A 1 cm space was filled with 1.1 g cm^{-3} bulk density soil (9.98 g) at the top of the compacted zone (1.6 g cm^{-3} BD) to help the pre-germinated seedlings to establish before hitting the compacted soil layer. After radicle emergence (4-6 mm in length), seedlings were gently placed in topsoil (upper 1 cm non-compacted) in a 3D-printed column (33 mm diameter x 100 mm height) for compaction experiments described below.

3.2.4. Soil compaction experiment

The methods for root compaction assays were performed as in the previous description (Pandey *et al.*, 2021a). The seeds were germinated in petri dishes with a moist sterilised filter paper and kept in the dark for 2 days at 28°C. The germinated seeds were then placed on the top of the soil, which was packed at various levels of compaction. The germinated seeds were then covered with a 1 cm loose soil top layer. The root tips were carefully removed after 4 days of growth by washing the soil media to maintain the root tips intact for confocal imaging. The root length was measured using ImageJ (<https://imagej.nih.gov/ij/>) (Schindelin *et al.*, 2012).

3.2.5. Expression profiling

Total RNA was isolated from wild-type (cv. Nipponbare) primary root tips (1 cm, encompassing the meristematic and elongation zones) of 7-day-old plants using TRIzol® reagent (Applied Biosystems) as per the manufacturer's protocol. Isolated RNA was treated with DNase1 enzyme (1 U μg^{-1} of total RNA) to remove genomic DNA contamination. 1 μg of total RNA from each of three independent biological replicates (each replicate comprised 10-15 root tips) was used for cDNA synthesis using RevertAid™ First strand cDNA synthesis kit (Thermo Fisher) according to the manufacturer's instructions.

SensiMix SYBR Hi-ROX (Bioline) master mix was used for qRT-PCR on a qTOWER real-time PCR System (Analytik Jena GmbH) with three biological replicates per treatment. Expression values were normalized with expression values of an endogenous control, *ubiquitin5* (Os01g0328400).

As previously described (Pandey *et al.*, 2021c), relative expression levels (fold change; FC) were calculated with respect to the control using the $2^{-\Delta(\Delta\text{Ct})}$ method.

Table 3.1 lists the primers used for rice ABA biosynthetic genes and their expression analysis. Student's *t*-test was used to evaluate all FCs statistically.

Table 3.1. List of primers used for expression analysis

Gene Name	Primer Sequence
<i>MHZ5-F</i>	GCTGAAGTCCCTTGAGGAGCCTC
<i>MHZ5-R</i>	CCCCCGAAGTGCCTATCGCAC
<i>MHZ4-F</i>	CTCTGTTCCGGCCTCGCGCA
<i>MHZ4-R</i>	TCAATGTGAGCGACCAATTGAAC
<i>AAO1-F</i>	TTCGCCATTTGTTTCGTAA
<i>AAO1-R</i>	CAGAGGAGGTTGCTCAAG
<i>AAO2-F</i>	CCCTTGACGCCAACAACACTG
<i>AAO2-R</i>	CCGCTTTCGCCACTTATT
<i>ZEP-F</i>	ATAGATGATGGCAACAAGGTAA
<i>ZEP-R</i>	TCAATGTCAGGAGGCACAA
<i>ABA1-F</i>	GGATGCCATTGAGTTTGGTT
<i>ABA1-R</i>	TGGCTGACTGAAGTCTCTCG
<i>ABA2-F</i>	AGCAAACCTGAAAGGTGTGGA
<i>ABA2-R</i>	AAAGCCACCATCCACCATGA
<i>NCED2-F</i>	CACTCCCTTCTCATTCCC
<i>NCED2-R</i>	AGCCCTTGTTTCAGGTAA
<i>CRTISO-F</i>	CAGTTGGTGGTGTGGTGGTAT
<i>CRTISO-R</i>	GGTCACATTAGCCTTGTAGCGTAT
<i>Ubiquitin5-F</i>	ACGATTGATTAAACCAGTCCATGA
<i>Ubiquitin5-R</i>	AACCAGCTGAGGCCCAAGA

3.2.6. ABA measurement from rice root tips

For endogenous ABA quantification, wild-type (cv. Nipponbare) primary root tips (1 cm) were harvested from non-compacted and compacted soil-grown plants. The root tips were gently washed for less than 2 minutes with autoclaved RO water to remove soil particles, immediately snap-frozen and stored at -80°C. Extraction (approximately 8-10 mg fresh weight per sample) was performed according to (Floková *et al.*, 2014); cold 10% methanol containing 1 M formic acid (v/v) was used as the extraction solvent. In brief, [²H₆] ABA was added as an internal standard prior to extraction, and samples were then extracted using 1 mL of extraction solvent, homogenized, and purified using Oasis HLB columns (30 mg/1 mL, Waters Corporation, Milford, MA, USA). For Figure 3.2A, ABA measurements were performed by ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) using an Acquity UPLC® System (Waters) equipped with an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm; Waters) coupled to a triple quadrupole Xevo™ TQ-S MS (Waters). For Figure 3.9B, analyses were conducted using a Phenomenex Polar C18 column (150 × 2.1 mm, 2.5 µm; Phenomenex, Torrance, CA, USA), using Milli-Q water (A) and ACN (B), both with 0.02% formic acid (v/v) as mobile phase, with measurements performed on a LC-MS/MS system comprising a 1260 Infinity II LC System coupled with 6495 Triple Quad LC/MS System, Jet Stream and Dual Ion Funnel technologies (Agilent Technologies, Santa Clara, CA, USA).

3.2.7. Radial sectioning and confocal imaging

Primary roots were removed from soil columns and immediately washed with autoclaved RO water in three consecutive water-filled dishes using fine brush strokes to remove soil particles. Washing and clearing of the root tips were performed as previously described (Pandey *et al.*, 2021a). 1-cm root tips of rice lines were fixed in freshly prepared 4% PFA and vacuum infiltration treatment was carried out for 2 h. Fixed root tips were washed with 1x PBS buffer three times and kept in ClearSee solution for 24 h. Fluorescence of the cleared root tips was imaged with a Leica TCS-SP8 confocal microscope using 10% argon and DPS1 lasers.

Root tip samples (apical 1.5 cm) were embedded in 3.5% melted agarose and 100- μm -thick transverse sections were cut from the elongation zone using a Leica Vibratome, stained with 1% Calcofluor white dye for 1 min on a glass slide, and imaged with a Leica TCS-SP5 confocal microscope using 10% diode laser excited at 404 nm wavelength to obtain cross-sectional images.

3.2.8. X-ray Computed Tomography (CT) imaging

Equally germinated seedlings (2-day-old) of wild-type and *osaba1*, *osaba2*, *mhz4* and *mhz5-1* mutants were grown in 3D-printed columns (33 mm diameter x 100 mm height) filled with sandy loam soil at 1.1 g cm⁻³ BD and 1.6 g cm⁻³ BD for non-compacted and compacted soil conditions, respectively. The plants were grown in a growth chamber at 28°C with a 12-hour photoperiod at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR and 70% relative humidity. Five-day-old seedlings were scanned using a Phoenix v|tome|x M[®] 240 kV X-ray μCT system (Waygate Technologies [a Baker Hughes business], Wunstorf, Germany) at the Hounsfield Facility (University of Nottingham, Sutton Bonington Campus, UK). The scans consisted of the collection of 2520 projection images in FAST mode (continuous rotation), with an X-ray tube energy and current of 140 kV and 200 μA , respectively. The detector exposure time was 131 ms, and the voxel resolution was 57 microns. Scan time was 5 min. Reconstruction of these 2520 images were performed using a GE Datos rec software. Polyline measurement tool was used to trace the length of primary roots grown in $\pm$ compacted soil conditions in VGStudio MAX software V2.2 (Volume Graphics GmbH, Germany).

3.2.9. ABA treatment in the hydroponic system

Five-day-old equally germinated seedlings (*oseil1*, *osein2*, *mhz5-1* mutants and Nipponbare wild-type; *osaux1-1*, *osaux1-3* mutants and Dongjin wild-type) were transferred to a 2 L capacity beaker containing autoclaved RO water and 10 μM ABA (Sigma) and grown for 2 days in a growth chamber at 28°C with a 12-hour photoperiod at 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR and 70% relative humidity. After 48 h, primary root tips (1 cm) were harvested, washed with RO water, and placed in 4% PFA for 1 h. Subsequently, root tips were washed in 1x PBS buffer two times and thereafter cleared in ClearSee solution for 15 days.

Cleared root tips were then treated with 1% Calcofluor dye (Calcofluor White Stain, Sigma) and imaged using a confocal microscope (Leica, TCS-SP5), as described previously (Pandey, *et al.*, 2021b).

3.2.10. Ethylene treatment

Rice seeds (*oseil1*, *osein2*, *mh5-1* mutants and Nipponbare wild-type; *osaux1-1*, *osaux1-3* mutants and Dongjin wild-type) were surface sterilised for 10 minutes with 25% bleach (diluted from 19% CaOCl₂ stock), then washed five times with sterilised water. In a rice growth chamber (12-hour photoperiod, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 70% relative humidity), sterilised seeds were germinated on a moist sterilised filter paper in the dark for 2 days at 28°C.

Pre-germinated rice seedlings were transferred on a 10" x 15" moist (with sterilised water) germination paper (catalogue no. PG1015; Hoffman Manufacturing, Inc, Oregon, USA) and rolled. Then, 3-day-old equally germinated rice seedlings were transferred into a gas-tight jar GasPak Plus™ System (BBL-71040 Anaerobic System Envelopes with Palladium Catalyst; Becton Dickinson, Cockeysville, Maryland, USA) which was injected with a defined dose of ethylene (20 ppm) from ethylene (C₂H₄) gas cylinder (N.03-grade; minimum purity 99.9%) which obtained from BOC Ltd (Guildford, Surrey, UK) (Figure 3.1). And they were grown for 3 days in gas-tight jars at 28°C with a 12-hour photoperiod, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, and 70% relative humidity.

After three days of ethylene treatment, primary root tips (1 cm) were harvested and subsequently, ten root tips from the control and ethylene treatments were cleared in ClearSee solution for 15-20 days for confocal imaging of longitudinal sections. The remaining five root tips per treatment were imbedded in a 3.5% agarose block for radial sectioning and imaging with a Leica TCS-SP5 confocal microscope as described previously (Pandey, *et al.*, 2021b).

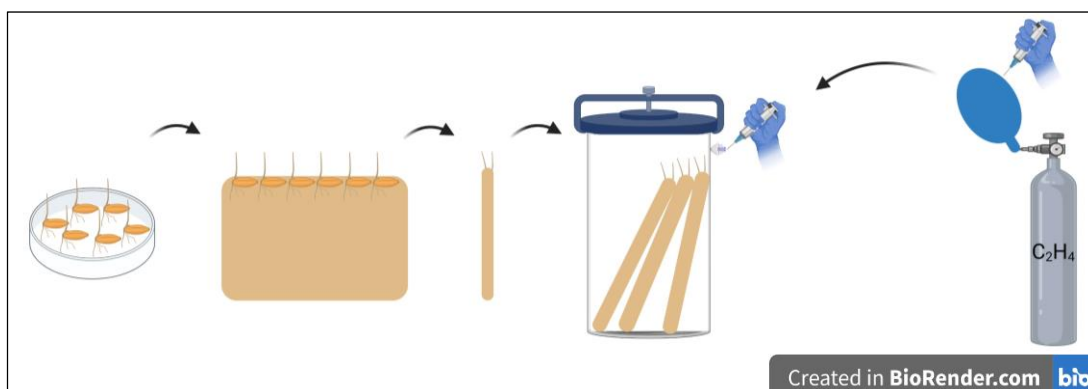


Figure 3.1. Schematic of ethylene-based (C_2H_4) root growth bioassay where germinated rice seedlings at equivalent stage of growth were transferred to a moist paper roll, then placed in a gas-tight jar which was injected with a defined dose of ethylene (20 ppm). This figure was created using BioRender.com.

3.2.11. Propidium iodide (PI) staining and cell length analysis

Seeds were germinated in the dark at $28^{\circ}C$ for 4 days, and germinated seedlings were grown in compacted and non-compacted soil for 4 days. Primary roots were harvested, cleaned with sterilised water, immersed in $1\ \mu M$ PI for 3 min, and imaged using Leica Laser Scan Microscope (TCS-SP5) at an excitation wavelength of 561 nm and emission wavelengths of 600–650 nm. Confocal images were analysed via ImageJ (<https://imagej.nih.gov/ij/>) (Schindelin *et al.*, 2012). Cell length analysis was performed as described previously (Pandey *et al.*, 2021a).

3.3. Results

3.3.1. Soil compaction induces ABA biosynthetic gene expression and hormone levels

Several hormone signals have been reported to function downstream of ethylene (Růžicka *et al.*, 2007; Swarup *et al.*, 2007; Qin and Huang, 2018; Jacobsen *et al.*, 2021) to inhibit root elongation, including auxin and ABA (Mulholland *et al.*, 1996; Li *et al.*, 2017; Miao *et al.*, 2021). However, the interaction of these hormones has not been studied in roots during growth in compacted soil. To address this, ABA levels were measured in rice primary root tip tissues harvested from plants grown in either non-compacted (1.1 g cm⁻³ bulk density [BD]) or compacted (1.6 g cm⁻³ BD) soil conditions. Root samples were then profiled using ultra-high-performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) (see methods). Hormone profiling revealed that ABA levels increased three-fold in root tips growing in compacted versus non-compacted conditions (Figure 3.2A).

To reveal why root tips grown in compacted soil have increased ABA levels, the effect of soil compaction on transcript abundances of nine ABA biosynthetic genes (*MHZ5*, *CRTISO*, *ABA1*, *ABA2*, *MHZ4*, *AAO1*, *AAO2*, *NCED2* and *ZEP*) was measured in root tip tissues (Figure 3.2B). Reverse transcription-quantitative PCR (RT-qPCR) analysis revealed that the majority (seven out of nine) of the ABA biosynthetic genes profiled were upregulated in wild-type root tips grown in compacted versus non-compacted soil conditions.

In contrast, none of the ABA biosynthetic genes were upregulated significantly in compacted soil conditions in the *mhz5-1* background (Figure 3.3). This effect is consistent with earlier observations on the impact of the *mhz5-1* mutation on the regulation of ABA biosynthetic genes under non-compacted conditions (Yin *et al.*, 2015).

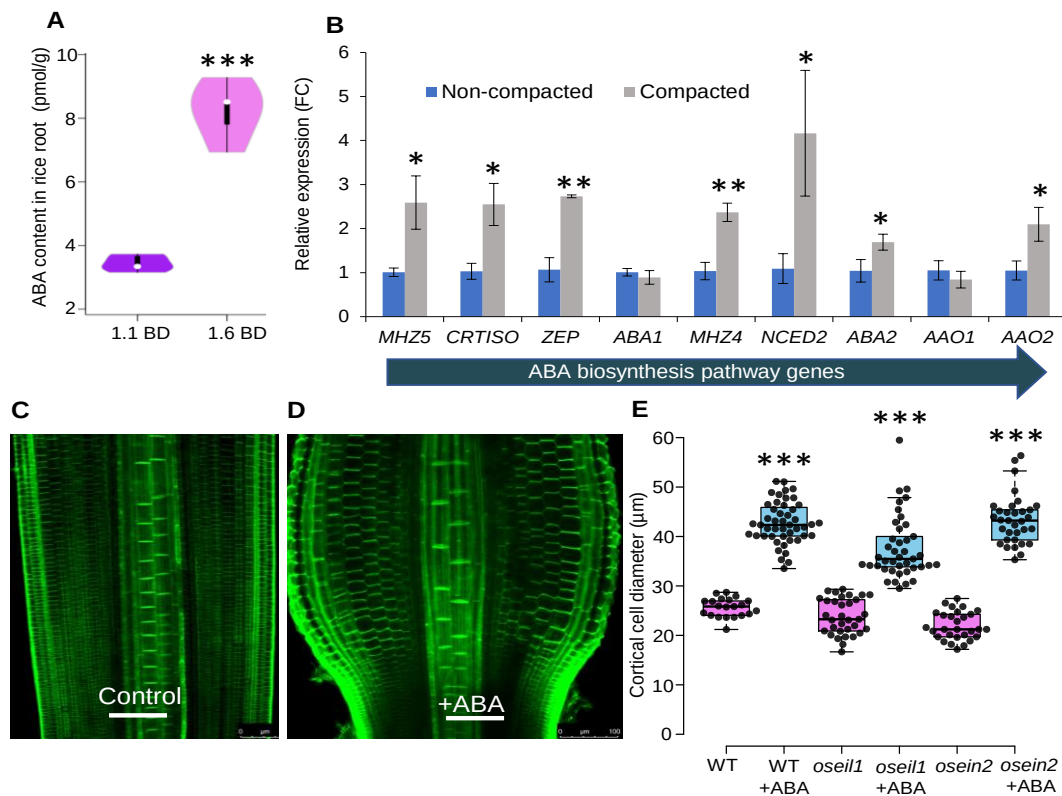


Figure 3.2. Soil compaction enhances ABA biosynthesis and radial expansion of rice primary root cortical cells. **(A)** ABA levels in root tips of wild-type (WT; cv. Nipponbare) grown in non-compacted (1.1 g cm⁻³ BD) and compacted (1.6 g cm⁻³ BD) soil in violin plots. **(B)** Bar graph showing relative expression (in fold change, FC) of ABA biosynthetic genes in root tips grown in compacted as compared to non-compacted soil, $n = 3$ replicates. Horizontal arrow shows the direction in the pathway of genes encoding within the ABA biosynthetic pathway in rice. **(C, D)** Representative images of control and ABA-treated root tips (elongation zone) showing a radial expansion of root cortical cells after ABA treatment (10 μM for 24 h); green fluorescence represents staining with 1% Calcofluor dye. Scale bars represent 100 μm. **(E)** Quantitative box plot showing the radial expansion (diameter of cortical cells) of ABA-treated root tips of wild-type (WT), *oseil1* and *osein2* mutants. Violin and box plots were generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014) **(A and E)**. *, ** and *** represent p -values of ≤ 0.05 , 0.01 and 0.001, respectively; calculated by Student's t -test between compacted and non-compacted treatments **(A, B)** and between genotypes with or without ABA treatment **(E)**.

