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**GENETIC AND ENVIRONMENTAL
FACTORS AFFECTING ROOT RESPONSES
TO PLANT NUTRITION**

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ABSTRACT

Abstract

Plants productivity is linked to root capacity to explore and exploit soil resources such as water, mineral nutrients or microbial associations. The study of root system development and its functionality under stress is essential to solve agricultural challenges. In nature, roots grow underground in darkness and in a temperature buffered environment. However, most of the *in vitro* studies expose roots to direct illumination or to high temperature in heat stress assays. To overcome these problems, we have designed new devices that maintain the root system in darkness (D-Root) or in temperature buffered gradient (TGRooZ).

Phosphate (Pi) is an essential nutrient and its deficiency is common in agricultural soils. Here, we report that root illumination alters the plant Pi starvation response (PSR) at morphological, transcriptional, and physiological levels. Using the D-Root, we show that Pi-starved plants increase the cis-zeatin:trans-zeatin (cZ:tZ) ratio. Transcriptomic analyses show that tZ represses cell cycle and PSR genes, whereas cZ induces genes involved in cell and root hair elongation and differentiation. In fact, cZ-treated seedlings show longer root system and root hairs compared with tZ-treated seedlings, increasing total absorbing surface. Mutants with low cZ levels do not allocate free Pi in roots during Pi starvation. Based on our data, we propose that Pi-starved plants increase the cZ:tZ ratio to maintain basal cytokinin responses. Therefore, cZ acts as a PSR hormone that stimulates root and root hair elongation, enlarging absorbing surface, and increases root Pi. Furthermore, we performed a genomic-wide association study (GWAS) on the Iberian Peninsula collection of *Arabidopsis thaliana* using the D-root to uncover new genetic regulators. Thus, we identified genomic regions implicated in PSR related traits. GWAS on Pi concentration revealed that our associated genes are enriched in inositol phosphate synthesis route, suggesting its involvement in natural variation. Overall analysis of common genes and their gene networks led to the identification of WRKY18 as a putative transcription factor involved in PSR.

In natural environments, fungal endophytes have been described as plant growth promoting (PGP) microorganisms that enhance plant nutrition. In this thesis, we aimed to identify, under Pi starvation, PGP fungal endophytes from a collection of natural isolates of *A. thaliana*. We identified Ps-1 as a PGP fungus that increases shoots, root system architecture and total phosphorous under Pi deficiency. Ps-1 increases the number of lateral roots, suggesting that influences the root clock. Furthermore, Ps-1 enhances *Hordeum vulgare* growth, suggesting broader host compatibility. Thus, our data indicate that Ps-1 is a PGP fungal endophyte under Pi deficiency in diverse hosts.

Climate change is increasing extreme heat events that negatively impact on plant development. Most of the experiments designed to study plant adaption to heat stress apply high temperatures to both shoot and root. In soils, roots grow in a temperature descendent gradient that is buffered in a day-nihgt cycle. Thus, we engineered the TGRooZ device that simulates this gradient to better analyze how heat affects plant responses. We demonstrate that whole plants exposure to high temperatures impairs growth. However, when shoots are at high temperature and roots in TGRooZ, roots grow efficiently and sustain shoot growth. Furthermore, gene expression, plant nutrition and microbiome composition of TGRooZ-grown roots are significantly different from high temperature-grown roots, correlating with higher functionality. Our data indicate that, by using TGRooZ in heat stress studies, we can improve our knowledge on plant responses to high temperatures.

Altogether, this work remarks the importance of studying the root system in conditions mirroring the soil environment to provide novel insights and improve the applicability from laboratory studies to the field.

RESUMEN

Resumen

La productividad de las plantas está vinculada a la capacidad de las raíces para explotar los recursos del suelo, como son el agua, los nutrientes o la microbiota. En la naturaleza, las raíces crecen bajo tierra en oscuridad y en un gradiente de temperatura. Sin embargo, los estudios *in vitro* exponen las raíces a iluminación directa o altas temperaturas en ensayos de estrés térmico. Para crear condiciones experimentales más parecidas a las naturales, hemos diseñado nuevos sistemas que mantienen el sistema radicular en oscuridad (D-Root) o en un gradiente de temperatura (TGRooZ).

El fosfato (Pi) es un nutriente esencial, y su deficiencia es común en suelos. Aquí demostramos que la iluminación de las raíces altera la respuesta de la planta a la deficiencia de Pi (PSR). Utilizando el D-Root, mostramos que la deficiencia de Pi aumenta el ratio cis-zeatina:trans-zeatina (cZ:tZ). Basándonos en análisis transcriptómicos, hemos demostrado que tZ reprime el ciclo celular y genes de PSR, mientras que cZ induce genes implicados en elongación y diferenciación celular y de pelos radicales. De hecho, las plántulas tratadas con cZ muestran un sistema radicular y pelos radicales más largos que las tratadas con tZ. Los mutantes con bajos niveles de cZ no acumulan Pi en raíces durante la deficiencia de Pi. Basándonos en estos datos, proponemos que la deficiencia de Pi aumenta la proporción cZ:tZ para mantener la respuesta basal de citoquininas. Por lo tanto, sugerimos que cZ actúa como una hormona mediadora de la PSR, aumentando el Pi en la raíz y ampliando la superficie de absorción. Además, realizamos un estudio de asociación del genoma completo (GWAS) en la colección de la Península Ibérica de *Arabidopsis thaliana* utilizando el D-root para descubrir nuevos reguladores genéticos. Identificamos regiones genómicas implicadas en los rasgos relacionados con la PSR. El GWAS de concentración de Pi muestra que nuestros genes asociados están enriquecidos en la ruta de síntesis del inositol fosfato, sugiriendo su implicación en la variación natural de este rasgo. El análisis global de los genes comunes y sus redes condujo a la identificación de WRKY18 como un putativo factor implicado en la PSR.

En la naturaleza, los hongos endófitos pueden actuar como promotores del crecimiento vegetal (PGP). En esta tesis, nos propusimos identificar, en una colección de aislados naturales de *A. thaliana*, hongos endófitos PGP en condiciones de deficiencia de Pi. Identificamos a Ps-1 como un hongo PGP que aumenta la parte aérea, el sistema radicular y el fósforo total bajo deficiencia de Pi. Ps-1 aumenta el número de raíces laterales, sugiriendo que influye en el reloj de la raíz. Además, Ps-1 aumenta el crecimiento de *Hordeum vulgare*, sugiriendo compatibilidad con más especies. En conjunto, nuestros datos indican que Ps-1 es un hongo PGP en diversos huéspedes.

El cambio climático está incrementando los eventos de calor extremo. La mayoría de los experimentos diseñados para estudiar la adaptación de las plantas al estrés térmico aplican altas temperaturas a parte aérea y raíces. En los suelos, las raíces crecen en un gradiente descendente de temperatura. Para simular este gradiente, hemos diseñado el dispositivo TGRooZ. Así, demostramos que la exposición de la planta entera a altas temperaturas perjudica su crecimiento. Sin embargo, bajo el gradiente de temperatura producido en TGRooZ, las raíces crecen eficientemente y mantienen el crecimiento de la parte aérea a alta temperatura. Además, la expresión génica, la nutrición mineral y la composición del microbioma son significativamente diferentes. Nuestros datos indican que utilizando TGRooZ en estudios de estrés térmico podemos mejorar nuestro conocimiento sobre las respuestas a las altas temperaturas.

En conjunto, este trabajo subraya la importancia de estudiar el sistema radicular en condiciones que reflejen el entorno del suelo para proporcionar nuevos conocimientos y mejorar la transferencia de laboratorio al campo.

ACRONYM AND ABBREVIATIONS

Acronym and abbreviations

Abbreviation

22SR	Whole plants (shoot and roots) growing at 22°C
32SR	Whole plants (shoot and roots) growing at 32°C
	Plants growing with 32°C in shoots and a temperature gradient
32TGRooZ	in roots
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
AAS	Atomic Absorption Spectroscopy
ABA	Absciscic acid
ACC	l-aminocyclopropane-1-carboxylic acid
AM	Arbuscular mycorrhizae
ANK	Ankyrin repeat
ANOVA	Analysis of Variance
ASV	Amplicon sequence variant
<i>C. tofieldiae</i>	<i>Colletotrichum tofieldiae</i>
CLD	Compact letter display
CK	Cytokinin
CV	Coefficient of variation
cZ	cis-Zeatin
cZR	cZ riboside
DEG	Differentially expressed genes
DGR	Dark-grown roots
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin riboside
eLR	Emerged lateral root
FDR	False discovery rate
FW	Fresh weight
GLM	Generalized lineal model
GO	Gene ontology

GWAS	Genome-wide association study
<i>H. vulgare</i>	<i>Hordeum vulgare</i>
IAA	Indol-3-acetic acid
ICP-MS	Inductively coupled plasma mass spectrometry
In	Indium
InsP	Inositol phosphate
InsP₆	inositol hexakisphosphate or phytate
InsP₇	5PP-InsP ₅
InsP₈	1,5(PP) ₂ -InsP ₄
iP	isopentenyladenine
iPR	isopentenyladenosine
IPTs	ADP/ATP isopentenyltransferases
ITPK4	1,3,4-trisphosphate 5/6-kinase 4
JA	Jasmonic acid
KIG	Known ionome genes
LGR	Light-grown roots
LR	Lateral root
LRP	Lateral root primordia
MAF	Minimum allele frequency
MIPS2	<i>myo</i> -inositol 1-phosphate synthase 2
MS	Murashige-Skoog
OZ	Oscillation zone
PBS	Pre-branching site
PCoA	Principal coordinates analysis
PDA	Potato dextrose agar
PERMANOVA	Permutational multivariate analysis of variance
PGP	Plant growth promoting
PHL	PHRI-Like
P	Phosphorous
Pi	Ortho-phosphate
PIP5K8	phosphatidylinositol 4-phosphate 5-kinase 8

PP-InsP	Inositol pyrophosphates
PRL	Primary root length
PSR	Phosphate starvation response
PtdInsP	Phosphatidylinositol phosphates
PUE	Phosphorous use efficiency
QC	Quiescent center
QTL	Quantitative trait loci
ROS	Reactive oxygen species
RSA	Root system architecture
RT	Room temperature
SA	Salicylic acid
SD	Standard deviation
SNP	Single nucleotide polymorphisms
TF	Transcription factor
TGRooZ	Temperature Gradient in the Root Zone
tRNA	Transfer RNA
tZ	trans-Zeatin
tZR	tZ riboside
VOC	Volatile organic compound

1. INTRODUCTION

1. Introduction

Plants are essential primary producers for ecosystem function and food production. The root system anchors plants to the soil and mediates the direct interactions with the soil and its resources (namely water, mineral nutrients or microbial associations). Based on these characteristics, it is becoming evident that a proper root functionality is essential for plant growth and productivity. Root system architecture (RSA) refers to the spatial configuration of the root system in the soil, and it can be modulated by the soil properties (Lynch, 1995; de la Fuente Cantó *et al.*, 2020) as well as in response to different biotic or abiotic stresses. Nutrient availability, heat stress or microbial associations can also influence root growth, modifying primary root length (PRL), lateral root (LR) development or root hairs formation and elongation, thus altering the total root surface (Gruber *et al.*, 2013; Finkel *et al.*, 2020; Tiwari *et al.*, 2022). Climate change, in many cases, exacerbates the impact of different biotic stresses, such as drought, heat or salinity, increasing the negative impact on soil quality. Soil erosion, nutrient leaching, loss of organic matter or salinization are among the most important problems associated with increasing temperature, CO₂ concentration, or variations in the precipitation regime (Pareek, 2017; Borrelli *et al.*, 2020). Taking into consideration the essential function of the roots, the study of root system development and functionality is becoming of vital importance from an agronomic point of view (Kochian, 2016). Thus, the improvement of root system might represent a sustainable tool to solve some climate challenges and crop production issues (Yang & Tilman, 2020; Lynch, 2022).

1.1. Root system expansion: brief view of root development

Root development shapes root three-dimensional organization in the soil. Until now, the majority of root development studies have been done in the model plant *Arabidopsis thaliana*, a dicotyledonous plant with a dominant primary root from where

LR branch emerged to design the final root system (Motte *et al.*, 2019). The root apical meristem contains the stem cell niche, a group of undifferentiated cells that continuously divide to originate the different root tissues and to sustain root growth. Inside the root apical meristem, it is located the quiescent center (QC), a reservoir of stem cells that rarely divide (Fig. 1). This low division rate and the undifferentiated state is maintained by the expression of a set of genes that slows down cell proliferation, including WOX5 (Petricka *et al.*, 2012). As root grows and matures, four developmental zones can be distinguished longitudinally: meristematic, transition, elongation and differentiation zone (Fig. 1). The meristematic zone is where cell division primarily occurs to increase the pool of cells that contribute to root growth. Cell types with different fate are continuously dividing in the meristem without entering in a differentiated state (stem cells), to form the different cell layers (Petricka *et al.*, 2012; Perilli *et al.*, 2012). The transition and elongation zone is where the stem cells lose their capacity to divide and primarily grow longitudinally, through the vacuolar expansion (Petricka *et al.*, 2012). The differentiation zone is characterized by the complete differentiation of cell types, and a fast elongation, and it is where root hairs start to form (Petricka *et al.*, 2012). Thus, root growth is the result of two processes: cell division/differentiation in the meristematic zone, and cellular elongation in the elongation zone (Motte *et al.*, 2019). Both zones are regulated by plant hormones, being key auxins, cytokinin (CK) and reactive oxygen species (ROS) and their spatial distribution (Tsukagoshi *et al.*, 2010; Silva-Navas *et al.*, 2016; Motte *et al.*, 2019).