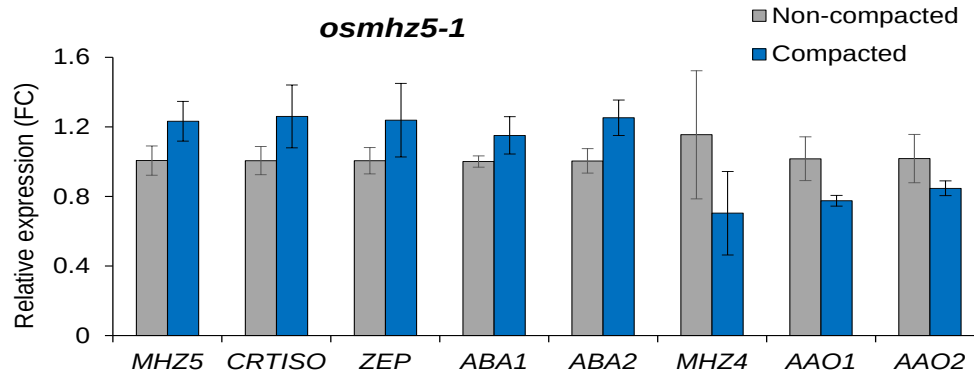


Figure 3.3. Compaction is unable to induce ABA biosynthetic genes in rice *mhz5-1* mutant primary root tips. Bar graph showing relative expression of ABA biosynthetic genes (*MHZ5*, *CRTISO*, *ZEP*, *ABA1*, *ABA2*, *MHZ4*, *AAO1* and *AAO2*) in *mhz5-1* mutant root tips (1 cm) under non-compacted (1.1 g cm⁻³ BD) and compacted (1.6 g cm⁻³ BD) soil conditions. The experiment was performed for three biological replicates, each replicate comprising ~10 root tips. Data are means $\pm$ SE.

3.3.2. ABA biosynthesis is upregulated by ethylene during a root compaction response

To examine the regulatory relationship between ethylene and ABA during root responses to compaction, wild-type (cv. Nipponbare) and ethylene-insensitive mutants *oseil1* and *osein2* (Ma *et al.*, 2013) were treated with either ethylene or ABA. Whilst roots of *oseil1* and *osein2* mutants did not respond to ethylene treatment, wild-type roots underwent a doubling in width largely due to the radial expansion of cortical cells (Figure 3.4A-G).

In contrast, ABA treatment (10 μ M for 24 h) caused cortical cells in all of these three rice lines to undergo radial expansion, mimicking the effect of ethylene on the wild-type (see Figure 3.2D versus control in Figure 3.2C for wild-type images, plus mutant images in Figure 3.5; cortical cell diameters $\pm$ ABA treatments are shown in Figure 3.2E). This observation suggested that ABA can trigger cortical cell radial expansion, and this signal acts downstream of the ethylene pathway in modulating root growth in response to compaction.

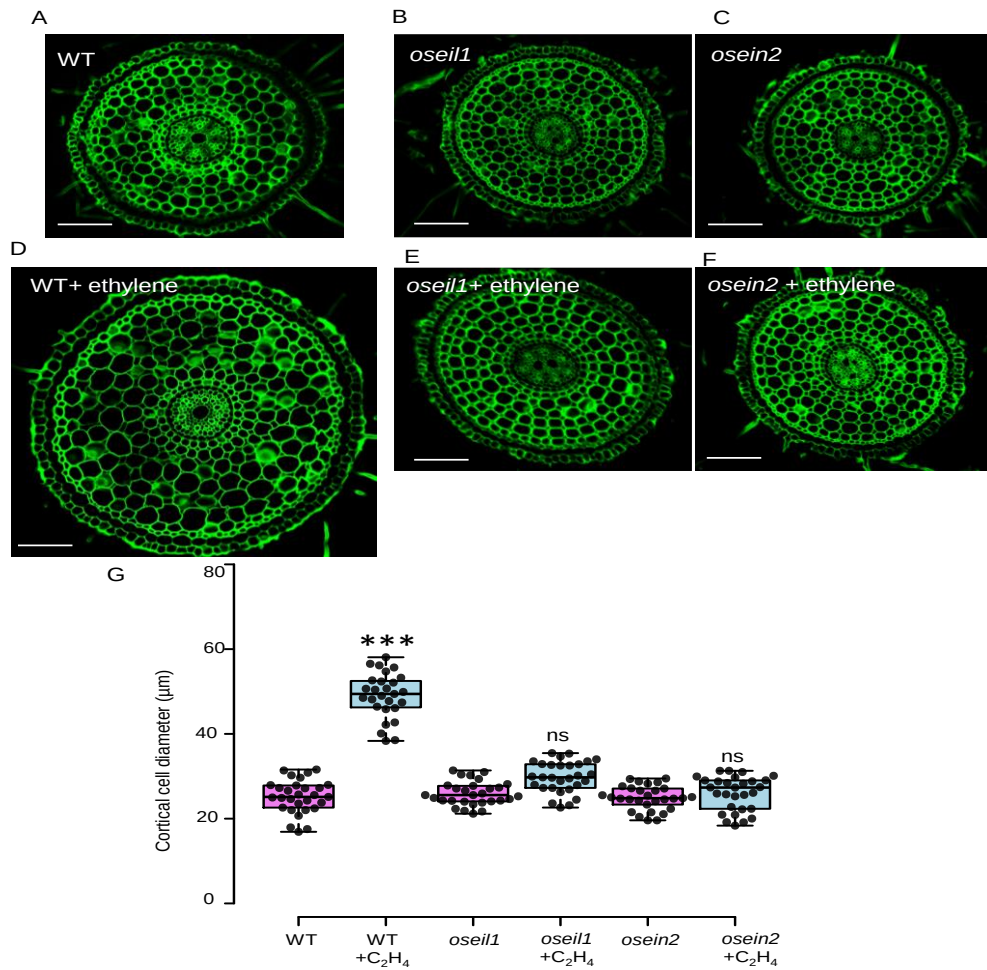


Figure 3.4. Ethylene promotes the radial expansion of wild-type rice root cortical cells. **(A-C)** Representative radial section images of wild-type (WT) and, *oseil1* and *osein2* rice mutants as controls. **(D-F)** Representative images of wild-type, *oseil1* and *osein2* after ethylene treatment (20 ppm for 3 days), showing radial expansion (diameter) of cortical cells in the wild-type but not the mutant root tips. Scale bars represent 100 μm in panels **A-F**. **(G)** Quantitative box plot showing the radial expansion (diameter) of control and ethylene-treated root tips of wild-type and, *oseil1* and *osein2* mutants. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). *** represents p -value ≤ 0.001 , calculated by Student's t -test. ns: non-significant.

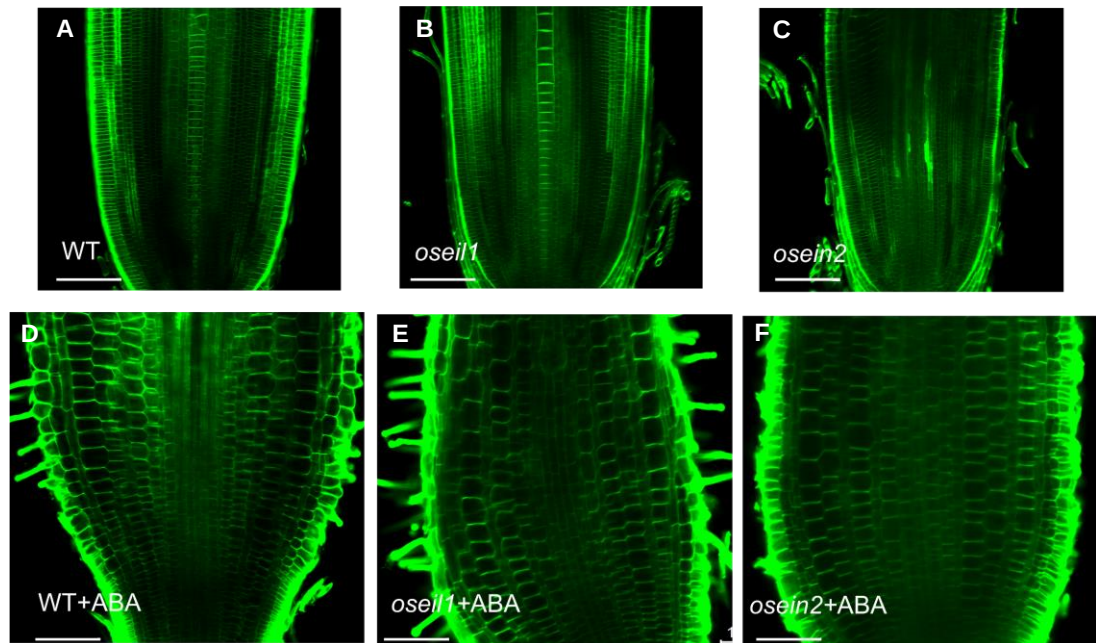


Figure 3.5. ABA acts as a downstream signal to ethylene signalling to promote the radial expansion of rice root cortical cells. **(A-C)** Representative images of the primary root tips of wild-type (WT) and, *oseil1* and *osein2* mutants as controls. **(D-F)** Representative images of wild-type and, *oseil1* and *osein2* after ABA treatment (10 μM for 24 h), showing the radial expansion of cortical cells in the root tips. Root tips were cleared using ClearSee and stained with 1% Calcofluor stain for confocal imaging. Scale bars represent 100 μm.

Next, to further confirm that ABA also acts downstream of ethylene in roots grown in compacted soil, the expression of ABA biosynthetic genes in wild-type rice root tips treated by ethylene was examined. The top ABA biosynthesis genes were selected to see the impact of ABA. This revealed that elevated transcript levels were detected for all three ABA biosynthesis genes profiled (*MHZ5*, *CRTISO* and *ZEP*; Figure 3.6), suggesting that enhanced ethylene response, triggered by soil compaction conditions (Pandey *et al.*, 2021b), promotes ABA biosynthesis.

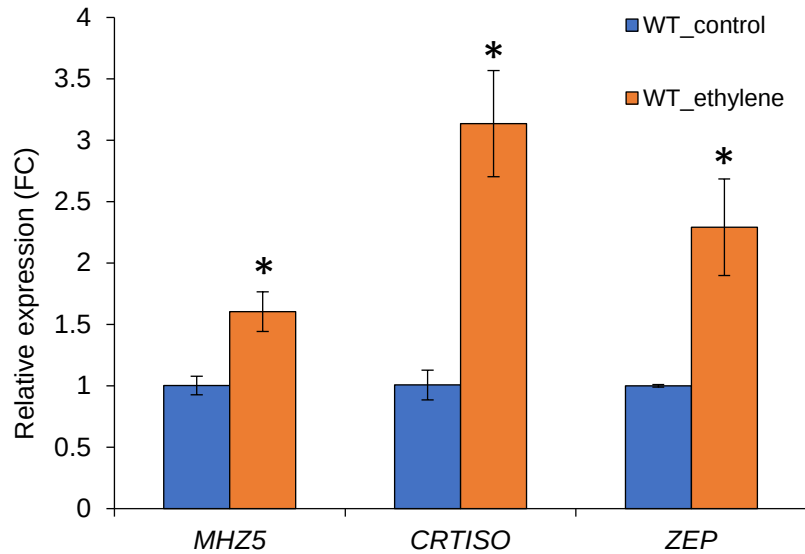


Figure 3.6. Ethylene promotes the induction of ABA biosynthesis genes in rice primary root tips. Bar graph showing relative expression (fold-change, FC) of ABA biosynthetic genes (*MHZ5*, *CRTISO* and *ZEP*) in root tips (1 cm) of wild-type (WT; cv. Nipponbare) after 20 ppm of ethylene treatment for 48 h compared with untreated controls. The experiment was performed for three biological replicates, each comprising ~10 root tips. Data are means $\pm$ SE. * represents p -value $\leq$ 0.05, calculated by Student's t -test.

3.3.3. ABA promotes the radial expansion of root cortical cells under compacted soil conditions

To determine whether ABA is essential for the radial expansion of root cortical cells in compacted soil conditions, the phenotypes of several rice ABA biosynthetic mutants were examined. *MHZ5-1* encodes an ethylene-inducible ABA biosynthetic enzyme (Figure 3.6) which, when mutated, displays an ethylene-insensitive root growth phenotype (Yin *et al.*, 2015). Wild-type and *mhz5-1* lines were grown in either non-compacted (1.1 g cm^{-3} BD) or compacted (1.6 g cm^{-3} BD) soil conditions, then confocal imaged (see 3.2.7). In contrast to the wild-type (Figure 3.7A and C), *mhz5-1* roots did not exhibit a significant increase in root diameter when grown in compacted soil (Figure 3.7B and D). Wild-type roots exhibited a doubling in cortical cell width under compacted soil conditions (Figure 3.7C and E), which was blocked in *mhz5-1* (Figure 3.7D and E).

Nevertheless, external treatment with ABA in a hydroponic system-induced radial expansion of cortical cells in *mhz5-1* (and wild-type) roots more than two-fold (Figure 3.7F-J) confirms that the *mhz5-1* remains sensitive to ABA. In conclusion, ABA biosynthesis is required to promote root radial expansion in response to soil compaction conditions (Figure 3.7J).

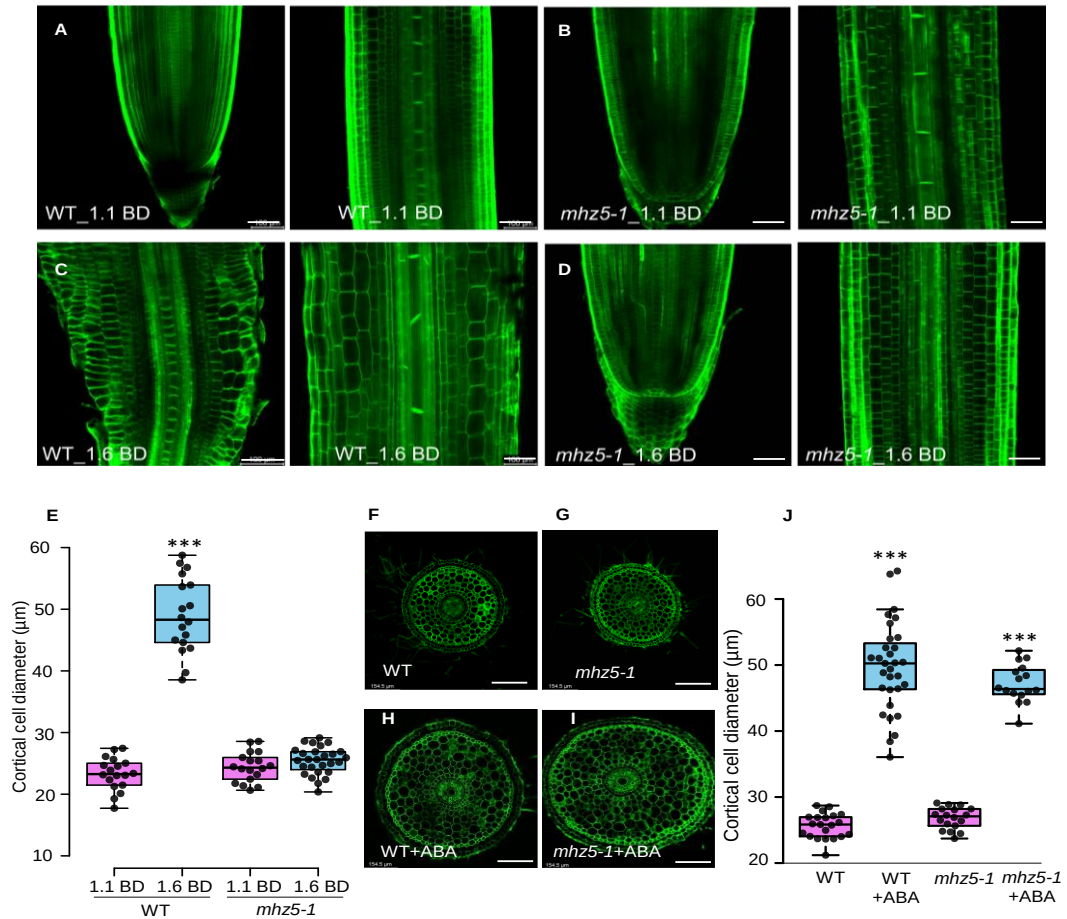


Figure 3.7. ABA promotes the radial expansion of rice primary root cortical cells in compacted soil. (**A-D**) Representative images of wild-type (WT) and *mhz5-1* (ABA-deficient mutant) root tips (meristematic and elongation zones) showing cortical cells in (**A, B**) non-compacted soil (1.1 g cm⁻³ BD) as compared with (**C, D**) compacted soil (1.6 g cm⁻³ BD). Scale bars = 100 μm (**E**) Boxplot showing the radial expansion of root cortical cells in wild-type and *mhz5-1* root tips (elongation zone) in non-compacted and compacted soils. (**F-I**) Representative radial sections of wild-type and *mhz5-1* root tips were grown under control (**F, G**) and 10 μM ABA (**H, I**) treatments for 48 h. Scale bars = 154.5 μm. (**J**) Boxplot of wild-type and *mhz5-1* root tips showing the radial expansion of root cortical cells after ABA treatment. All experiments were repeated three times. Box plots were generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). *** represents *p*-value of ≤ 0.0001, calculated by Student's *t*-test between non-compacted and compacted treatments (**E**) and between control and ABA treatments (**J**).