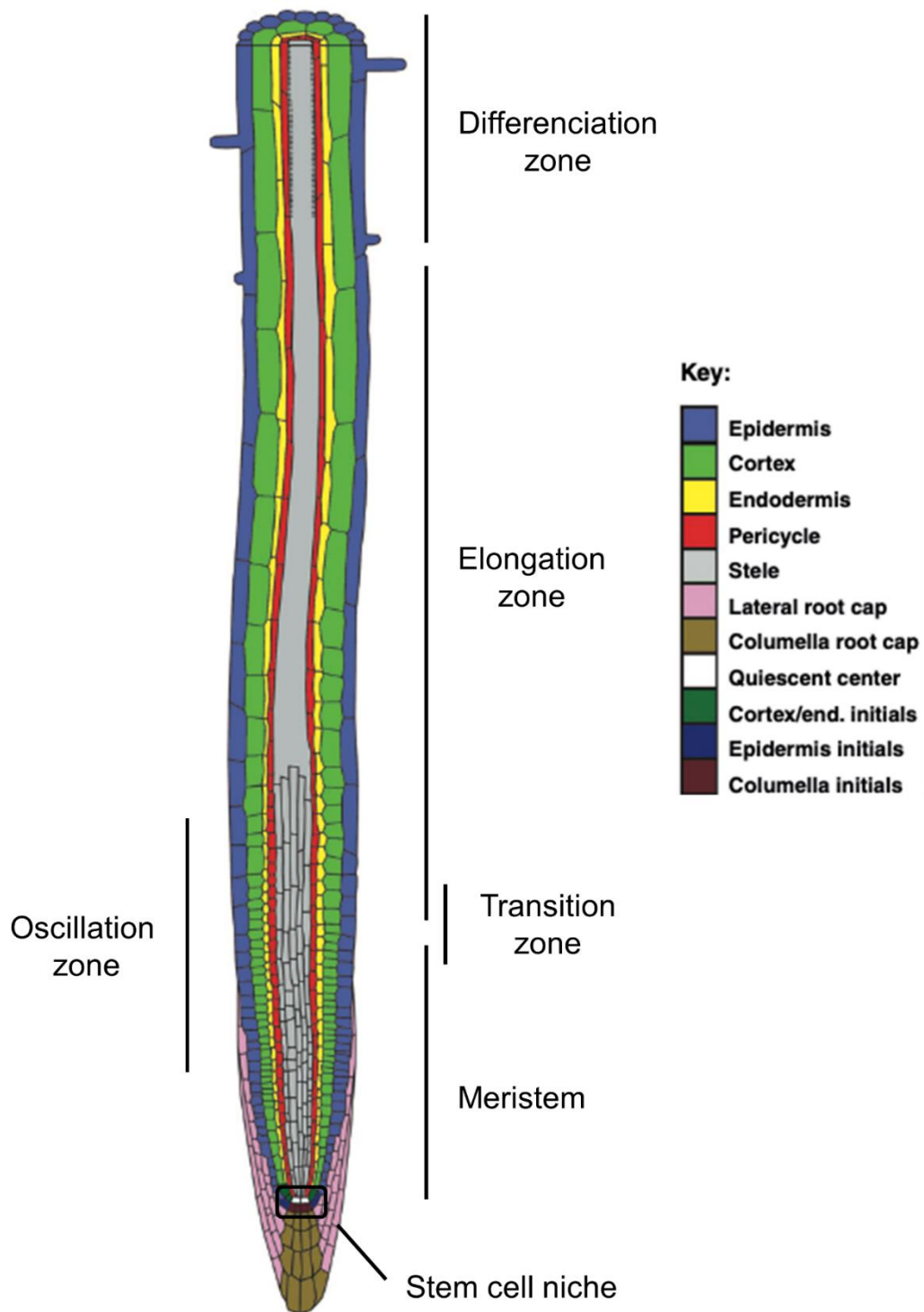


Fig. 1. *A. thaliana* root developmental zones and cellular types. Root organization of *A. thaliana*. There are four characteristic developmental zones longitudinally: meristem, transition, elongation and differentiation zones. From the stem cell niche emerge the different cellular layers composing the root that are color coded. The oscillation zone is located between the meristem and elongation zone, where cells are specified as lateral root founder cells by the root clock. Modified from: Ubeda-Tomás, *et al.* 2020.

Root branching, an essential feature of the root system for RSA expansion and soil exploration, occurs through sophisticated genetically controlled mechanisms. This process can be summarized in two distinct steps: LR specification and LR primordia (LRP) morphogenesis (Stoeckle *et al.*, 2018) (Fig. 2). LR specification is the process where a group of cells in the xylem pole pericycle are primed as lateral founder cells (Fig. 2a). This process depends on the root clock, which oscillates periodically in the so-called oscillation zone (OZ) in the meristem (Fig. 1) (Moreno-Risueno *et al.*, 2010). In the OZ, the root clock can be visualized by the *DR5::LUC* reporter that contains a synthetic auxin response promoter fused to the LUCIFERASE gene. Thus, *DR5::LUC* is expressed in response to auxin signaling, and its expression waves in the OZ in the basal meristem along with a large number of associated genes that are expressed in phase (i.e. oscillating at the same time that *DR5*). In addition, other set of genes show an opposite expression peak, called antiphase genes. The expression maximum of in-phase genes, including *DR5::LUC*, marks a few cells that forms a pre-branching site (PBS) in each oscillation (Fig. 2a). These PBS have the capacity to form fully functional LRs (Perez-Garcia *et al.*, 2022). Inside the PBS, the xylem pole pericycle cells are the initial set of cells with competence to originate LR (Fig. 2) (Motte *et al.*, 2019). Once specified, LR founder cells express *SKPB2* (S-Phase Kinase-Associated Protein2B), a F-box protein that negatively regulates cell cycle, inhibiting LR initiation (Manzano *et al.*, 2012). The second process, LRP morphogenesis, starts with LR founder cells nuclear migration followed by anticlinal and periclinal cell divisions that will form the new LR primordium (LRP) (Banda *et al.*, 2019) (Fig. 2b). To allocate space for the new cells that conform the LRP, *EXPASIN A1* (*EXPA1*) expression promotes the cell wall loosening in the initial divisions (Ramakrishna *et al.*, 2019). The process ends with the new LRP mechanically rupturing through endodermis and subsequent cell layers until its final emergence, forming an emerged LR (eLR) (Banda *et al.*, 2019). The phytohormone auxin plays a key role in the formation of LRs, through the selective degradation of Aux/Indol-3-acetic acid (IAA) repressors by the F-box family TIR1 and AFBs (Lavy & Estelle, 2016). Aux/IAA degradation releases positive AUXIN RESPONSE FACTOR (ARF) transcription factors (TFs). Among the different ARF members, *ARF7* and *ARF19* have a key role during the initiation of LR founder cell division to give rise to new lateral root primordia (LRP)

(Okushima *et al.*, 2007). Environmental signals, such as nutritional deficiencies or abiotic stresses, can modulate RSA by altering these processes (Motte *et al.*, 2019).

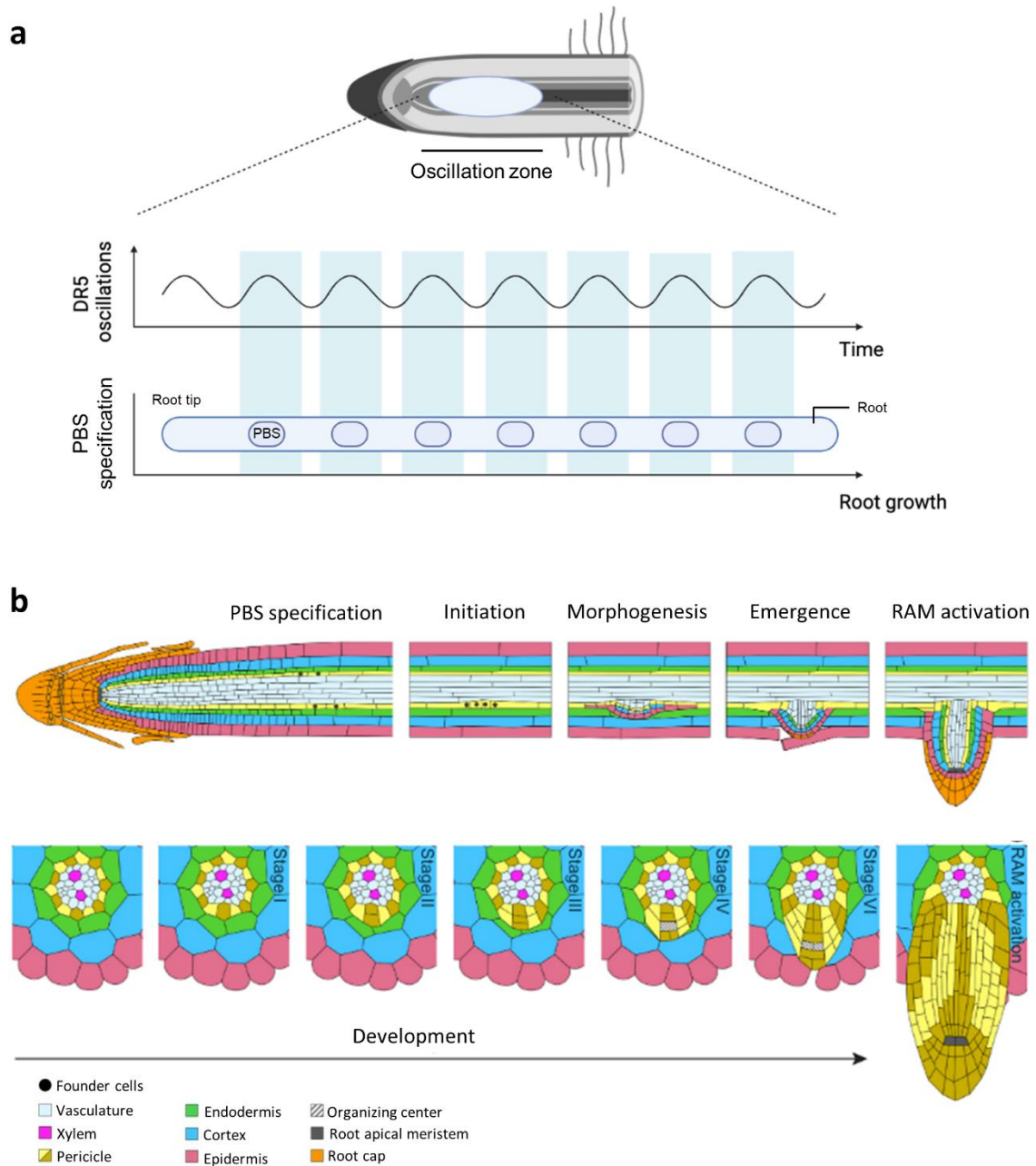


Fig. 2. Root branching: Root clock oscillations and LR emergence processes. **a** Root clock processes. In the oscillation zone (OZ), there is a transcriptional oscillation of genes that correlates with DR5 expression. The gene oscillations occurring in this zone specify the cells in each DR5 expression peak, producing the PBS patterning. Notice how the oscillation maximum in the root clock, represented in blue background, specifies PBS as root develops. **b** LR developmental processes from specification to emergence view along the root longitudinally (top) or radial cross-sections (bottom). The initiation starts

in PBS marked founder cells in the xylem pole pericycle cells. As the initiation process begins (Stages I-II), LR starts its morphogenesis from cellular divisions, acquiring the characteristic domo shape (Stages III-IV). When the LRP is mature enough, the developmental processes cause it to break the epidermis and emerge (Stages VI-VII). To completely form a functional LR, the root apical meristem is established (Stage VII). The colors indicate different cell types. Modified from: Banda, *et al.* 2019.

1.1.1. Light influence on RSA development

In vitro studies in model plants, mainly in *A. thaliana*, have provided rapid and in-depth molecular knowledge on how the development of the root system is regulated (Motte *et al.*, 2019; Serrano-Ron *et al.*, 2021). In nature, roots grow mainly underground, where light is generally blocked by the first 0.5 cm of the soil (Tester & Morris, 1987); therefore, root growth occurs mainly in darkness. However, most of the *in vitro* studies have been performed using standard laboratory growth systems and transparent agar-containing Petri dishes. This way, the entire plant, including the root system, is cultivated with constant illumination and temperature. In these conditions, the photosensors involved in the light root avoidance response are expressed in the roots (Silva-Navas *et al.*, 2016), indicating that direct illumination of the root system could have a major impact on root physiological responses through photoreceptor signaling.

Shoot light sensing also plays a role in root development. Photomorphogenesis is the developmental response of plants to the exposure to light, independently of photosynthesis (Arsovski *et al.*, 2012; Lee *et al.*, 2017). Light is sensed by the plant in the shoot and then either directly transmitted through internal tissues (stem-piped light) or the signal transduced through mobile molecules to roots (Lee *et al.*, 2017; van Gelderen *et al.*, 2018b; Yang & Liu, 2020). *HY5* seems to be a key factor in coordinating the communication between above- and belowground parts, by moving it-self from the shoot to the root and sending other signals to the root, to control growth and nutrient uptake (Chen *et al.*, 2016; van Gelderen *et al.*, 2018a; Burko *et al.*, 2020). Interestingly, most of *HY5* responses impacting root development are not affected by root illumination (Zhang *et al.*, 2019b). However, other photomorphogenesis factors, such

as PHYB, are closely related to thermomorphogenesis processes that could be drastically different in roots in natural conditions compared to *in vitro* illumination or changes in root temperature (D E Lima *et al.*, 2021). PHYB and the DET1-COPI-HY5 network, which is involved in photomorphogenesis, coordinates plant growth in response to temperature (Delker *et al.*, 2014; Legris *et al.*, 2016) and plant Pi uptake (Sakuraba *et al.*, 2018). In this context, the root elongation induced by moderate-high temperatures is dependent on light intensity (Fei *et al.*, 2019). In nature, roots grow across a soil temperature gradient, which is achieved by the geothermal properties of soil, which largely differs from homogeneous temperatures normally used in *in vitro* cultures. These observations point out that root illumination or other unnatural conditions, such as root temperature, may affect some of the signals transported from the aerial part of the plants to the roots and vice versa, especially in experiments performed at extremely high temperatures.

White light is composed of different wavelengths that have a direct role in root growth. Blue light produces shorter roots with less LR (Moni *et al.*, 2015; Silva-Navas *et al.*, 2015), red light mediates positive phototropism (Kiss *et al.*, 2003) and ultraviolet-B light reduces LR density (Yang *et al.*, 2020). These studies demonstrate that root-expressed photoreceptors perceive light to generate different alterations of phytohormone signaling, organization of the cytoskeleton and, eventually, primary and LR formation and length. In addition, direct illumination of roots produces a stress that affects the normal root development and the responses to other environmental clues. Thus, root illumination produces an unbalanced amount of ROS (Yokawa *et al.*, 2011; Silva-Navas *et al.*, 2015). Furthermore, light has a negative effect on root growth and response to nitrate deficiency or salinity compared with dark-grown roots (DGR) (Silva-Navas *et al.*, 2015; Conesa *et al.*, 2020). Altogether, these data show that root illumination can influence RSA and acts as a stress that could alter some physiological responses (Fig. 3). Therefore, the use of devices such as the D-root to maintain roots in the dark to perform *in vitro* experiments could provide novel insights into how roots response and adapt to changes in the environment.

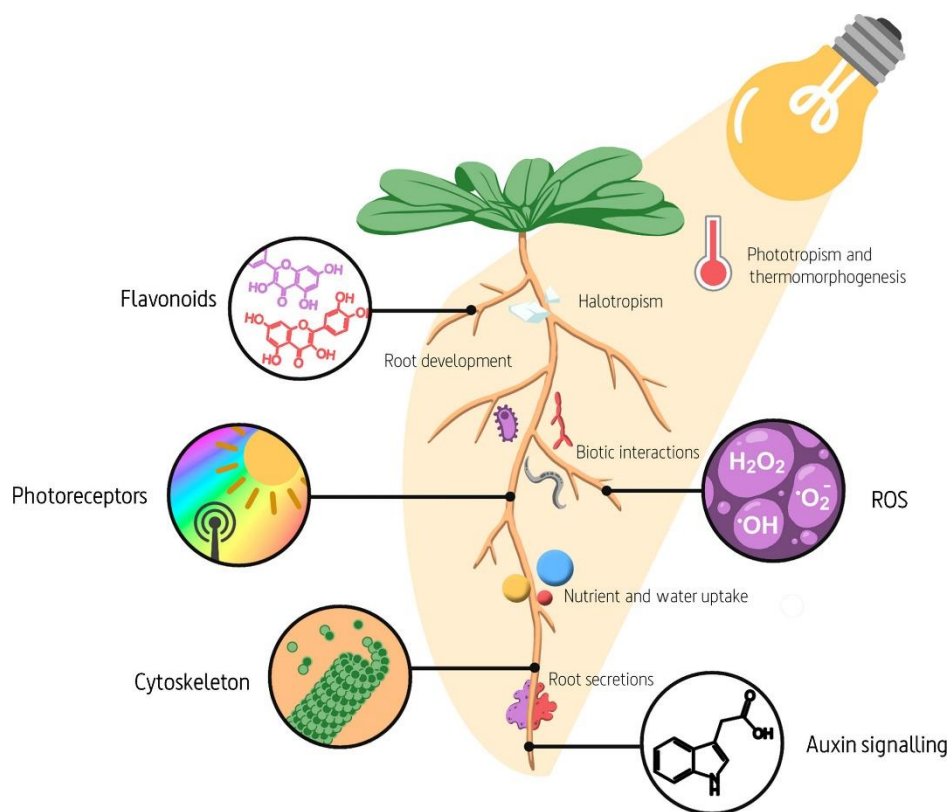


Fig. 3. Root illumination affects root processes. Graphical representation of root processes and environmental interactions altered by the direct effect of root illumination under standard experimental conditions. Bubbles highlight root processes that are altered by illumination. In the illuminated area, we highlight the factors from the plant–environment interaction biased by light in roots. ROS, reactive oxygen species.