3.3.4. ABA biosynthetic mutants exhibit the increased root penetration capability in compacted soil

Next, the impact of disrupting ABA biosynthesis on root penetration in compaction soil was studied. To examine this, non-invasive X-ray micro-CT imaging (Pandey *et al.*, 2021a) of wild-type and ABA biosynthetic mutant *mhz5-1* roots grown in non-compacted and compacted soil conditions (Figure 3.8A-D). CT imaging in compacted soil revealed that *mhz5-1* root elongation was much less inhibited (~15% reduction) compared to the wild-type (~40% reduction) (Figure 3.8K). Hence, cortical cell swelling in response to soil compaction does not appear to aid rice root penetration capacity. Instead, ABA-dependent root swelling appears to reduce rice root elongation when exposed to compacted soil.

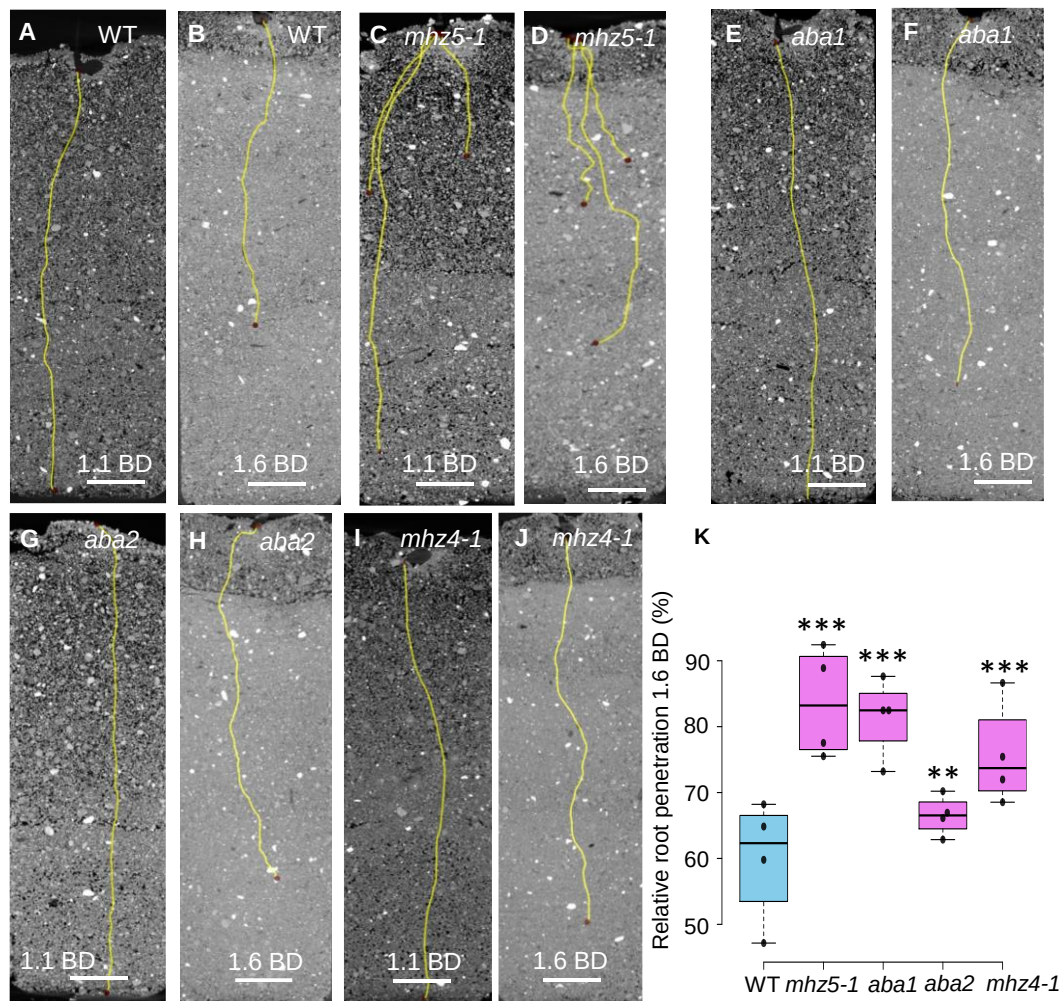


Figure 3.8. ABA-deficient mutants have better root penetration ability than wild-type rice in compacted soil. Representative CT (Computed Tomography) images of primary root length in (A, B) wild-type (WT; cv. Nipponbare) as compared with (C, D) *mhz5-1*, (E, F) *osaba1*, (G, H) *osaba2* and (I, J) *mhz4-1* ABA-deficient mutants in non-compacted (1.1 g cm⁻³ BD) and compacted (1.6 g cm⁻³ BD) soil. (K) Box plot showing root penetration ability of wild-type and, *mhz5-1*, *osaba1*, *osaba2* and *mhz4-1* mutants in compacted soil expressed as % of root growth in non-compacted soil. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). For CT imaging, four replicates were imaged, and the experiment was repeated twice. ** and *** represent *p*-values of ≤ 0.01 and 0.001 , respectively; calculated by Student's *t*-test. Scale bars represent 10 mm in panels A-J.

To independently test this hypothesis, the root penetration ability of several other rice ABA biosynthetic mutants was examined (Figure 3.9A) (Agrawal *et al.*, 2001; Ma *et al.*, 2014; Liao *et al.*, 2018). The *osaba1* mutant is unable to catalyse the oxidation of zeaxanthin to produce trans-violaxanthin and has much less ABA than the wild-type (Agrawal *et al.*, 2001), while the *osaba2* mutant is unable to produce abscisic aldehyde that is converted to ABA by AAO (abscisic aldehyde oxidase) (Liao *et al.*, 2018). The *mhz4* mutant, which also contains less ABA (61% of wild-type), is homologous to the *Arabidopsis aba4* mutant (Ma *et al.*, 2014). *Arabidopsis ABA4* encodes neoxanthin synthase, which converts trans-violaxanthin to neo-violaxanthin, and the *aba4* mutant does not accumulate ABA under stress (North *et al.*, 2007). CT imaging revealed that all the ABA biosynthetic mutants examined (*osaba1*, *osaba2*, *mhz4* and *mhz5-1*) exhibited higher root penetration ability than the wild-type in compacted soil (Figure 3.8C-K). Hence, rice mutant analysis confirmed that ABA functions to reduce root elongation and increase the penetration ability when exposed to compacted soil conditions.

To further clarify the role of ABA in regulating root penetration activity, ABA levels were quantified in root tips of the ABA biosynthetic mutants were grown in non-compacted (1.1 g cm^{-3} BD) and compacted soil (1.6 g cm^{-3} BD) conditions. UHPLC-MS/MS analysis revealed that, in contrast to wild-type, compacted soil conditions did not induce significantly higher ABA levels in the mutants (Figure 3.9B). ABA biosynthetic mutants also exhibited less radial expansion of cortical cells than the wild-type in compacted soil conditions (Figure 3.10A-H). By comparing ABA accumulation (Figure 3.9B) and radial expansion (Figure 3.10A-I) datasets, higher ABA levels appear to correlate with greater radial expansion of cortical cells in compacted soil conditions.

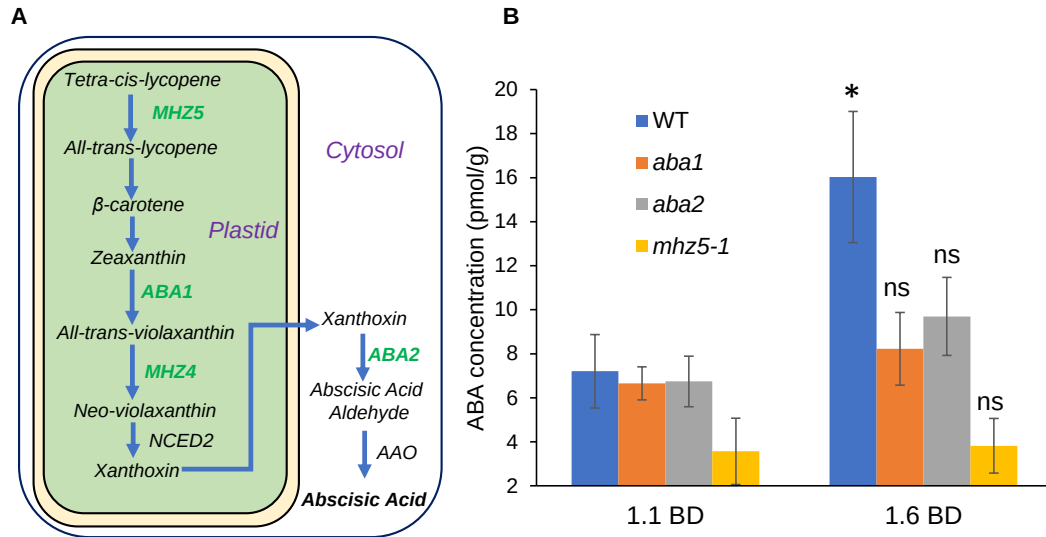


Figure 3.9. Compaction is unable to induce increased ABA levels in primary root tips of rice ABA biosynthetic mutants. **(A)** Schematic representation of ABA biosynthesis pathway in rice roots showing the position of enzyme-encoding genes. Loss-of-function mutants of ABA biosynthetic genes used in this study are highlighted in green font. **(B)** Bar graph showing ABA level (pmol g⁻¹ fresh weight) in root tips of wild-type (WT; cv. Nipponbare) and ABA biosynthetic mutants *aba1*, *aba2* and *mhz5-1* in non-compacted (1.1 g cm⁻³ BD) and compacted (1.6 g cm⁻³ BD) soil conditions. 3-5 replicate samples were used for ABA quantification. Each replicate (10 mg fresh weight) consisted of ~6 root tips (1 cm) in non-compacted soil and ~4 root tips in compacted soil. * represents p -value ≤ 0.05 , calculated by Student's t -test comparing genotypes between respective bulk density levels. ns: non-significant.

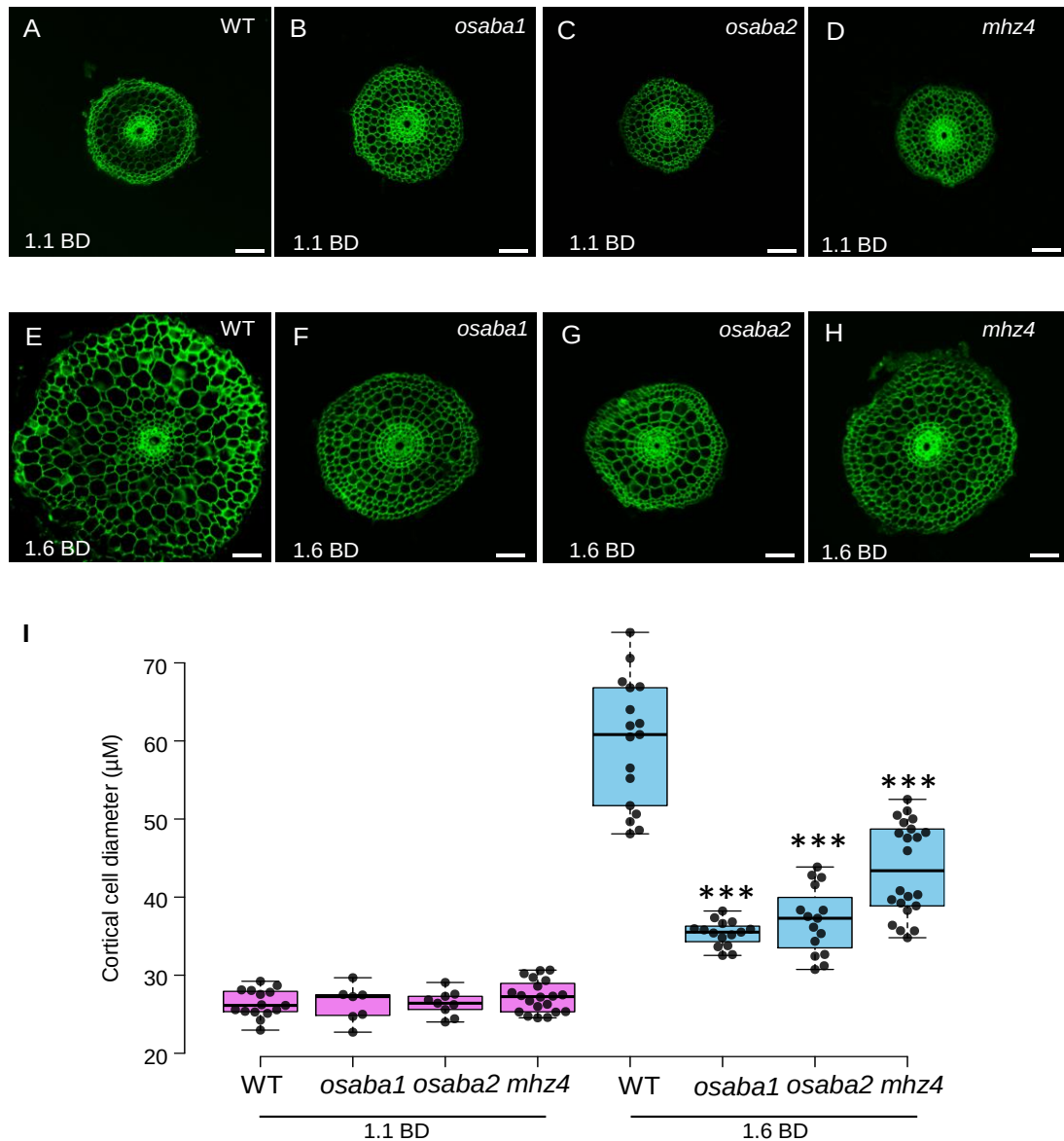


Figure 3.10. Rice ABA biosynthesis mutants exhibit a less radial expansion of primary root tip cortical cells in compacted soil. Representative radial section images of primary root tips of wild-type (WT) and, *osaba1*, *osaba2* and *mhz4* mutants in (A-D) non-compacted (1.1 g cm⁻³ BD) and (E-H) compacted (1.6 g cm⁻³ BD) soil. Scale bars represent 100 μm. (I) Quantitative box plot showing the radial expansion (diameter) of root cortical cells in primary root tips of wild-type and, *osaba1*, *osaba2* and *mhz4* mutants in non-compacted and compacted soil. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). *** represents *p*-value ≤ 0.0001, calculated by Student's *t*-test between genotypes in compacted compared with non-compacted conditions.

3.3.5. Ethylene regulates epidermal and cortical cell expansion via auxin and ABA in a sequential fashion

Huang *et al.*, (2022) describe the importance of auxin as a signal functioning directly downstream of the ethylene response pathway during a rice root compaction response. The authors report that transcription factor OsEIL1 binds directly to the promoter of the auxin biosynthesis gene *OsYUC8* to upregulate its expression which, acting in conjunction with auxin influx carrier *OsAUX1*, is required to trigger the inhibition of root elongation during compaction. Hence, ABA could be regulated indirectly by ethylene via auxin. Consistent with this possibility, a loss-of-function mutation in *OsAUX1* was able to disrupt ethylene induction of key ABA biosynthesis genes (Figure 3.12). Moreover, (unlike ethylene) ABA treatment was able to promote the radial expansion of cortical cells in *osaux1* mutant roots (Figure 3.11). However, ABA did not fully restore *osaux1* radial expansion to the wild-type (cv. Dongjin) level was showed, suggesting that auxin (delivered via *OsAUX1*) is required to modify root epidermal properties to enable cortical cells to expand radially in response to ABA.

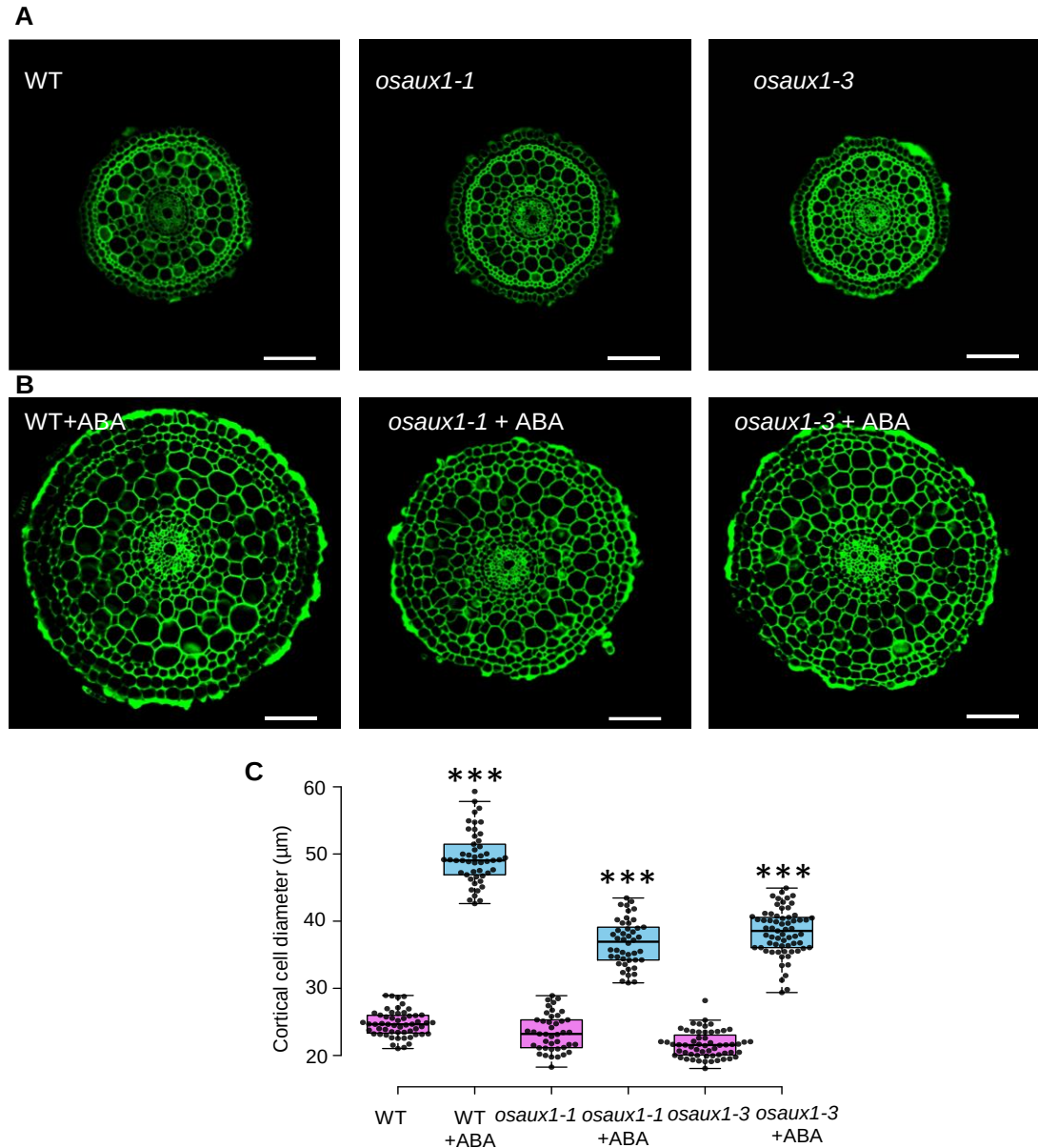


Figure 3.11. ABA acts as a downstream signal to auxin to promote the radial expansion of rice primary root cortical cells. **(A)** Representative radial section images of wild-type (WT; cv. Dongjin) and, *osaux1-1* and *osaux1-3* mutants as controls. **(B)** Representative images of wild-type and *osaux1-1* and *osaux1-3* mutants after ABA treatment (10 μM for 24 h) showing the radial expansion of cortical cells in the root tips. Scale bars represent 100 μm in panels **A-B**. **(C)** Quantitative box plot showing cortical cell diameters with and without ABA treatment in the root tips of wild-type and, *osaux1-1* and *osaux1-3* mutants. Box plot was generated using BoxPlotR (<https://shiny.chemgrid.org/boxplotr/>) (Spitzer *et al.*, 2014). *** represents p -value of ≤ 0.001 , calculated by Student's t -test.

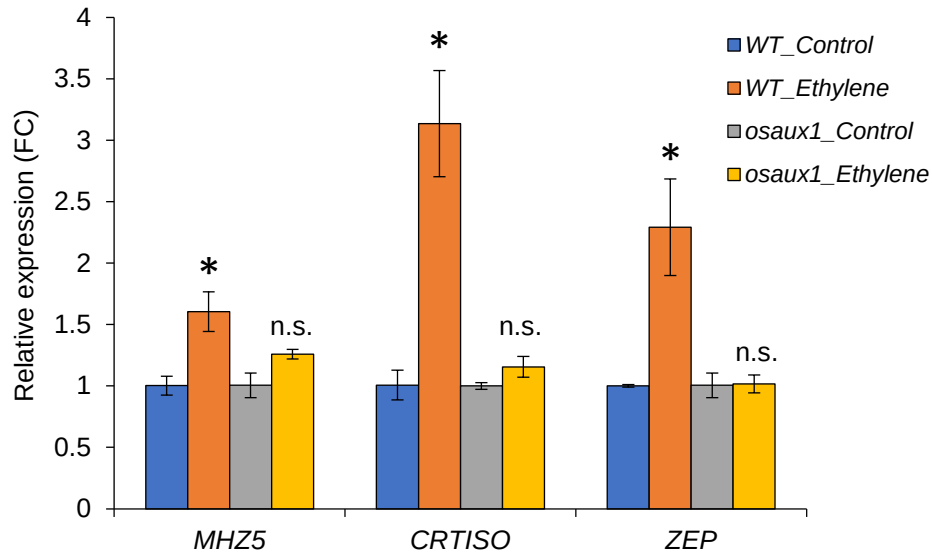


Figure 3.12. Ethylene is unable to promote induction of ABA biosynthetic genes in the *osaux1* mutant primary root. Bar graph showing relative expression (fold-change, FC) of ABA biosynthetic genes (*MHZ5*, *CRTISO* and *ZEP*) in root tips (1 cm) of wild-type (WT; cv. Dongjin) and ethylene-insensitive *osaux1* mutant after treatment with 20 ppm ethylene for 48 h. Relative expression was calculated with respect to untreated wild-type roots. The experiment was performed in three biological replicates, each comprising ~10 root tips per genotype and treatment. * represents p -value ≤ 0.05 , calculated by Student's t -test. n.s.: non-significant.

3.4. Discussion

This chapter reports that rice roots employ a hormone signal (i.e., ABA) distinct from ethylene to promote one of the key root responses to soil compaction, cortical cell radial expansion (summarized in Figure 3.13). Profiling levels of ABA biosynthesis transcripts and the hormone signal itself revealed that soil compaction stress led to a significant upregulation of their mRNAs and ABA. This upregulation of ABA biosynthesis does not appear to be a direct consequence of the elevated ethylene response in roots exposed to soil compaction. Huang *et al.*, (2022) report that ChIP-PCR assays did not detect OsEIL1-GFP binding to the promoter of the ethylene-inducible ABA biosynthesis *MHZ5* gene. Hence, the ethylene response pathway appears to upregulate ABA biosynthesis in root tips indirectly during compacted soil conditions.

Soil compaction stress may enhance root ABA levels (in addition to induction by ethylene) by decreasing soil water availability. Although soil pores can be filled with water molecules in compacted soil conditions, the water is less accessible to roots as it is tightly held (by greater matric forces) in smaller pores in compacted soil than in larger pores in non-compacted soil, making it more difficult for plant roots to extract (Bulmer and Simpson, 2005). Indeed, soil water release curves indicate lower water potential (moisture is less accessible to the plant) of compacted compared with non-compacted soils at the same soil water content (Smith *et al.*, 2001). Thus, small void size and lower water potential render the water molecules less accessible for roots in compacted soils, which is likely to mimic mild drying conditions and therefore can enhance ABA levels in root tip tissues.

Irrespective of the mode(s) of ABA induction during compaction, this hormone signal appears essential for rice root cortical cell radial expansion. ABA biosynthesis mutants containing lower levels of this hormone exhibit a reduction in radial swelling of root cortical cells when growing in compacted soil. Increased synthesis of ABA in response to compaction stress causes wild-type root tips to swell.

Classically, root tip swelling was reasoned to facilitate root penetration of compacted soil by widening the pores in the soil (Correa *et al.*, 2019). However, root tip swelling is attenuated in rice ethylene and ABA-mutants that exhibit improved penetration abilities in compacted soil (Pandey *et al.*, 2021b; Figure 3.8). This suggests that roots that remain narrower are better able to penetrate compacted soil. Conversely, wild-type rice roots that swell in compacted soil appear less able to penetrate. The induction of root tip swelling by ethylene (via ABA) in compacted soil may serve to aid a localised slowing down of root growth, enabling plant resources to be diverted to other parts of the plant's root system that may be experiencing less-compacted soil conditions.

ABA appears to act downstream of ethylene and auxin signalling during rice root compaction responses. A sequential regulatory cascade of these hormone signals appears to aid changes in root growth at both the cellular and tissue scales. At the cell scale, coordinating ethylene, auxin and ABA responses during a soil compaction response ensures ABA-mediated radial cell expansion occurs after auxin (and ethylene) inhibition of cell elongation (Huang *et al.*, 2022). At the tissue scale, cortical cells cannot expand radially in response to ABA without modification of outer epidermal cells by auxin. This sequential regulatory behaviour appears to be mediated through ethylene response pathway component EIL1 binding auxin biosynthesis gene YUC8 directly, whereas EIL1 controls ABA biosynthesis gene expression indirectly (as discussed above and schematised in Figure 3.13).

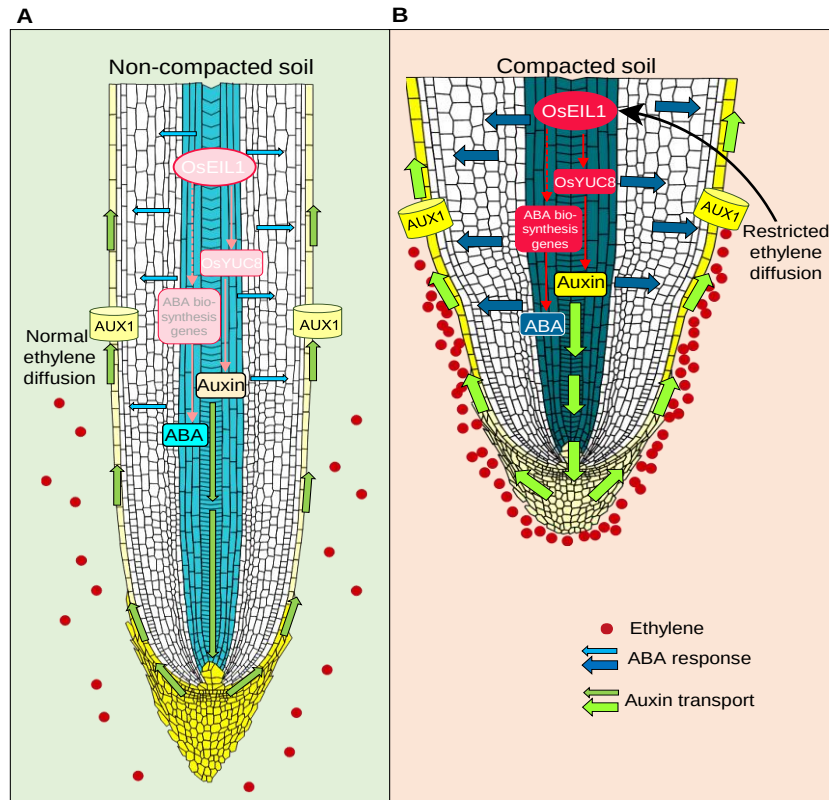


Figure 3.13. Ethylene orchestrates induction of both auxin and ABA biosynthesis to regulate inhibition of elongation and promotion of the radial expansion of rice roots in compacted soil. Schematic representation of root responses in compacted soil vs. non-compacted soil. Compacted soil conditions promote higher ethylene response due to restricted diffusion of ethylene in smaller air-filled soil pores. Enhanced ethylene response promotes *OsEIL1* activation, which directly upregulates auxin biosynthesis through *OsYUC8*. Higher auxin response in epidermal cells of meristematic and elongation zones inhibits epidermal cell elongation (and thus root elongation). In parallel, a higher ethylene response promotes (*indirectly*) higher ABA biosynthesis in vascular tissues causing radial expansion of root cortical cells, thereby also contributing to the inhibition of root elongation in compacted soil conditions. Red dots are gaseous ethylene molecules, red ellipse indicates enhanced ethylene response in compacted soil conditions. Intensity of colour (light to darker) and width of the arrow indicate a higher response/level for auxin (yellow) and ABA (sky blue to royal blue). Auxin transport is denoted by green arrows. This figure was generated using Adobe Illustrator CC 2015 software (Adobe, San Jose, California, USA).

4. Chapter 4. Investigating the relationship between suberin biosynthesis and lateral root development under environmental stress conditions

4.1. Introduction

Roots play a key role in foraging for resources such as nutrients and water in soil (de Kroon, 2007; Morris *et al.*, 2017). Lateral roots are critical for increasing total root surface area by >99%. Once lateral roots emerge into the soil, the new organ is often exposed to environmental stresses such as water and nutrient limitations. Few studies have addressed the post-emergence stage of lateral roots and their adaptive responses, largely focusing instead on the regulation of organ initiation and emergence (Swarup *et al.*, 2008; Bao *et al.*, 2014; Orman-Ligeza *et al.*, 2018). Schneider *et al.*, (2020) recently reported that when maize plants were exposed to water deficit, their lateral roots became longer. Genome-wide association studies (GWAS) were utilised to uncover several loci regulating lateral root length using detailed field phenotypic analysis of >450 genotypes in a maize diversity panel. One of the top gene candidates encoded a very-long-chain fatty acid enzyme which is involved in several processes including suberin synthesis and lateral root development (Trinh *et al.*, 2019).

Suberin is an apoplastic biopolymer deposited in specialised plant tissues during secondary cell wall differentiation. The cork oak tree, *Quercus suber*, has the most well-known occurrence of suberin in its periderm. More than half of the suberin is found in this tissue located at the plant-environment interface (Graça and Pereira, 2000). Suberin is a cell wall polymer that is highly persistent and primarily composed of long-chain hydroxylated fatty acids. It generates a lipophilic barrier in roots as part of the Casparian strip that gates the apoplastic flow of ions, gases, and water (Franke *et al.*, 2012). Despite being physiologically important, knowledge about suberin formation has only increased in the past decade, largely through studies in *Arabidopsis* (Höfer *et al.*, 2008).

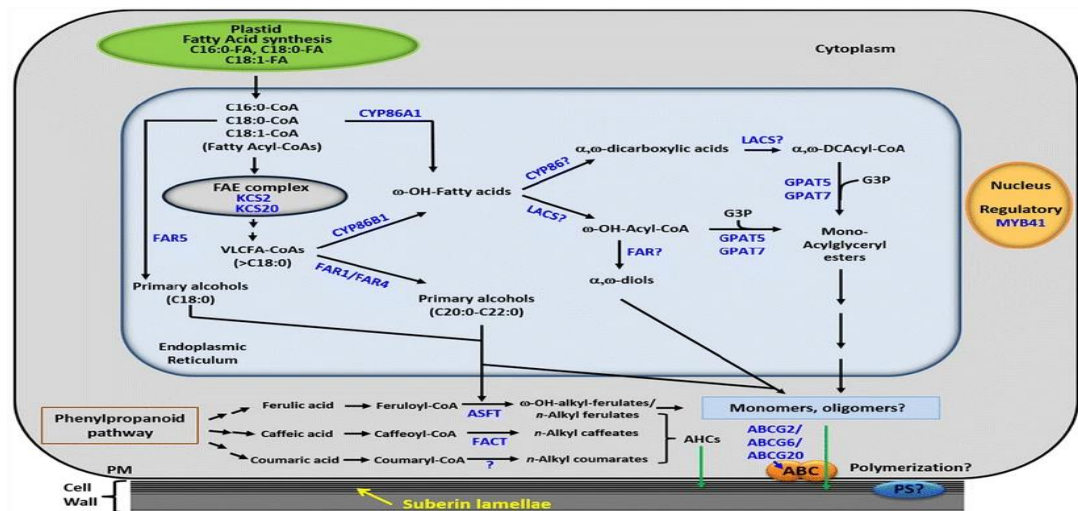


Figure 4.1. A schematic of the suberin biosynthetic pathway. Blue colours indicate *Arabidopsis thaliana* protein names (adapted from Vishwanath *et al.*, 2015).

Characterisation of suberin in *Arabidopsis* has identified several genes required for its biosynthesis including for fatty acid hydroxylation cytochrome P450 oxygenases (CYP) and fatty acid elongation 3-ketoacyl-CoA synthases (KCS). *KCS2*, *CYP86A1*, and *CYP86B1* appear to be particularly important for suberin synthesis. For instance, the isolation of *Arabidopsis* mutants with low suberin composition resulted in the identification of the *cyp86a1* mutant which increased root permeability for water and solutes, leading to a two-fold increase in mineral ion transport and uptake (Franke *et al.*, 2012; Vishwanath *et al.*, 2015).

Suberin deficient roots have also been created using a transgenic approach. Naseer *et al.*, (2012) used an endodermis-specific promoter to express CUTICLE DESTRUCTION FACTOR 1 (CDEF1), a plant-encoded cutinase (Takahashi *et al.*, 2010; Ursache *et al.*, 2018), to prevent the accumulation of suberin. This line was critical in assessing suberin's physiological roles in roots (Naseer *et al.*, 2012; Barberon *et al.*, 2016; Li *et al.*, 2017; Andersen *et al.*, 2018; Salas-González *et al.*, 2021). Despite a severe disruption in suberin accumulation, apoplastic gating remained intact. Hence, suberin lamellae are not required for the formation of an apoplastic barrier and form after Casparian strips (CSs), as previously described by (Alassimone *et al.*, 2010; Naseer *et al.*, 2012; Barberon, 2017).

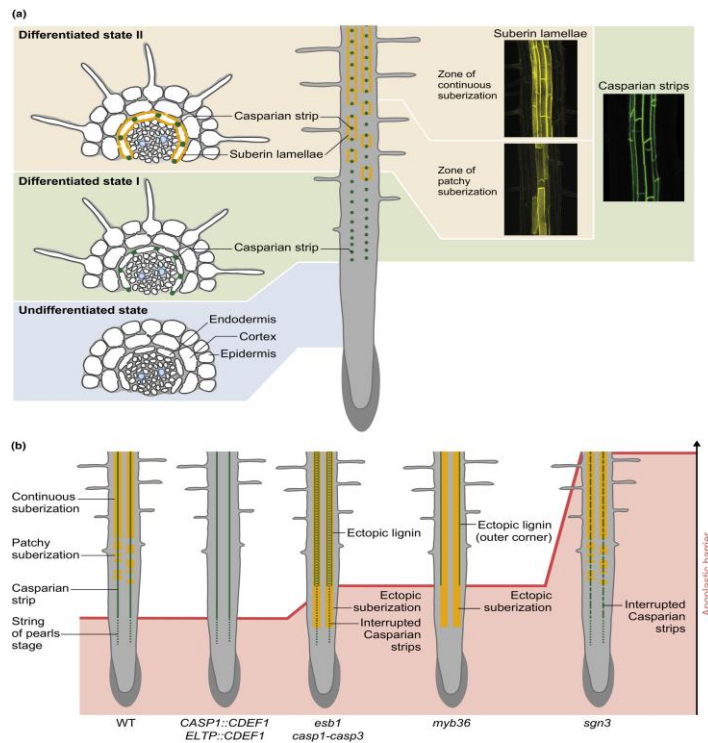


Figure 4.2. Differentiation of the endodermis. **(a)** Endodermis differentiation is depicted in schematic form. **(b)** Endodermis differentiation in various Arabidopsis genotypes is depicted schematically (adapted from Barberon, 2017).