1.2. Phosphate starvation response (PSR) in plants

Phosphorous is one of the most important nutrients for plant growth and development. The absorbable form for plants is ortho-phosphate (H_2PO_4^- and HPO_4^{2-} ; hereby Pi), but its concentration in soil is limited (around $2 \mu\text{M}$ in most cases) and tends to accumulate in the soil top layers (Raghothama, 1999). Additionally, soil P can be present in organic forms and inorganic complexes, which are not available for plant uptake. To overcome this problem and enhance plant productivity, Pi fertilization is widely used. However, Pi fertilization use efficiency is low, since more than 70% can be loosen due to soil fixation, microbial activity or leaching (López-Arredondo *et al.*, 2014).

Furthermore, Pi resources are limited, as they are non-renewable. This limitation is rising the prices of fertilizers, presenting a challenge for future agriculture productivity and a food security problem (Fig. 4a) (Elser, 2012). Thus, improving plant Pi availability and use efficiency is becoming a key issue from the economic and sustainable point of view (Fig. 4b). Understanding plant molecular and physiological processes that underlay Pi starvation responses is fundamental to develop new breeding strategies to improve nutrient efficiency.

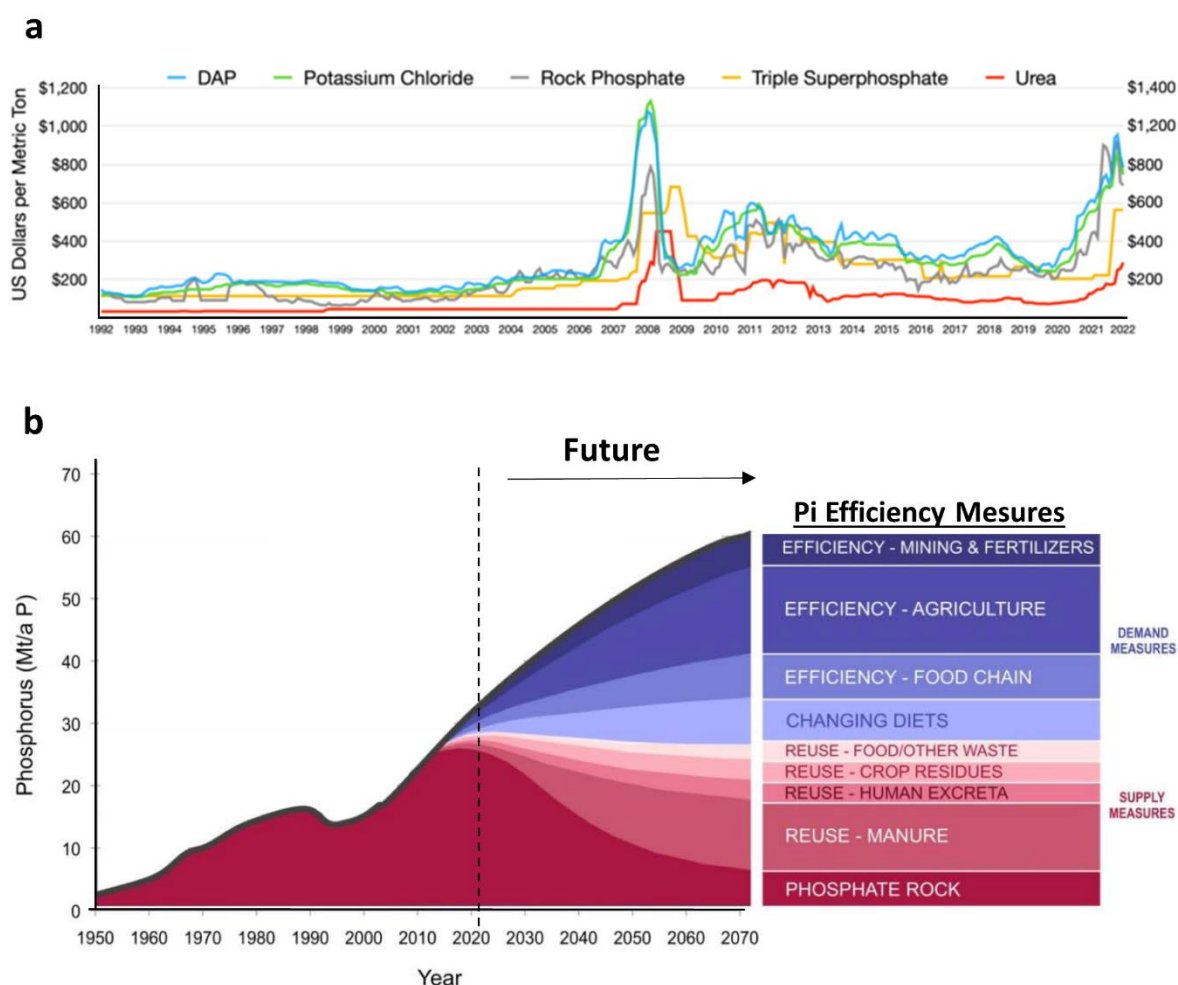


Fig. 4. Phosphorous fertilization. **a** Prices of nitrogen (urea and dap), potassium (potassium chloride) and phosphorous (dap, rock phosphate and triple superphosphate) fertilizers from 1992 to 2022. DAP – Diammonium phosphate. Triple superphosphate – Fertilizer made by mainly $\text{Ca}(\text{H}_2\text{PO}_4)_2$. (Source: Wikimedia by Wikideasl. Data: www.indexmundi.com). **b** Phosphorous current demand and projected usage. Measures for sustainable phosphorous usage in demand and supply are specified. Notice the agriculture efficiency projected impact. (Modified from: Cordell & White, 2013).

1.2.1. PSR in plants: signaling and molecular components

Plants have evolved complex regulatory mechanisms and crosstalk signaling pathways to maintain their cellular and organs Pi homeostasis (Baek *et al.*, 2017). In low Pi level environments, plants activate a Pi starvation response (PSR) aimed at mobilizing and uptaking Pi from the rhizosphere and to reallocate endogenous Pi from storage tissues (Fig. 5). This PSR involves metabolic readjustments, morphological changes and the activation of specific transcriptional programs, mainly regulated by the PHR1-miR399-PHO2 module (Rubio *et al.*, 2001; López-Bucio *et al.*, 2003; Bari *et al.*, 2006; Bustos *et al.*, 2010; Péret *et al.*, 2011; Secco & Whelan, 2014). Cellular Pi starvation signaling is mediated by the SPX-InP₈-PHR complex (Fig. 6) (Dong *et al.*, 2019; Ried *et al.*, 2021). When Pi levels are high, cellular ATP is high, and inositol hexakisphosphate (InsP₆ or phytate) is phosphorylated sequentially to form inositol pyrophosphates (PP-InsPs), 5PP-InsP₅ (InsP₇) and 1,5(PP)₂-InsP₄ (InsP₈), respectively, in an energy dependent process (Wang *et al.*, 2021). InsP₈ mediates the interaction between SPX and PHR proteins, thus sequestering PHR and PHR1-Like (PHL) TFs that are responsible of PSR transcriptional activation (Paz-Ares *et al.*, 2022). On the contrary, when Pi levels are low, cellular ATP is low and InsP₈ levels are depleted (Dong *et al.*, 2019). Then, PHR, and its closest homologous PHL, are released from SPX inactivation and bind to genes containing PIBS motives in their promoter, activating their expression to modulate the PSR (Paz-Ares *et al.*, 2022). In response to low Pi levels, miR399 is expressed to enhance the PHO2 mRNA degradation. PHO2 is an ubiquitin-conjugating E2 enzyme involved in PHO1 degradation, a xylem loading Pi transporter. Thus, under Pi deficiency, Pi xylem transport is restricted through this mechanism, increasing the root:shoot Pi ratio to allocate Pi in the roots. Under Pi deprivation, plants also induce the expression of high affinity transporters of the *Phosphate Transporter* (PHT) super-family, encoding transporters for epidermal cells in contact with soil (PHTIs) as well as transporters for the subcellular compartment distribution (López-Arredondo *et al.*, 2014).

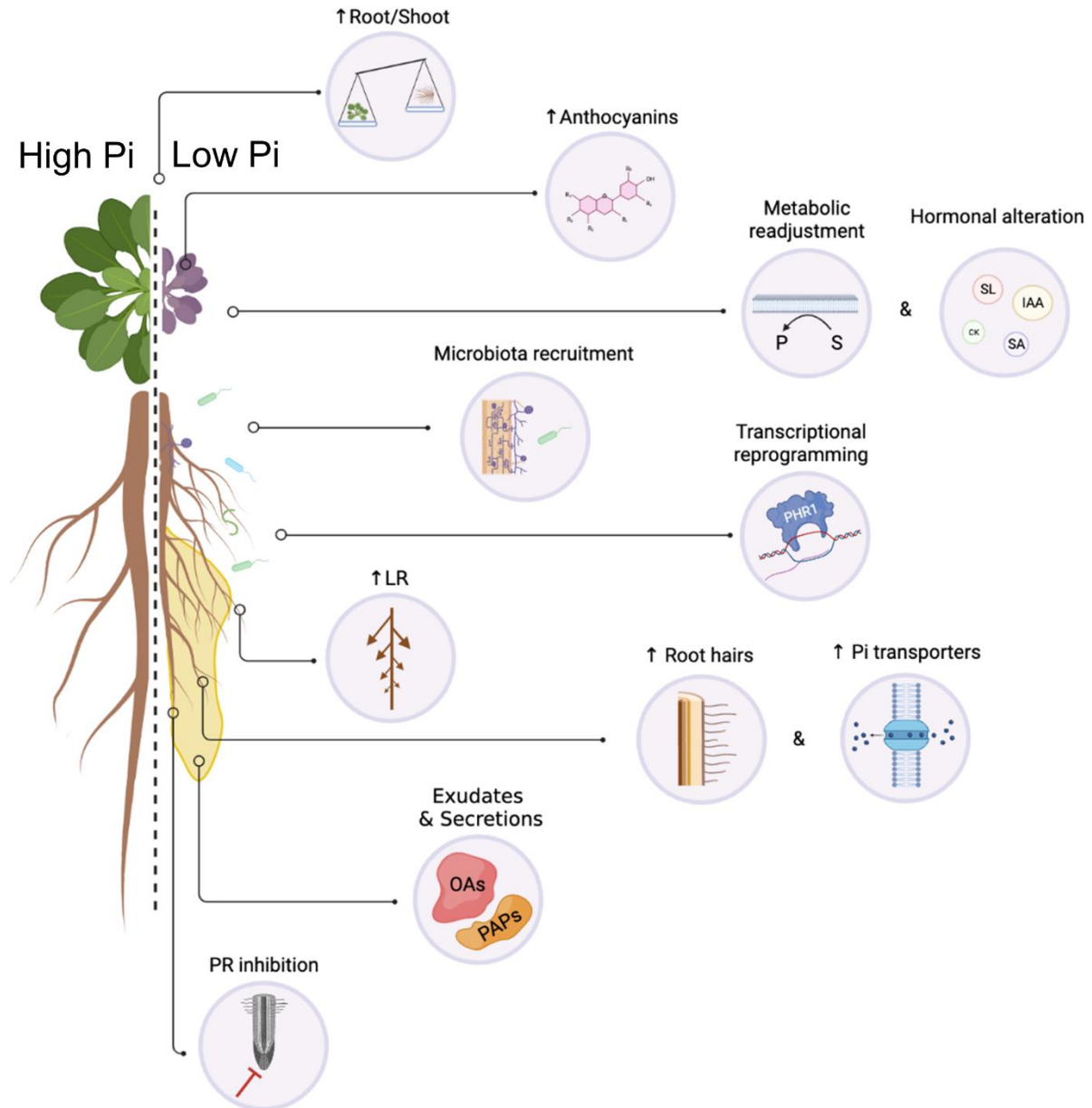


Fig. 5. PSR response in plants. Graphical synthesis of plant PSR processes, highlighted in bubbles. *A. thaliana* plant growing under high phosphate conditions (left) and under low phosphate (right). Phosphate deficiency in *A. thaliana* is described to block the principal root elongation. In addition, secretion of phosphatases and exudates are released to increase Pi availability. Roots also increase the root hair production and elongation and induce specific Pi transporters. To search for Pi, root branching is enhanced through increasing the number of LR. To deploy PSR, there is an extensive transcriptional reprogramming mediated, among others, by PHR1. Microbial recruitment is modified in PSR and mediates some beneficial interactions such as arbuscular mycorrhizae symbiosis. Metabolic readjustment occurs to scavenge Pi, i.e. phospholipids are substituted with sulpholipids and there is an increase in anthocyanin synthesis. PSR responses are coordinated by phytohormones through their level alterations. As root development is enhanced in phosphate starvation, root:shoot ratio increases in plants.