By studying Casparian strip mutants, Li *et al.*, (2017) discovered a novel function for suberin in the regulation of nutrient transport in lateral roots.

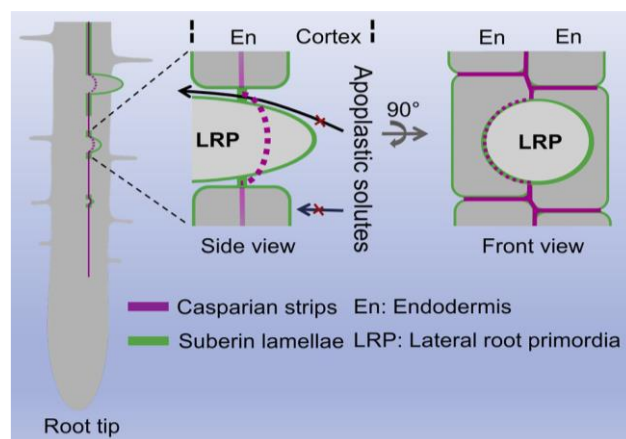


Figure 4.3. The function of suberin in nutrient transport via the spatial organization of root endodermal barriers (adapted from Li *et al.*, 2017).

In recent years, the cellular and molecular basis of lateral root growth in the model plant *Arabidopsis thaliana* has been extensively studied. Many genes have been identified that control lateral root emergence, patterning, and initiation process. Fatty acid elongation and synthesis processes are associated in one candidate gene related with the phenotypic expression of Average Lateral Root Length (LL) under water-stress conditions (Zm00001d021200). *KCS4*, a 3-ketoacyl-CoA synthase family member involved in the biosynthesis of very-long-chain fatty acid (VLCFA), is encoded by the Arabidopsis orthologue of this gene. Additionally, VLCFA-containing lipids are shown to be essential in cell plate formation during cytokinesis. Mutants with VLCFA depletion show delayed cell plate formation and a 50% reduction in root growth (Bach *et al.*, 2011). Based on the experimental evidence in Arabidopsis, the orthologue maize gene potentially has functional relevance to the LL trait.

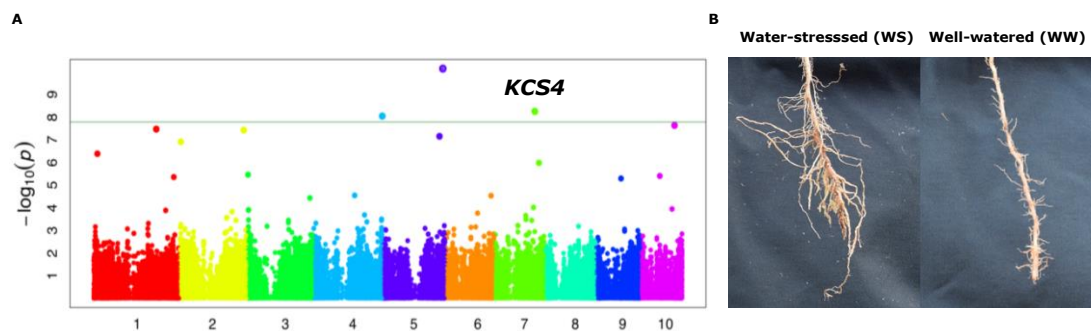


Figure 4.4. GWAS results for Average Lateral Root Length (LL) for maize plants grown in water-stress conditions in Arizona, USA. **(A)** Zm00001d021200 (rs7_145766958) Arabidopsis orthologue of *KCS4*. **(B)** Representative images of maize lateral roots on a crown root under water-stressed (WS) and well-watered (WW) conditions. These figures were retrieved from rootplasticitygwas.nottingham.ac.uk/ (Schneider *et al.*, 2020).

KCS4 was predominantly expressed in developing embryos, germinated and mature pollen grains, leaf veins and, apical meristems of roots and shoots. In *kcs4* complement lines, *KCS4* promoter-driven *KCS4* expression restored the growth-retarded phenotype to the wild-type (WT; Col-0), whereas *KCS4* disruption inhibited root growth and pollen tube elongation (Figure 4.5) (Kim *et al.*, 2021).

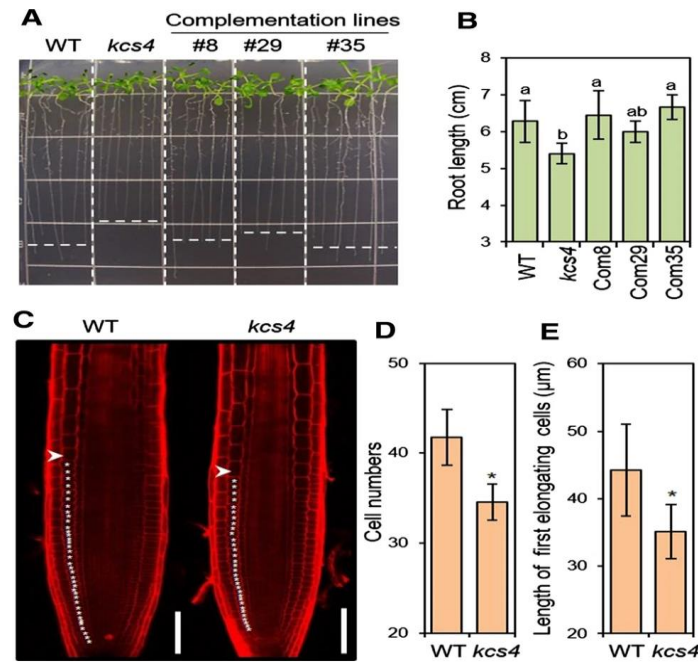


Figure 4.5. Three *kcs4* complementation lines, *kcs4*, and wild-type (WT; Col-0) were tested for their root growth. Seedlings of three *kcs4* complementation lines, *kcs4*, and wild-type (WT; Col-0) were imaged using a Leica light microscope. $n = 60$ (A) and (B). Cortical cell number and length in *kcs4* and wild-type (WT; Col-0) root meristem zones (C–E). (C) PI staining of the root meristem zone in 4-day-old *kcs4* and wild-type (WT; Col-0). Arrows and white stars indicate the first elongated cortical cells and cortical cells, respectively. The graphs in (D) and (E) show statistical analyses of the cell numbers and length of first elongating cells in the roots of *kcs4*, and wild-type (WT; Col-0). Scale bars represent 75 μm (adapted from Kim *et al.*, 2021).

Water stress regulates several root adaptive responses including compaction (focus of Chapter 3). In Chapter 4, I investigate how water stress controls lateral root outgrowth. Lateral root branching and length play essential roles during adaptation to water stress. To identify potential regulators of this adaptive process, GWAS predictions on a root phenotype dataset generated for a maize diversity panel subjected to water stress were analysed. One promising loci predicted by GWAS encoded a suberin synthesis gene *KCS4*. I explore its role in this adaptive response. Loss-of-function *kcs4* mutants exhibited significantly higher lateral root (LR) length compared to wild-type (WT; Col-0). Moreover, *kcs4* roots display a similar longer lateral root phenotype as suberin degradation line *pCASP1::CDEF*, suggesting there is a link between root surface area and suberin-based water-impermeable barrier formation in roots.

Spatial expression showed that *KCS4* is endodermis and xylem pole pericycle (XPP)-specific. Arabidopsis plants expressing XPP-specific *pXPP::KCS4::GFP* and endodermal-specific *pCASP1::KCS4::GFP* were created using GreenGate cloning. The relationship between suberin biosynthesis and lateral root development under environmental stress conditions was investigated.

4.2. Materials and methods

4.2.1. Plant materials

Arabidopsis *kcs4* (encoding *KCS4*; TAIR id: AT1G19440) SALK T-DNA (SALK_095739) mutant line (ecotype Col-0) was used in this study. In the SALK collection (Alonso *et al.*, 2003), only one homozygous T-DNA insertion line for *KCS4* was available, and it was obtained from The Nottingham Arabidopsis Stock Centre (NASC) (Scholl *et al.*, 2000).

4.2.2. Growth conditions and methods

Arabidopsis seeds were surface sterilised for 5 minutes with 20% bleach (diluted from 19% CaOCl₂ stock), and 1 minute with diluted triton and then rinsed five times with sterile water. Arabidopsis growth chamber (maintained at 21°C, 12-hour photoperiod, 300 µmol m⁻² s⁻¹ PAR, 70% relative humidity) was used to grow all the Arabidopsis seedlings.

Arabidopsis seedlings were vertically grown on ½ Murashige and Skoog Basal Salt Mixture (MS) (catalogue no. M5524; Sigma-Aldrich) (Murashige and Skoog, 1962), pH = 5.7 and 1% (10 g/L) bacto agar (Scientific Laboratory Supplies, Nottingham, UK). In a row of 3 cm from the top of 245 mm × 245 mm × 25 mm square plate, ten sterilised Arabidopsis seeds were transferred to each MS media plate, and then, they were sealed with a 3M surgical tape. The germination date was recorded for each Arabidopsis seed.

4.2.3. Molecular works

4.2.3.1. DNA extraction

In liquid nitrogen, ~100 mg of Arabidopsis leaves were ground into a fine powder and DNA extraction was performed using ISOLATE II Plant DNA Mini Kit (Bioline, London, UK) and finally, DNA was stored at -20°C.

4.2.3.2. RNA extraction

Total RNA was isolated from primary root tips of 10-day-old plants using TRIzol® reagent (Applied Biosystems). Ten-day-old Arabidopsis roots were powdered in liquid nitrogen with 500 µl of TRIzol, transferred to a 1.5 ml Eppendorf tube, vortexed, and left for 5 min at room temperature (25°C). Following the addition of TRIzol, 200 µl chloroform was added to the same Eppendorf tube, which was then spun at maximum speed (13000 rpm) for 5 min at 4°C. A total of ~500 µl of supernatant was collected and vigorously vortexed in a new tube containing 250 µl of ethanol.

The samples were kept at room temperature (25°C) for more than 10 minutes before being centrifuged at 10000 rpm for 15 sec using the RNeasy pink column (Qiagen, Hilden, Germany). After adding 350 µl RW1 buffer to RNeasy and centrifuging at 10000 rpm for 15 sec, the flow-through was discarded. Then, 10 µl of DNase I stock solution was added to 70 µl of Buffer RDD, which was gently mixed and centrifuged for a few sec. DNase I incubation mix (80 µl) was directly applied to the RNeasy column membrane and allowed to sit for 15 min on the benchtop.

Then, 350 µl of Buffer RW1 was added to an RNeasy column and centrifuged for 15 sec at 10000 rpm. After filling the column with 500 µl of Buffer RPE and spinning it for 15 sec at 10000 rpm, the flow-through was discarded. The column was refilled with 500 µl of Buffer RPE, centrifuged at 10000 rpm for 2 min, and the collection tube was discarded. The spin-column was then placed in a new 2 ml collection tube and centrifuged at maximum speed (13000 rpm) for 1 min.

Finally, the column was transferred to a new 1.5 ml collection tube, 30 µl RNase-free water was added, and the samples were centrifuged at 10000 rpm for 1 min before being frozen at -20°C to elute.

4.2.3.3. Polymerase chain reaction (PCR)

A variety of polymerase chain reaction conditions were performed, and these are listed in Table 4.1 and Table 4.2. Reactions were performed in 20 μ L volumes (Table 4.1).

Table 4.1. PCR reaction mixture

Reagent	Volume per reaction of 20 μ l
5x buffer	4 μ l
10 μ M forward primer	0.5 μ l
10 μ M reverse primer	0.5 μ l
10 mM dNTPs	0.4 μ l
<i>Taq</i> DNA Polymerase (1 U/ μ L) (Invitrogen)	1 μ l
Template DNA	1 μ l
Distilled water (dH ₂ O)	12.6 μ l

Table 4.2. PCR cycling conditions

Temperature °C	Time	Cycle
94°C	2 min	1
94°C	30 sec	
Annealing temperature	30 sec	35
72°C	1 min	
	(per kb)	
72°C	5 min	1
4°C	∞	∞

4.2.4. Propidium iodide (PI) staining

Primary roots were harvested, cleaned with sterilised water, immersed in 1 μ M PI for 3 min, and imaged using Leica Laser Scan Microscope (TCS-SP5) at an excitation wavelength of 488 nm and emission wavelengths of 510 nm. Confocal images were analysed via ImageJ (<https://imagej.nih.gov/ij/>) (Schindelin *et al.*, 2012).

4.2.5. Suberin staining

In Fluorol Yellow 088 solution, vertically grown (on ½ MS, pH = 5.7) Arabidopsis seedlings that are 4 and 8-day-old are incubated, as previously described by (Lux *et al.*, 2005). Suberin stained primary roots were imaged using a Leica TCS-SP8 confocal microscope.

4.2.6. CRISPR/Cas9 design

In the SALK collection (Alonso *et al.*, 2003), only one homozygous T-DNA insertion line for *KCS4* was available from The Nottingham Arabidopsis Stock Centre (NASC) (Scholl *et al.*, 2000) termed SALK T-DNA (SALK_095739). To generate independent *KCS4* alleles, CRISPR/Cas9 editing technology was employed as previously described (Tsutsui and Higashiyama, 2017). CRISPR target sequences were designed using <https://crispor.tefor.net/> (Concordet and Haeussler, 2018) (see Figure 4.6; Supplementary Table 1).

Each of these individual CRISPR vectors was assembled (see Figure 4.7), creating gRNA transgenes which were then transformed into *Arabidopsis* using the *Agrobacterium*-mediated floral dip technique (Clough and Bent, 1998). Hygromycin was used to select transgenic seeds that contained the assembled constructs.

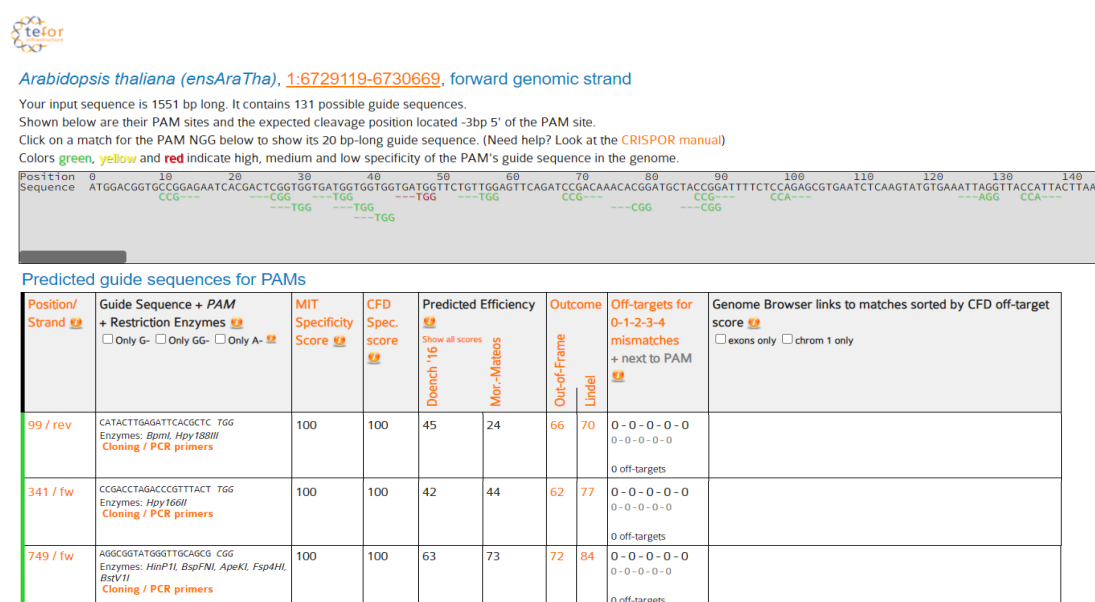


Figure 4.6. CRISPOR's main output. A table with one guide target per row, off-target locations and numbers, and annotated with predictive scores. CRISPR target sequences were designed using <https://crispor.tefor.net/> (Concordet and Haeussler, 2018) (see Supplementary Table 1).

The Hygromycin resistance cassette, *RBCS* terminator, linker GFP, and B-dummy entry vectors previously provided in the GreenGate kit (Addgene) were pre-assembled (Lampropoulos *et al.*, 2013).

To amplify the complete *KCS4* gene sequence, primers were designed (see Supplementary Table 2). The primers contain overhangs that enable the *KCS4* sequence to be ligated into the empty entry vectors. Because *BsaI* enzymes are used in the GreenGate cloning process to cut selected sequences, the genomic sequence that is added to an entry vector must be *BsaI*-free. There were no *BsaI* sites found in the *KCS4* gene in this case (Figure 4.8).

Each of these individual GreenGate entry vectors was assembled, creating either XPP-specific *pXPP::KCS4::GFP* and endodermal-specific *pCASP1::KCS4::GFP* transgenes which were then transformed into *Arabidopsis* using the *Agrobacterium*-mediated floral dip technique (Clough and Bent, 1998). Hygromycin was used to select transgenic seeds that contained the assembled constructs. Transgenic lines were recovered for both the XPP-specific *pXPP::KCS4::GFP* and endodermal-specific *pCASP1::KCS4::GFP* transgenes (see 4.3.7.1.1 and 4.3.7.1.2, respectively).

Table 4.3. GreenGate entry vectors

Module	Entry Vectors	
	<i>pXPP::KCS4::GFP</i>	<i>pCASP1::KCS4::GFP</i>
Promoter	pGG-A-XPP = pGGA000 containing 2513 bp sequence of XPP promoter	pGG-A-CASP1 = pGGA000 containing 1206 bp sequence of CASP1 promoter
N-tag	pGGB003 (B-dummy)	pGGB003 (B-dummy)
Gene	pGG-C-KCS4 = pGGC000 containing 1548 bp sequence of <i>KCS4</i> gene	pGG-C-KCS4 = pGGC000 containing 1548 bp sequence of <i>KCS4</i> gene
C-tag	pGGD001 (Linker-GFP)	pGGD001 (Linker-GFP)
Plant terminator	pGGE001 (<i>RBCS</i> terminator)	pGGE001 (<i>RBCS</i> terminator)
Plant resistance cassette	pGGF005 (Hygromycin)	pGGF005 (Hygromycin)
Empty destination vector	pGGZ003 (Plant resistance at LB)	pGGZ003 (Plant resistance at LB)

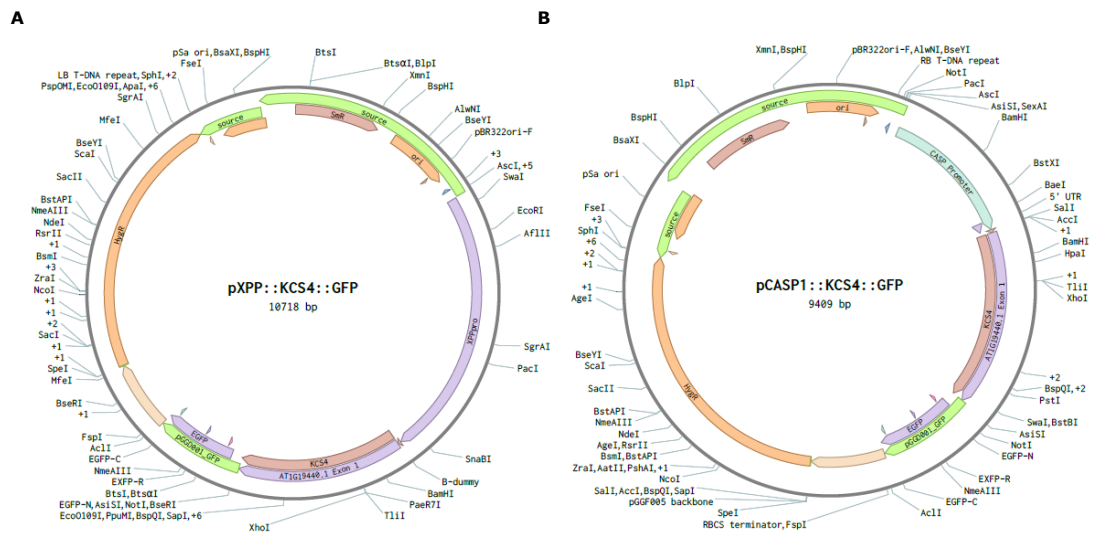


Figure 4.8. GreenGate cloning-created constructs. **(A)** *pXPP::KCS4::GFP*. **(B)** *pCASP1::KCS4::GFP*. Composition of the final destination vectors used in Arabidopsis transformation. For each segment shown here, entry vectors were created and ligated together to form the final destination vector. GreenGate vectors were designed using a web-based design tool Benchling [Biology Software] (2020); retrieved from <https://benchling.com>.

4.3. Results

4.3.1. *KCS4* belongs to a family of Arabidopsis root expressed 3-ketoacyl-CoA synthases

GWAS studies in maize have identified the suberin synthesis gene *KCS4* as a key gene regulating root length during water stress (Schneider *et al.*, 2020). To determine whether this regulatory function is conserved in other plant species, I characterised the function of suberin and specifically *KCS4* in Arabidopsis. To understand the spatial expression of *KCS4* (AT1G19440), bioinformatic analysis of organ expression in public data repositories ePlant (<https://bar.utoronto.ca/eplant/>) (Klepikova *et al.*, 2016; Waese *et al.*, 2017) revealed that the gene is root expressed (Figure 4.9).

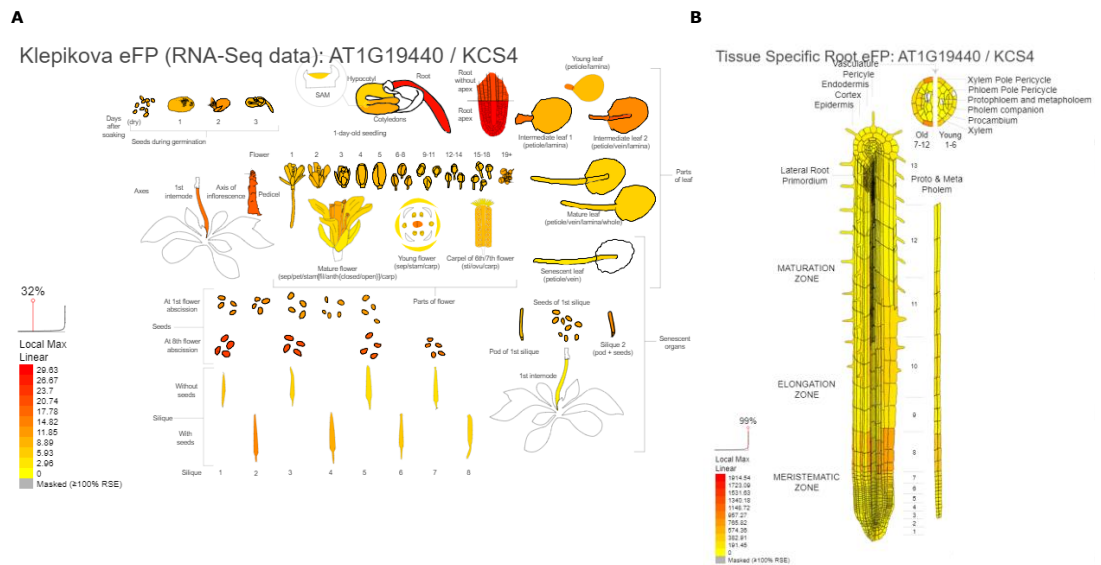


Figure 4.9. *KCS4* (AT1G19440) tissue and organ specificity expression. Expression data was retrieved from ePlant <https://bar.utoronto.ca/eplant/> (Klepikova *et al.*, 2016; Waese *et al.*, 2017).

Tissue specificity-wise, phloem, phloem pole pericycle (PPP), xylem pole pericycle (XPP), endodermis, cortex, and hair cell (HC) in the elongation zone (slice 7) have the highest levels of *KCS4* expression. Also, in the meristematic zone (slices 5 and 6), xylem pole pericycle (XPP) has the highest level of expression. *KCS4* is also XPP expressed in the elongation zone (Figure 4.10).

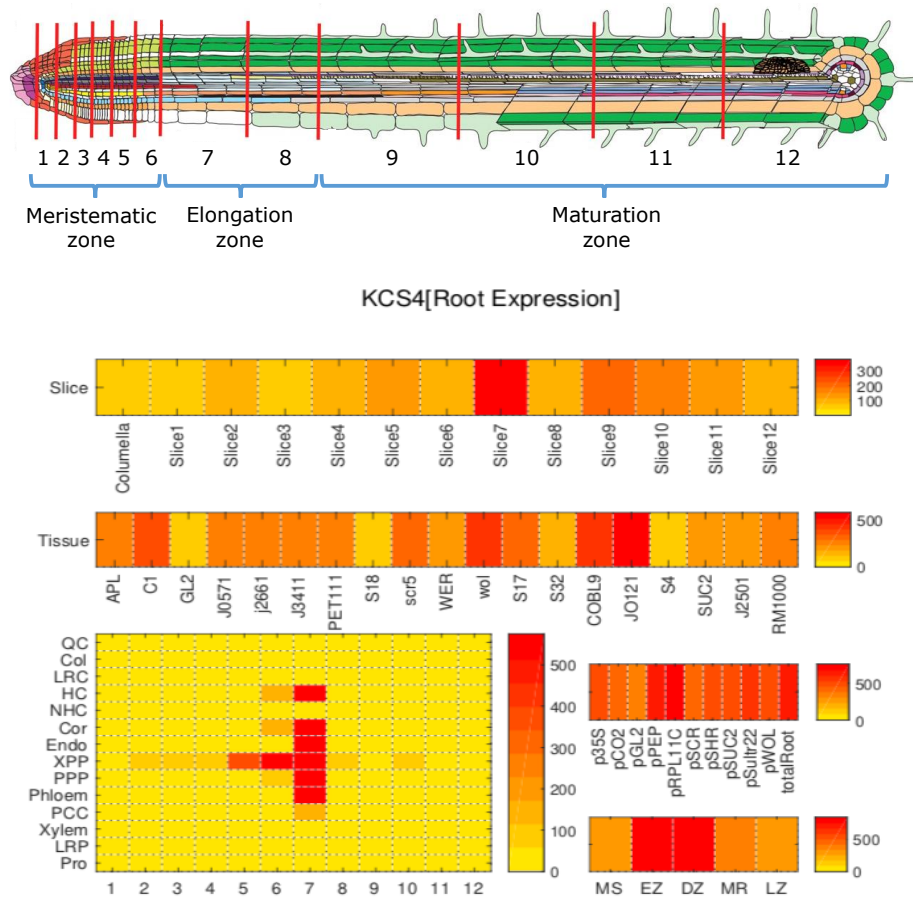


Figure 4.10. Spatial expression of *KCS4*. The eFP browser was used to obtain transcript expression data from (Brady *et al.*, 2007). Data was compiled into expression tables by Dr. Rahul Bhosale. The x-axis displays the expression of the root along its longitudinal axis: meristematic zone (1-6 slices), elongation zone (7-8 slices), and maturation zone (9-12 slices). The expression in each cell file is shown on the Y-axis: Pro: procambium, LRP: lateral root primordia, xylem, PCC: phloem companion cells, phloem, PPP: phloem pole pericycle, XPP: xylem pole pericycle, Endo: endodermis, Cor: cortex, NHC: non-hair cell, HC: hair cell, LRC: lateral root cap, Col: columella, QC: quiescent centre.

4.3.2. *KCS4* SALK insertion line verification

To assess *KCS4* function in Arabidopsis, the SALK T-DNA (SALK_095739) insertion line was selected for this study. The line contained a single SALK T-DNA (SALK_095739) insertion within its only exon. The genotyping was performed to identify the homozygote lines for SALK_095739. Loss-of-function mutant *kcs4* was confirmed to be a homozygous knockout (KO) (Figure 4.11).

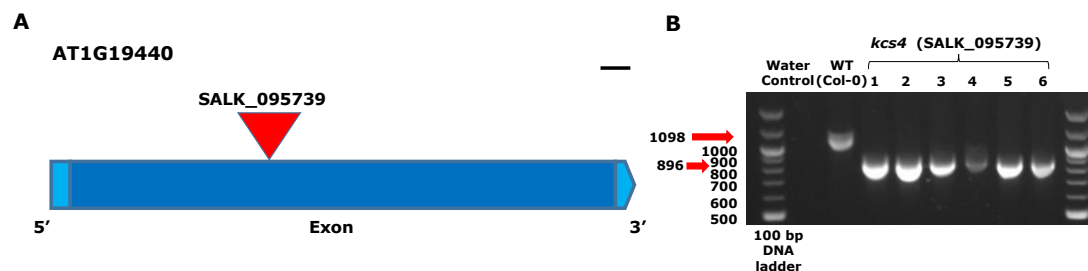


Figure 4.11. Genotyping to identify homozygous knockout in *KCS4*. **(A)** Gene structure of AT1G19440 (*KCS4*). Location of T-DNA insertion (SALK_095739) within its only exon is +499 bp after the transcription start site. Dark blue box represents an exon. Scale bar represents 100 bp. **(B)** Electrophoresis gel image showing lines 1-6 were the homozygote lines. The size of the amplified fragment of wild-type (WT; Col-0) and *kcs4* lines (using primers KCS4-1F, KCS4-1R and LBb1.3; see Supplementary Table 3) were 1098 bp and 896 bp, respectively.

4.3.3. *KCS4* SALK insertion line disrupts gene expression

PCR analysis was performed to identify whether line SALK_095739 did not contain a *KCS4* transcript. I profiled *KCS4* transcript levels in wild-type (WT; Col-0) and the SALK_095739 line. PCR analysis was performed using the primers KCS4-2F and KCS4-2R (see Supplementary Table 4). In contrast to wild-type (WT; Col-0), the *KCS4* transcript was not detected in SALK_095739 seedlings (Figure 4.12). Hence, the SALK_095739 line represented a *KCS4* null mutant.

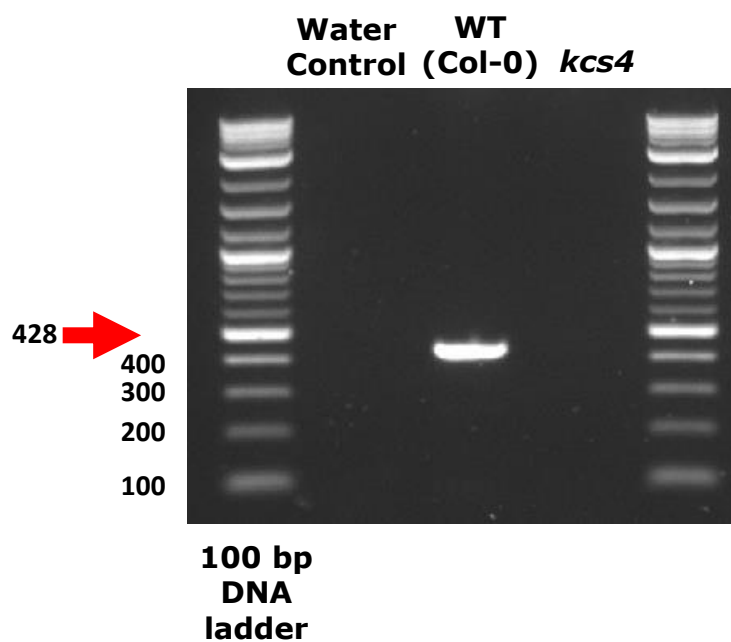


Figure 4.12. PCR analysis of *KCS4* mRNA in wild-type (WT; Col-0) and SALK_095739 lines. The size of the amplified fragment from the *KCS4* transcript (using primers KCS4-2F and KCS4-2R; see Supplementary Table 4) was 428 bp.

4.3.4. Loss-of-function *kcs4* seedlings exhibited increased lateral root (LR) length compared to WT (Col-0)

To assess whether loss of *KCS4* impacted LR length, the root system architecture of 13-day-old seedlings of several *Arabidopsis* lines was phenotyped (Figure 4.13). Loss-of-function *kcs4* mutants exhibited significantly higher lateral root (LR) length compared to wild-type (WT; Col-0). Moreover, *kcs4* roots display a similar lateral root phenotype to suberin degradation line *pCASP1::CDEF*. Both *kcs4* mutant and *pCASP1::CDEF* lines showed an increase of two-fold in lateral root (LR) length compared to wild-type (WT; Col-0).

The first 5 lateral roots length of loss-of-function *kcs4* mutant and suberin degradation line *pCASP1::CDEF* are significantly higher (~87% and ~65% increase, respectively) compared to wild-type (WT; Col-0) (Figure 4.13). Primary root length, lateral root numbers, and lateral root density of *kcs4* were also increased compared to wild-type (WT; Col-0) (Figure 4.13).

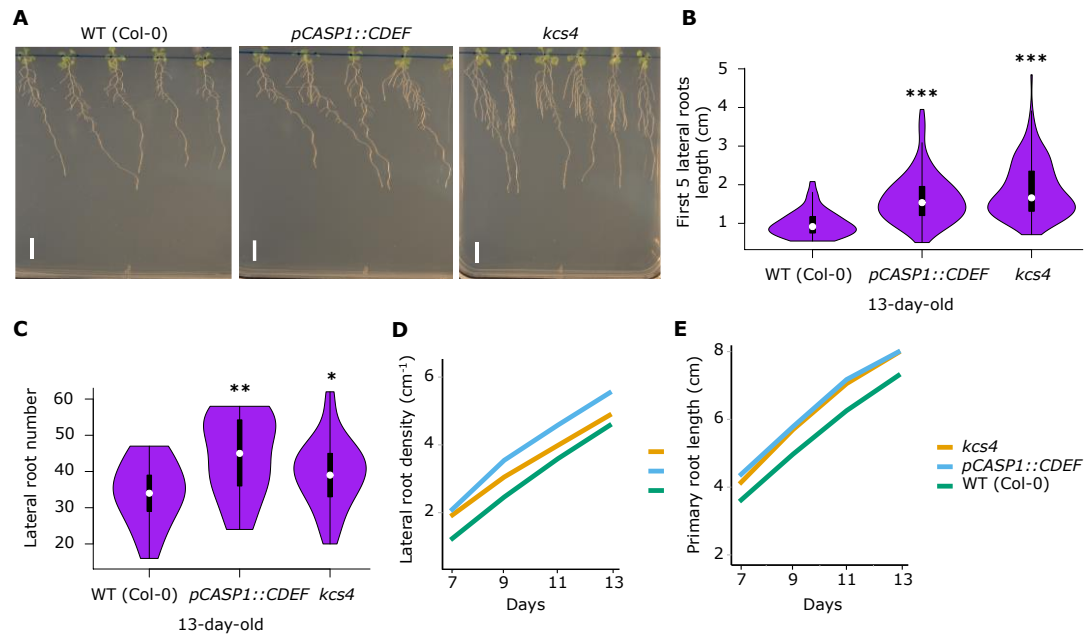


Figure 4.13. Root phenotypes of wild-type (WT; Col-0), suberin-defective *pCASP1::CDEF*, and *kcs4* lines. **(A)** Representative images of wild-type (WT; Col-0), *pCASP1::CDEF*, and *kcs4* grown on $\frac{1}{2}$ MS, 1% sucrose, pH = 5.7 for 13 days. Scale bars represent 10 mm. **(B)** First 5 lateral roots length. **(C)** Lateral root number. **(D)** Lateral root density. **(E)** Primary root length. **(B-C)** Violin plots were generated using BoxPlotR (shiny.chemgrid.org/boxplotr/) (Spitzer *et al.*, 2014). **(D-E)** Plots were generated using PlotTwist (huygens.science.uva.nl/PlotTwist/) (Goedhart, 2020). *, ** and *** represent *p*-values of ≤ 0.1 , 0.01 and 0.0001, respectively; calculated by Student's *t*-test.