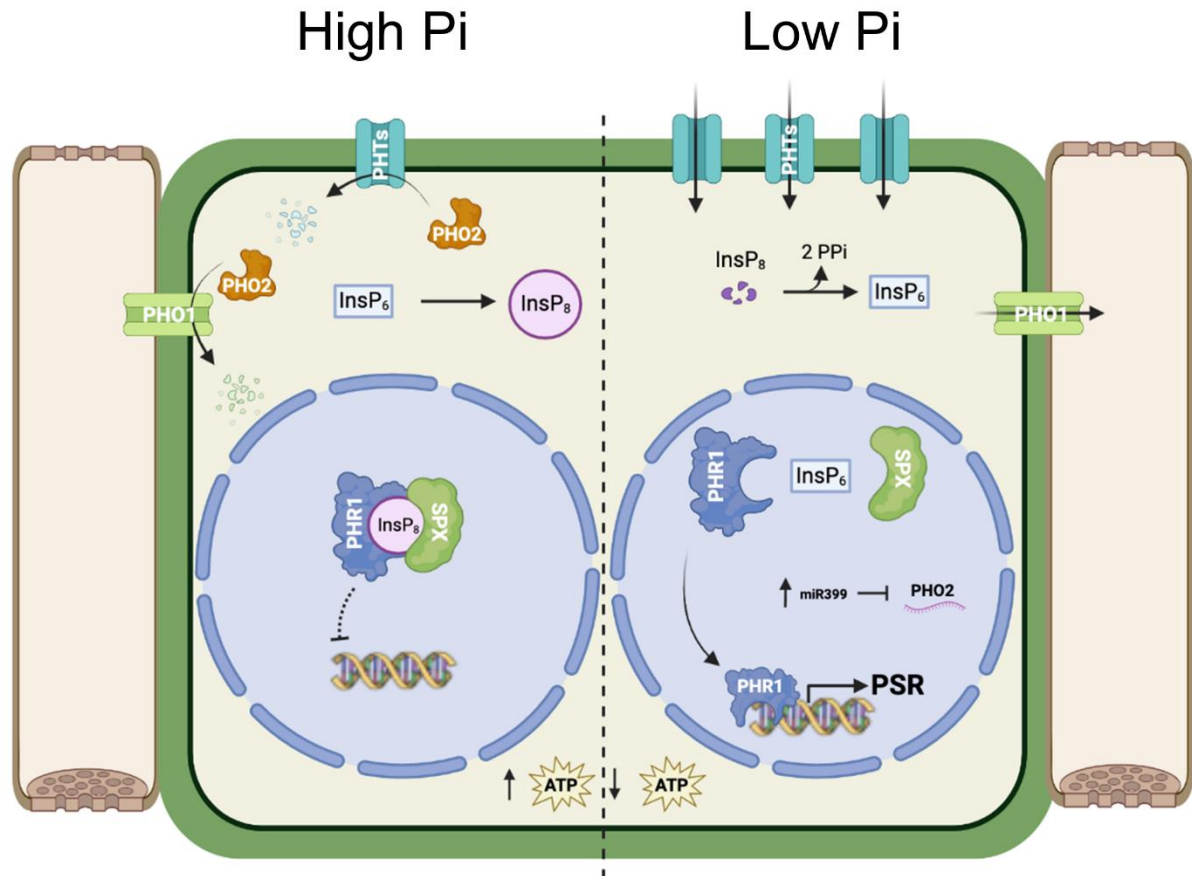


Fig. 6. Cellular PSR signaling in plants. Representation of PSR cellular signaling in plant cells. Under high Pi, InsP₆ is converted to InsP₈ that allows the binding of PHR1/PHL TFs with SPX proteins. This PHR-InsP₈-SPX complex prevents the TF regulation of transcription. PHO2 mediates the degradation of PHTs and PHO1 Pi transporters. Under low Pi conditions, PP-InsP as InsP₈ are degraded to InsP₆ generating PPi and obtaining energy. As InsP₈ is degraded, PHR/PHLs are liberated producing the PSR transcriptional response. As the transcriptional response occurs, miR399 is induced and mediates PHO2 mRNA degradation thus preventing the Pi transporter degradation.

Inositol phosphates (InsP) regulate and interconnect Pi signaling and energetic processes, making these compounds key elements in PSR as signaling mediators (Lorenzo-Orts *et al.*, 2020; Wang *et al.*, 2021). InsP₈ synthesis starts with glucose-6-phosphate to form myo-Inositol, reactions catalyzed by MIPS enzymes (Fig. 7) (Donahue *et al.*, 2010; Cridland & Gillaspay, 2020). Myo-inositol is subsequently phosphorylated in two different pathways that converge in the formation of InsP₅: the lipid-dependent pathway, in which phosphatidylinositol phosphate (PtdInsP), component of the cellular membranes, is the substrate of different enzymes as the

Phosphatidylinositol-4-phosphate kinases (PIP4K and PIP5K gene families), or the lipid-independent pathway, in which myo-inositol is directly phosphorylated by INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 4 (ITPK4) (Cridland & Gillasp, 2020; Wang *et al.*, 2021). The successive phosphorylation of InsP₅ to InsP₈ is mediated by different enzymes, all of which are necessary to active PSR (Kuo *et al.*, 2018; Dong *et al.*, 2019; Riemer *et al.*, 2021). The intermediate products of this synthesis pathway have multiple functions; PtdInsPs intermediates are specific components of the subcellular membranes and regulators of membrane trafficking (Krishnamoorthy *et al.*, 2014), phytate has a role as seeds Pi reservoir or as cofactor of TIRI-Auxin binding (Tan *et al.*, 2007) and PP-InsP (InsP₇ and InsP₈) is an energy reservoir (Lorenzo-Orts *et al.*, 2020). The auxin binding TIRI is an important regulator under Pi starvation, as TIRI-ARF19 regulates both LRP formation and development (Pérez-Torres *et al.*, 2009).

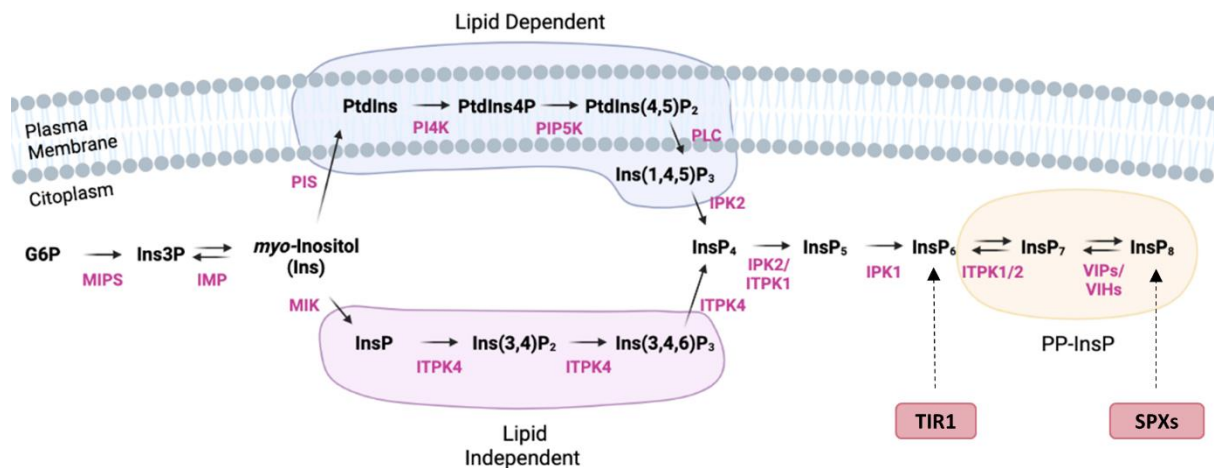


Fig. 7. Schematic pathway of InsP and PP-InsP synthesis in *A. thaliana*. InsPs are synthesized by two pathways: Lipid dependent (blue background) and lipid independent (purple background). PP-InsP are synthesized from InsP₆ (phytate) (yellow background). Enzymes, represented in purple, are indicated below the reaction arrows: MIPS: inositol-3-phosphate synthases; IMP: inositol monophosphatase; PIS: phosphatidylinositol synthase; PI4K: phosphatidylinositol 4-kinase; PIP5K: phosphatidylinositol-4-phosphate 5-kinase; PLC: phospholipase C; IPK2: inositol polyphosphate 6/3-kinases; MIK: myo-inositol kinase; ITPK4: inositol 1,3,4-trisphosphate 5/6-kinase; ITPK1: inositol 1,3,4-trisphosphate 5/6-kinase; IPK1: inositol tetra-/pentakisphosphate 2-kinase; VIP/VIHs: inositol hexakisphosphate and diphosphoinositol-pentakisphosphate. Arrows: forward reaction catalyzed by the enzyme. Double arrows: reversible reaction catalyzed by the enzyme.

1.2.2. RSA responses to Pi deprivation

PSR involves many morphological changes aimed at enlarging the root surface area to search for new patches of Pi in the soil (Fig. 5). As Pi is an immobile nutrient, it tends to locate at the top part of the soil. In *A. thaliana*, *in vitro* response to PSR involves the reduction of the primary root growth and the increase in LR density and root hairs. The reduction of the primary root occurs through the LPR1/2-PDR2 module of local Pi sensing, that is dependent on the iron:phosphate ratio. Under Pi deficiency, LPR1 (a multicopper oxidase) oxidizes Fe^{2+} to Fe^{3+} , leading to ROS production and callose deposition in the root meristem. The callose deposition leads to a reduction of root meristem activity that results in reduction of root growth (Ticconi *et al.*, 2009). However, this PR inhibition has been questioned, as it seems, at least in part, consequence of a malate-mediated photo-Fenton reaction triggered by blue light (Zheng *et al.*, 2019). In that sense, root illumination also induces iron accumulation on normal growth conditions that could accentuate this effect under low Pi availability (Silva-Navas *et al.*, 2015). PSR also promotes the emergence of LRs by increasing auxin accumulation and the expression of its receptor *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) in LRP (Crombez *et al.*, 2019) and enhances LR growth (López-Arredondo *et al.*, 2014). To maximize the root surface to capture Pi, plants stimulate root hair development, which can be responsible of the majority of Pi uptake (López-Arredondo *et al.*, 2014). Low Pi, mediated by ARF19, induces *RSL2* and *RSL4* TF expression that are responsible for root hair development (Bhosale *et al.*, 2018).