4.3.5. Suberin marker *GPAT5::mCITRINE-SYP122* is expressed around LRP and in emerged LRs

GPAT5 (glycerol-3-phosphate acyltransferase 5) is one of a group of suberin biosynthesis enzymes that has been extensively studied (Barberon *et al.*, 2016). The pattern of suberin deposition in Arabidopsis roots has been shown to closely match *GPAT5*. To monitor changes in suberin during seedling root development, Arabidopsis reporter lines expressing suberin marker *GPAT5::mCITRINE-SYP122* (Barberon *et al.*, 2016) were confocal imaged. This revealed a highly dynamic pattern of suberin distribution during WT root development. High expression of the *GPAT5::mCITRINE-SYP122* reporter was detected in the mature root, but this was notably excluded from emerging LR primordium. However, reporter expression was detected in epidermal cells overlying emerging LR primordium. Finally, reporter expression was detected in a ring at the base of an emerged LR primordia.

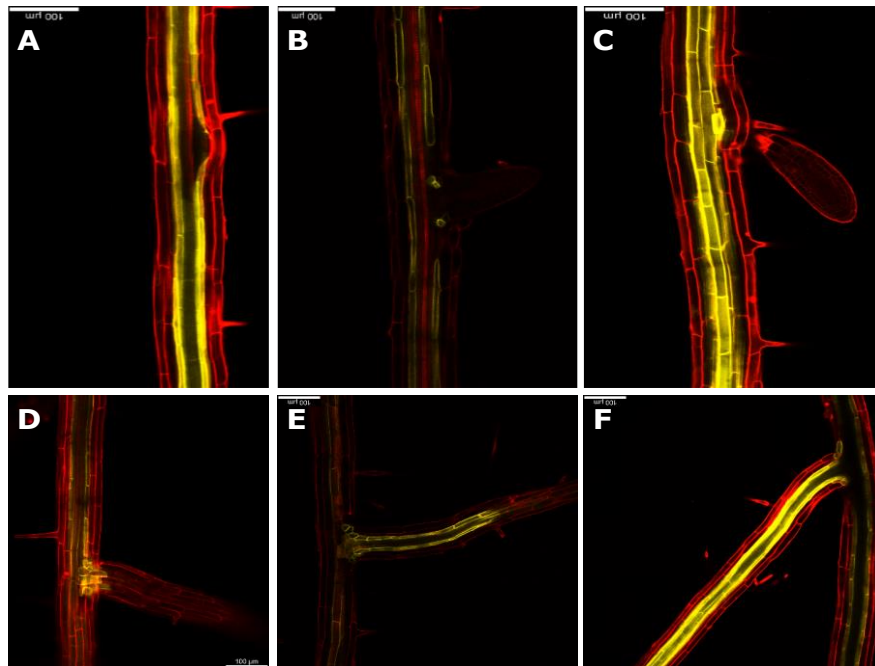


Figure 4.14. Suberin marker *GPAT5::mCITRINE-SYP122*. Representative images of transgenic Arabidopsis homozygous lines expressing *GPAT5::mCITRINE-SYP122* grown on ½ MS for 9 days (**A-F**), stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP5 confocal microscope. (**A**) High expression in the mature root, excluded from emerging LR primordium. (**B**) Expression in epidermal cells overlying emerging LR primordium. (**C**) No expression in cell membrane of emerging LR primordium. (**D**) Expression in a ring under an emerged LR primordia. (**E-F**) High expression in mature lateral root. Scale bars represent 100 μ m.

4.3.6. Suberin imaging in *KCS4* SALK knockout (KO) and suberin-defective line *pCASP1::CDEF*

Changes in suberin distribution in the loss-of-function *kcs4* mutant and suberin-degradation line *pCASP1::CDEF* compared with wild-type (WT; Col-0) were monitored using suberin-specific stain Fluorol Yellow 088 (Figure 4.15). This stain revealed similar changes in suberin distribution as the *GPAT5::mCITRINE-SYP122* reporter in WT seedling roots. Strikingly, the stain revealed a complete loss of suberin in the *pCASP1::CDEF* line in both primary and lateral roots. However, suberin staining in roots of the *kcs4* line appeared spatially similar to WT. Nevertheless, it cannot be ruled out that there are quantitative changes in suberin levels during *kcs4* mutant root development.

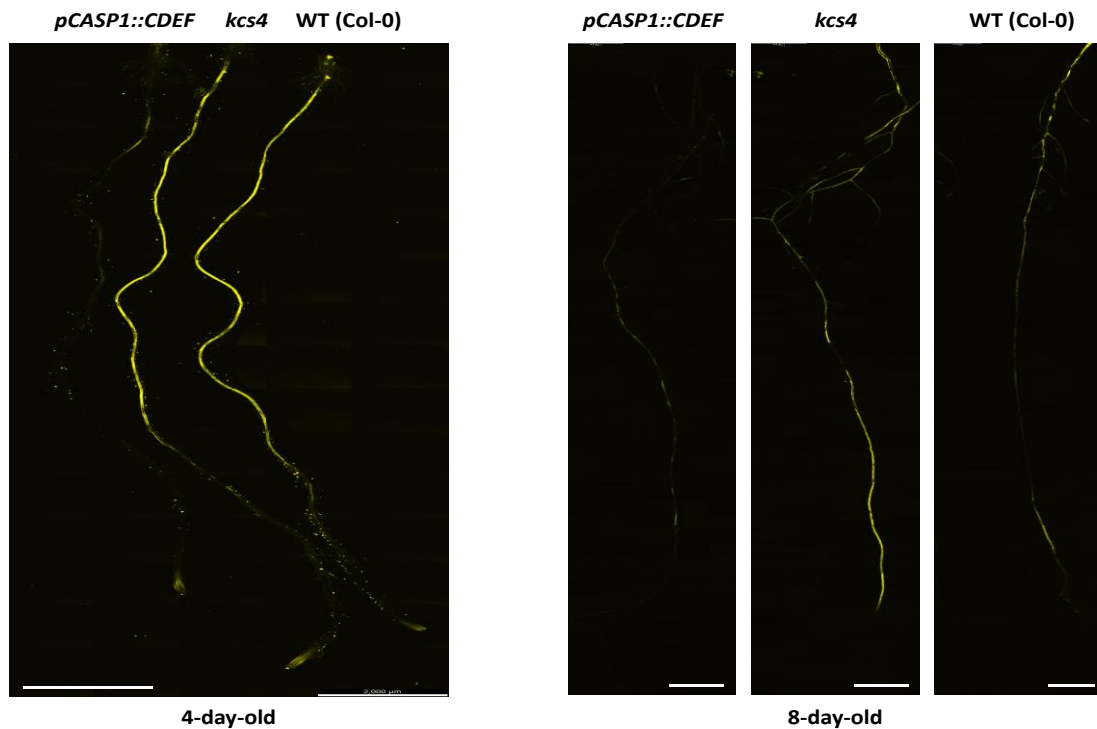


Figure 4.15. Suberin-specific stain Fluorol Yellow 088 revealed major changes in roots in *pCASP1::CDEF* (but not *kcs4*). Representative images of transgenic Arabidopsis *KCS4* SALK knockout (KO) and suberin-defective line *pCASP1::CDEF* grown on ½ MS for 4 and 8 days (**A-B**), stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP8 confocal microscope. (**A**) No suberization was detected in roots in suberin-defective line *pCASP1::CDEF*, whereas high suberin levels were detected in the mature root of *kcs4* mutant and wild-type (WT; Col-0). (**B**) No suberization was detected in suberin-defective line *pCASP1::CDEF* during LR development whereas suberin decreased in the mature root and lateral root of wild-type (WT; Col-0) and *kcs4* mutant backgrounds. Scale bars represent 2000 μm.

4.3.7. Functional genomic studies of *KCS4* dependent LR development

4.3.7.1. Creating tissue-specific *KCS4* expression lines

To identify the root cell types in which *KCS4* functions to control lateral root development, a tissue-specific rescue strategy of the *kcs4* mutant was developed. Transcriptomic analysis revealed that *KCS4* expression is endodermis and xylem pole pericycle (XPP)-specific (Figure 4.10). To determine in which of these tissues *KCS4* was important, Arabidopsis plants expressing XPP-specific *pXPP::KCS4::GFP* and endodermal-specific *pCASP1::KCS4::GFP* were created (see 4.2.7).

4.3.7.1.1. *pXPP::KCS4::GFP* (XPP; xylem pole pericycle-specific) tissue-specific over-expression line

Transgenic Arabidopsis T2 lines expressing *pXPP::KCS4::GFP* were selected with Hygromycin, then stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP5 confocal microscope. In the wild-type (WT; Col-0) background, xylem pole pericycle-specific GFP expression was detected in the mature root. High expression was detected in stele cells close to the QC at the root tip which continued into the mature root (Figure 4.16A-E) and in mature lateral root primordia (Figure 4.16F). Radial cross-sections revealed a punctate pattern labelling structures between endodermal and pericycle cells layers (Figure 4.16D-E). These *pXPP::KCS4::GFP* lines are being crossed into the *kcs4* background to assess whether they can rescue the mutant LR length phenotype.

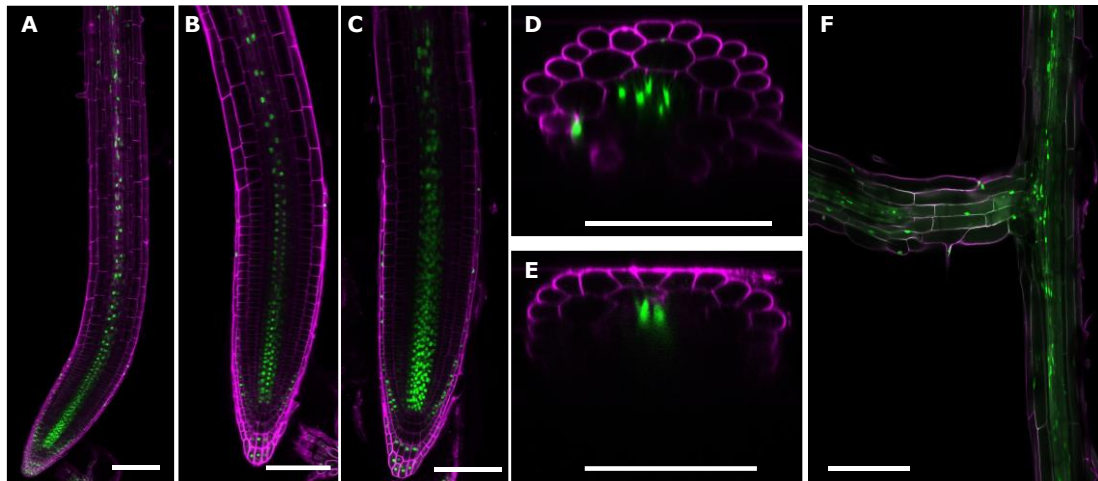


Figure 4.16. *pXPP::KCS4::GFP* tissue-specific over-expression line. Representative images of transgenic Arabidopsis T2 lines expressing *pXPP::KCS4::GFP* grown on ½ MS with Hygromycin B antibiotic for 7 days (**A-E**) and 14 days (**F**), stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP5 confocal microscope. In the wild-type (WT; Col-0) background, xylem pole pericycle-specific GFP expression in the mature root of *kcs4*. (**A-C**) High expression in vasculature. (**D-E**) Representative radial images. (**F**) Expression in mature lateral root primordia. Scale bars represent 100 μm.

4.3.7.1.2. *pCASP1::KCS4::GFP* (CASP1; endodermal-specific) tissue-specific over-expression line

Transgenic Arabidopsis T2 lines expressing *pCASP1::KCS4::GFP* were selected with Hygromycin, then stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP5 confocal microscope. In the wild-type (WT; Col-0) background, endodermal-specific GFP expression was clearly detected in the mature root. However, there was no expression in meristem and elongation zones (Figure 4.17A & C), mirroring CASP1 expression. These *pCASP1::KCS4::GFP* lines are being crossed into the *kcs4* background to assess whether they can rescue the mutant LR length phenotype.

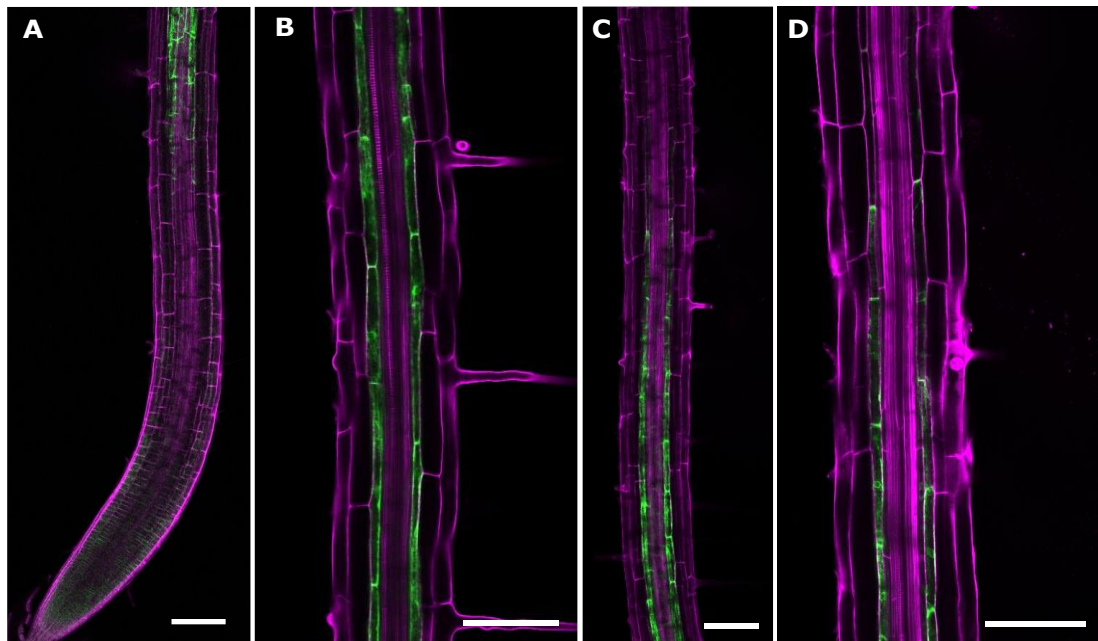


Figure 4.17. *pCASP1::KCS4::GFP* tissue-specific over-expression line. Representative images of transgenic Arabidopsis T2 lines expressing *pCASP1::KCS4::GFP* grown on ½ MS with Hygromycin B antibiotic for 7 days (**A-D**), stained with Propidium iodide (PI) and were imaged using a Leica TCS-SP5 confocal microscope. In the wild-type (WT; Col-0) background, endodermal-specific GFP expression in the mature root of *kcs4*. (**A & C**) No expression in meristem and elongation zones. (**B & D**) Expression in the mature root zone. Scale bars represent 100 μm.

4.3.7.2. Creating new *KCS4* alleles using CRISPR/Cas9

In the SALK collection (Alonso *et al.*, 2003), only one homozygous T-DNA insertion line for *KCS4* was available from The Nottingham Arabidopsis Stock Centre (NASC) (Scholl *et al.*, 2000) termed SALK T-DNA (SALK_095739). To generate independent *kcs4* alleles, CRISPR/Cas9 editing technology was employed (see 4.2.6). CRISPR target sequences were designed using <https://crispor.tefor.net/> (Concordet and Haeussler, 2018).

All three gRNA sequences (termed 99 gRNA, 341 gRNA and 749 gRNA) were able to generate positive edits in the *KCS4* gene. Sanger sequencing revealed several insertions/deletions one to four (bp) upstream of the PAM site (Figure 4.18). Three *KCS4* edited lines that carry a heterozygous G and A insertion were chosen for further phenotypic analysis to validate our SALK line results.

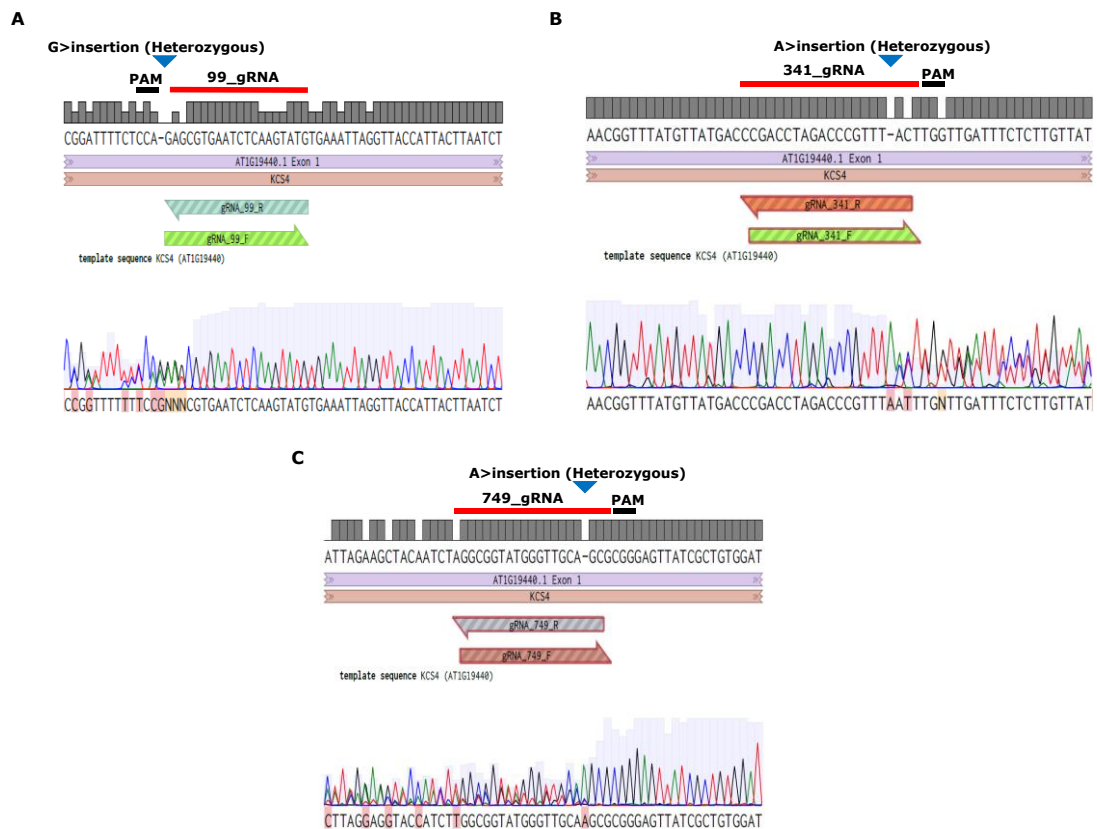


Figure 4.18. List of events with dendrograms containing *KCS4* 99 gRNA, 341 gRNA, and 749 gRNA positive edits. Dendrogram images of three *KCS4* edited lines that carry a heterozygous G and A insertion one to four (bp) upstream of the PAM site. CRISPR targets were identified within its only exon (AT1G19440) using a web-based design tool Benchling [Biology Software] (2020); retrieved from <https://benchling.com>.

4.4. Discussion

Lateral root branching and increased length play essential roles during adaptation to water stress to increase the plants' surface area and volume of soil being explored (Cai *et al.*, 2022). To identify potential regulators of this important adaptive response, GWAS predictions on a root phenotype dataset generated for a maize diversity panel subjected to water stress was analysed (Schneider *et al.*, 2020). One promising candidate gene predicted by GWAS in the latter study encoded the suberin synthesis gene *KCS4*. This thesis chapter employed functional genomic and gene editing approaches in *Arabidopsis* to assess whether *KCS4*'s lateral root length regulatory role is conserved across angiosperms.

Intriguingly, loss-of-function *kcs4* mutants exhibited significantly higher lateral root (LR) length compared to wild-type (WT; Col-0). Moreover, *kcs4* roots display a similar increased lateral root phenotype as suberin degradation line *pCASP1::CDEF*. Recent research using a cell scale water imaging approach revealed that *pCASP1::CDEF* and/or other Arabidopsis mutants disrupting suberin and/or Casparian strip integrity impacted water fluxes towards shoot tissues (Pascut *et al.*, 2021). These independent lines of evidence suggest that modification of root suberin levels causes a reduction in water fluxes to the shoot, triggering compensatory changes in lateral root growth to ensure sufficient water reaches leaves.

Luzarowska *et al.*, (2020) demonstrated *KCS4*'s ability to bind CoA, as well as its activity as an enzyme in the elongase complex. The specificity of *KCS4* and its precise role, on the other hand, are unknown. As a result, the authors hypothesised that *KCS4* is involved in abiotic-stress-induced lipid rearrangement in Arabidopsis. The elongase complex and other members of the KCS family localise to the ER, as previously shown by (Joubès *et al.*, 2008). Luzarowska *et al.*, (2020) were able to demonstrate *KCS4* localization to the endoplasmic reticulum using *KCS4*-YFP transient over-expression in *Nicotiana benthamiana*. The KCS family evolved from a single gene in algae to a three-member gene family in *Marchantia polymorpha*, a plant that colonised the terrestrial environment, through several gene duplications. In Arabidopsis, KCS is a gene family with 21 members (Guo *et al.*, 2016). Because the KCS family is important in plant stress response, it appears that the reason for a large number of KCS family members is Arabidopsis' adaptation to various natural environments (Weigel and Mott, 2009). The climate conditions were retrieved from the WorldClim database (<https://www.worldclim.org/>) (Fick and Hijmans, 2017).

Given that *KCS4* regulates TAG levels in conditions of depleted carbon reserves, it is possible that *KCS4* was selected for natural selection to improve adaptation in stressful environments (Luzarowska, 2020). FAs with varying saturation levels and acyl-chain lengths are specific to members of the large KCS family.

Furthermore, it appears that the 21 KCSs evolved to control VLCFA with varying temporal and spatial resolution as members of the KCS family express differently in different organs under different conditions (Joubès *et al.*, 2008). As a result, changes in the biosynthesis and accumulation pattern of cuticular waxes in response to changing environmental conditions play a significant role in plant stress resistance (Luzarowska, 2020).

A variety of biological and abiotic stresses can cause changes in cell wall composition. Drought, as previously demonstrated by (Moura *et al.*, 2010), can result in the formation of suberin. Furthermore, (Vandeleur *et al.*, 2009) discovered increased suberin deposition in the roots of drought-stressed grapevine (*Vitis vinifera* L.). Increased root suberin content was discovered to increase water utilisation during the vegetative period while decreasing daytime transpiration rate (Baxter *et al.*, 2009).

The presence of root apoplastic barriers, which prevent uncontrolled apoplastic transport, is linked to selectivity in root–rhizosphere communication (Tylová *et al.*, 2017). Lateral roots are extremely important in this regard because they provide the most surface area with the least amount of biomass investment (Postma *et al.*, 2014) and constitute a significant portion of a complex root system's absorptive surface (Waisel and Eshel, 2002).

Suberization of endodermal cells is a plastic response during nutrient stress and also varies in different hormone treatments. The level of preformed suberin is drastically affected when root grows in different soil environments. This plastic response (suberization) can help the plant roots modification under different abiotic stress conditions. Researchers should focus more on this plastic response to study the suberization response and root architecture modification to better understand the foraging strategies of plants (Barberon *et al.*, 2016).

5. Chapter 5: General discussion and future perspectives

Plant roots face many different stresses while growing in soil, of either a physical, abiotic and biotic nature, which impacts their ability to forage for resources like water and nutrients (Lynch *et al.*, 2021). Plants have evolved acclimative responses which enable roots to adapt to, and often overcome, the limitations imposed by these soil stresses. For example, in response to soil water limitation many species of plants form longer lateral roots (Schneider *et al.*, 2020; Cai *et al.*, 2022) which serves to increase their root surface area and volume of soil being explored to secure moisture. Another example includes root responses to soil compaction where, on encountering a hard layer, roots often slow or cease growing, enabling plants to divert their resources to other parts of their root system growing in less challenging conditions (Lynch *et al.*, 2021). Plant hormones like ABA and ethylene often play critical roles during root acclimative responses to soil stresses like water limitation and soil compaction (Pandey *et al.*, 2021b; Zhang *et al.*, 2021). However, do these hormone signals play common roles amongst all land plants or are there clear differences in how they are utilised during root acclimative responses?

Is the role of ethylene during root compaction responses universal among plant species?

Pandey *et al.*, (2021b) recently demonstrated that compacted soils inhibit *Arabidopsis* and rice root growth via enhanced ethylene signalling, rather than increased mechanical impedance as originally thought. A critical piece of evidence supporting a role for this hormone during root-soil compaction responses in these plant species was the ability of ethylene response mutants to overcome high soil impedance compared to wild-type (Pandey *et al.*, 2021b). Later studies using ethylene response inhibitor 1-MCP to improve maize root penetration in compacted soil further confirmed the role of ethylene during this acclimative response (Vanhees *et al.*, 2021).

However, characterisation of the tomato *Never-ripe* (*Nr*) mutant, which disrupts a receptor component of the primary ethylene signalling pathway, does not confer a compaction resistant phenotype seen in ethylene mutants of other species (Chapter 2). This represents a surprising result, given that disrupting the ethylene response pathway (including via the receptor) in another eudicot model (*Arabidopsis*), as well as cereal models rice, maize and wheat (Pandey *et al.*, 2021b; Vanhees *et al.*, 2021; Pandey, Moreno and Bennett, pers. comm.), confers soil compaction resistance. Several potential reasons exist why this is the case for the *Nr* mutant including its impact on root gravitropism, secretion of mucilage and microbiome, which could indirectly disrupt the ability of mutant roots to penetrate compacted soils (see Chapter 2, Discussion section). Ideally, additional tomato mutations in the primary ethylene response pathway should be characterised (such as EIN2 and EIN3; as performed in rice by Pandey *et al.*, 2021b) before reaching a definitive conclusion.

Why may ethylene not be important for tomato root compaction responses? Tomato originated in desert environments of South America where soils are very dry and sandy, which may impact the utility of an ethylene-based compaction mechanism. Under these circumstances, other hormones like abiotic stress signal abscisic acid (ABA) may be more important for tomato compaction responses.

Is ABA a common signal among plant species during compaction stress?

Abscisic acid (ABA) represents a water stress hormone, and its role in the regulation of plant drought responses has been extensively researched (Schachtman and Goodger, 2008; Cutler *et al.*, 2010). When soil dries out, ABA accumulates, and the endogenous ABA concentration of the plant can be used to predict soil water availability (Zhang and Davies, 1989). Creelman *et al.*, (1990) and Rowe *et al.*, (2016) showed that ABA acts as an inhibitor of plant growth under water deficit conditions. As previously described by Sharp and LeNoble, (2002), ABA is known to be an inhibitor of plant root and shoot growth under well-watered conditions.

Soil compaction imposes not just mechanical impedance on roots, but also water stress, as a result of the reduced moisture release properties of compacted soil (Lynch *et al.*, 2021). In a dry sandy soil environment, employing an abiotic stress signal like ABA, rather than a gaseous signal like ethylene, to trigger a root compaction response would appear logical. Tracy *et al.*, (2015) demonstrated ABA-biosynthesis tomato mutant *notabilis* (*not*) has a reduced ability to penetrate compacted soil. *Notabilis* (*not*) is an ABA-biosynthesis mutant caused by a null mutation in the gene *NCED1*, which encodes a *9-cis-epoxycarotenoid dioxygenase* (Burbidge *et al.*, 1999; Zhang *et al.*, 2021). Hence, ABA normally negatively regulates root compaction responses such as growth inhibition in tomato. In contrast, ABA biosynthesis mutants in rice exhibit less root growth inhibition and radial swelling in compacted soil (Chapter 3, this thesis; Huang *et al.*, 2022). Therefore, ABA normally positively regulates root compaction responses in rice. The contrasting effects of ABA in tomato and rice roots during soil compaction responses may reflect the distinct desert and flooded environments in which these species originated, respectively. Consistent with ABA playing a primary regulatory role in coordinating compaction responses in tomato roots, this signal's transduction pathway is positioned upstream of ethylene in this species (Tracy *et al.*, 2015). In contrast, the ethylene response pathway is positioned upstream of ABA in rice (Qin *et al.*, 2019; Chapter 2, this thesis).

In rice, ABA promotes radial expansion of cortical cells (Chapter 3, this thesis). Is there an advantage for the ABA-induced root-tip swelling during soil compaction? It is possible that in conditions of soil compaction where the root is growing into channels/cracks but otherwise cannot penetrate, tip swelling might play the classically presumed role of widening the channels, thereby allowing root penetration that otherwise could not occur. Future work will explore the relationship between these hormone-driven root growth responses to compaction and structural features of the soil, such as the presence of channels and cracks, the thickness of the compacted soil layer and levels of moisture. Nevertheless, where soil compaction is uniform, the rice root tip swelling response is not needed and in fact, is negatively associated with penetration.

Translating knowledge of key compaction signals to develop novel crops

Results obtained in my thesis provide encouragement that novel varieties of compaction-resistant crops can be selected based on reduced root levels or responses to key plant hormones like ABA and ethylene, respectively. However, the choice of target hormone appears to depend on the crop species. For example, mutating components of the primary ethylene response pathway, as demonstrated by Pandey *et al.*, (2021b) in rice, is not effective in tomato. Ideally, targeting genes downstream of ABA and ethylene primary hormone response pathways appears to be a better strategy as it avoids many of the negative effects losing such key signals would have on plant performance. For example, when a plant loses its sensitivity to ethylene, it often loses its ability to form mycorrhizal interactions. Torres de Los Santos *et al.*, (2011) described the pattern of mycorrhization in mutant tomato plants with altered ethylene biosynthesis and/or perception pathways.

Screens performed on tomato introgression lines (Chapter 2, this thesis) appears to have successfully identified candidate genes acting downstream of the ethylene (and likely ABA) primary response pathway(s) which confer compaction resistance. Two less ethylene-sensitive tomato introgression lines (IL2-1-1 and IL3-1) that are also compaction-resistant. Neither of these tomato ILs (IL2-1-1 and IL3-1) encode primary ethylene signalling components within their introgressed genomic regions (Chapter 2). However, these introgressed genomic regions do encode ethylene synthesis and/or downstream signalling components. In the case of IL2-1-1, the introgressed region contained 337 genes, two of which were classified as putative ethylene-related genes. Figure 2.13 illustrates that (Soly02g022920 and Soly02g030210) have been identified as encoding an ethylene-responsive nuclear protein and an AP2-like ethylene-responsive transcription factor candidate, respectively (Table 2.1). These genes also have Arabidopsis homologs (Table 2.2).

In the case of IL3-1, the introgressed region contained 191 genes, five of which were classified as putative ethylene-related genes. Figure 2.14 illustrates the five genes (Soly03g005500, Soly03g005510, Soly03g005520, Soly03g005600, and Soly03g006320) have been predicted to encode an ethylene-responsive transcription factor and a S-adenosyl-L-methionine-dependent methyltransferases superfamily protein (Table 2.1). These genes also have Arabidopsis homologs, several of which have ethylene-related functions (Table 2.2). The effects of these candidate ethylene-related genes in tomato roots will be studied in the future. Several approaches could be used to identify whether these candidate ethylene-related genes in tomatoes are regulating ethylene-insensitivity and soil compaction resistance. For example, gene editing could be employed to mutant individual candidate genes within the introgressed regions (assuming the loss-of-function allele exhibits a phenotype) to pinpoint the causative gene(s). Once identified, a gene-editing (and/or backcrossing) based approach could be used to modify elite tomato lines to confer root compaction resistance.

Suberin cannot function as an apoplastic barrier (Hosmani *et al.*, 2013) because it is deposited between adjacent endodermal cells separated by the cell wall (Kosma *et al.*, 2014; Li *et al.*, 2017). Moreover, suberin has been found to be necessary for the maintenance of an apoplastic barrier between lateral root primordia (LRP) and the endodermal cells that surround it (Li *et al.*, 2017). During the process of lateral root emergence, the cell wall of the endodermal cell that covers the LRP is degraded (Kumpf *et al.*, 2013). Suberin may be used to fill the gap between the adjacent plasma membranes of the LRP and the surrounding endodermal cells after the cell wall is removed (Li *et al.*, 2017).

Endodermal cell suberization is a plastic response to nutrient stresses that varies with hormone treatment. When roots grow in different soil environments, the level of preformed suberin changes dramatically. This plastic response (suberization) can aid in the modification of plant roots under various abiotic stress conditions (Barberon *et al.*, 2016).

It would be interesting to investigate how *kcs4* mutant affects the nutrient and water uptake under water stress conditions. Based on the higher lateral root branching in *kcs4* mutant, I hypothesize that it will enhance water acquisition capacity than WT. If successful, this suberin based branching response can be exploited to engineer other cereal crops for better water uptake capacity under water stress conditions. It is also important to note that ABA affects suberization process which eventually act as a water stress signal. However, it would be interesting to observe that how KCS4 transcriptional reporter line (XPP and CASP1 promoter driven) will respond to external water stress or by ABA treatments. If ABA can promote the suberization process by enhancing KCS4 expression, it can be directly linked that plant roots respond to water stress via changing their endodermal suberin content by enhancing KCS4 expression.

To better understand plant foraging strategies, researchers should focus more on this plastic response to study the suberization response and root architecture modification.

Supplementary information

Supplementary Table 1. Primers used for CRISPR/Cas9 constructs

Primers	5' to 3'
99 gRNA-F	ATTG CATACTTGAGATTACGCTC
99 gRNA-R	AAAC GAGCGTGAATCTCAAGTATG
341 gRNA-F	ATTG CCGACCTAGACCCGTTTACT
341 gRNA-R	AAAC AGTAAACGGGTCTAGGTCGG
749 gRNA-F	ATTG AGGCGGTATGGGTTGCAGCG
749 gRNA-R	AAAC CGCTGCAACCCATACCGCCT

Supplementary Table 2. Primers used for GreenGate entry vectors

Primers	5' to 3'
pGG-C- KCS4-F	ACCAGGTCTCTGGCT ATGGACGGTGCCGGAGAAT
pGG-C- KCS4-R	AACAGGTCTCTCTGA ATAACTTAAAGTTACCGGATACTTGTC

Supplementary Table 3. Primers used for *KCS4* SALK insertion line verification

Primers	5' to 3'
KCS4-1F	GTTTCTCGTCCTGGGTTTCTC
KCS4-1R	CCAACTCGAAACAAGCAGTTC
LBb1.3	ATTTTGCCGATTTCGGAAC

Supplementary Table 4. Primers used for PCR

Primers	5' to 3'
KCS4-2F	CCGACCTAGACCCGTTTACT
KCS4-2R	AGGCGGTATGGGTTGCAGCG

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