The PSR is interconnected with several hormonal signaling pathways (Martín *et al.*, 2000; Rouached *et al.*, 2010; Baek *et al.*, 2017). Among them, CKs play an important role in controlling the balance of root:shoot growth ratio. Pi starvation reduces CK concentrations and the expression of the CK receptor CYTOKININ RESPONSE 1 (CRE1/AHK4) (Kuiper & Steingröver, 1991; Franco-Zorrilla *et al.*, 2002). CK-signaling reduction leads to an increase in the root:shoot ratio by favoring root growth in detriment of shoot growth, similarly to PSR. CKs also downregulate genes induced by Pi starvation (Martín *et al.*, 2000; Franco-Zorrilla *et al.*, 2002), probably by increasing intracellular Pi (Wang *et al.*, 2006). In addition, *ARF16* negatively regulates Pi signaling

and uptake in response to CK (Shen *et al.*, 2014). These data indicate that CKs play an important role in the control of PSR and plant adaptation to low Pi concentrations. The CK receptors can perceive different CK types with different affinities (Heyl *et al.*, 2012). The most abundant CK type in *A. thaliana* is the trans-zeatin (tZ), which is synthesized by the ADP/ATP isopentenyltransferases (IPTs). Another zeatin isomer present in higher plants is the cis-zeatin (cZ), although its function is less understood (Romanov *et al.*, 2006; Heyl *et al.*, 2012). cZ is synthesized by IPT2 and IPT9, two transfer RNA (tRNA) IPT enzymes, using tRNA degradation products (Miyawaki *et al.*, 2006). cZ-type has been involved in the regulation of plant growth but also in the response to different biotic and abiotic challenges (Großkinsky *et al.*, 2013; Schäfer *et al.*, 2015).

1.2.3. Genetic approaches to identify PUE genes in plants

The increasing agronomic interest in breeding crops with higher phosphorous use efficiency (PUE) has led to the exploration of the genomic landscape of Pi acquisition and usage. Uncovering the molecular basis of PSR has led to a better understanding of plant response and breeding targets. However, genetic engineering of PHR transcriptional regulators or PHT transporters has yield different results and not always producing a higher plant yield (López-Arredondo *et al.*, 2014), suggesting that finer manipulation and better understanding of the regulation are needed to successfully achieve this goal.

As Pi is an immobile nutrient, it tends to be located at the top part of the soil. Breeding root phenotypes are aimed at improving RSA by increasing the total root surface, through the development of LR_s at the top and increasing root hairs to enhance Pi acquisition (Bates & Lynch, 2001; Lynch & Brown, 2001; Lynch, 2019; Liu, 2021). LR_s are key players in this response as they enhance root surface to explore new soil areas and increase the surface to develop higher number of root hairs (Heppell *et al.*, 2015). Quantitative trait loci (QTL) implicated in RSA modification for enhanced Pi acquisition have been reported for different species as oilseed rape, rice, sorghum, maize or bean (Rogers & Benfey, 2015; Gu *et al.*, 2016a; Duan *et al.*, 2021). In rice,

PSTOL1, which encodes a protein kinase, confers tolerance to Pi deficiency through expanding RSA and has been successfully translated into field (Gamuyao *et al.*, 2012). Other strategy to increase RSA and nutrient uptake has been hormonal manipulation, such as diminishing specifically root CK levels to enlarge RSA and improve P uptake (Ramireddy *et al.*, 2018; Paz-Ares *et al.*, 2022). Altogether, these examples illustrate the importance of PSR modulation of RSA towards plant Pi nutrition.

Natural variability is an important source of information on relevant traits for PUE (Paz-Ares *et al.*, 2022). Plant polymorphisms are the result of natural evolutionary processes tested over plant-life history that could improve our understanding of PSR (Alonso-Blanco *et al.*, 2009). For instance, *LPRI* effect in PRL was identified as a QTL from two recombinant lines in *A. thaliana* (Reymond *et al.*, 2006). Genome-wide association studies (GWAS) have proved to be a successful strategy to identify PUE genes in crops (Zhang *et al.*, 2014b; Luo *et al.*, 2019; Giovannetti *et al.*, 2019; Kohli *et al.*, 2020). In *A. thaliana*, different studies have analyzed PUE and RSA using natural variation (Narang *et al.*, 2000; Chevalier *et al.*, 2003). The genetic bases of Pi accumulation in shoots has been approached by GWAS, leading to the association of *PP2A-2* and identification of *PHT1* locus involvement in this response (specifically *PHT1;l-3*) (El-Soda *et al.*, 2019; Yi *et al.*, 2021). Similarly, the TF *MYB52* regulation of *PHT1;l* has been discovered through GWAS (Chien *et al.*, 2022). These findings shed light on the usefulness of GWAS approaches to identify genetic regulators of PUE.

1.3. Microbiome: bacteria and fungi in plant PSR

In natural environments, plants interact permanently with microbes competing for and/or exchanging resources. Plants have evolved strategies to signal and establish beneficial interactions with bacteria or fungi in exchange for carbonated compounds that are fixed during plant photosynthesis (Parniske, 2008; Singh *et al.*, 2022). Unavailable Pi soil (organic or salt-bound and precipitated Pi) can be transformed into plant available Pi through solubilization and mineralization processes by specific

microbes (Singh *et al.*, 2022). Under Pi starvation, plants PSR mediates signaling and assembly of beneficial microbiota that enhance their Pi nutrition (Hiruma *et al.*, 2016; Castrillo *et al.*, 2017; Almario *et al.*, 2017; Das *et al.*, 2022).

1.3.1. Fungal endophytes under Pi limiting conditions

Arbuscular mycorrhizae (AM) widespread obligate symbiosis are well known for their capacity to transfer Pi to the plant (Parniske, 2008). However, around 10-20% of the land plants are not able to establish AM symbiosis, including the Brassicaceae family (Hiruma *et al.*, 2018). Brassicaceae is a family with a high agronomic and economic value (Jabeen, 2020). Among this family, *A. thaliana* has been established as the model plant during the last 30 years due to its small genome size, the genetic resources, its genetic transformation, small size, etc (Woodward & Bartel, 2018; Koch, 2019). Despite this lack of AM symbiosis, Brassicaceae can host endophytic fungi and establish mutualistic interactions with them (Hiruma *et al.*, 2018; Poveda *et al.*, 2022). Fungal endophytes live inside plant tissues asymptotically and can establish mutualist relationships with the plant (Saikkonen *et al.*, 1998). These microorganisms are an essential part of plant microbiota, occurring in all plants and environments (Saikkonen *et al.*, 1998; Trivedi *et al.*, 2020) and spanning different fungal taxa, which can vary in their host range (Saikkonen *et al.*, 1998; Aly *et al.*, 2011). It has been well described that, under low Pi conditions, the root fungal endophytes *Colleotrichum tofieldiae*, *Serendipita indica* or *Helotiales sp.* F229 transfer Pi to the plant (Bakshi *et al.*, 2015; Hiruma *et al.*, 2016; Almario *et al.*, 2017), although their mechanisms of interaction with the plant and Pi transfer are not fully understood. This highlights the function of fungal endophytes under Pi deficiency as plant growth promoting (PGP) microorganisms capable of phosphorous transference similarly to AM in nonmycorrhizal plants, and the need of further studies to delve into the knowledge of these microorganisms (Hiruma *et al.*, 2018; Poveda *et al.*, 2022). Furthermore, in addition to nutritional enhancement, fungal endophytes can protect plants against other abiotic or biotic stresses. Thus, for example, *S. indica* increases tolerance to salinity (Lanza *et al.*, 2019), and endophytes of the genus *Trichoderma* act in the biocontrol of nematodes (Poveda *et al.*, 2020). These properties

remark the importance of fungal endophytes as PGP and their potential use as biotechnological solutions for crop production (Pozo *et al.*, 2021).

As already stated previously (Section 1.1.1., Fig. 3), root illumination, through the alteration of auxin signaling and the overproduction of ROS, may alter the plant interaction with other organisms. Furthermore, the broad presence of photoreceptors in plant-associated bacteria and fungi suggests that their exposure to light may have an impact on their growth and communication with the plants during *in vitro* studies (Bonomi *et al.*, 2012; Losi & Gärtner, 2021; Xue *et al.*, 2021). When exposed to different wavelengths light, some root-interacting bacteria and/or fungal species, such as *Bacillus thuringiensis*, *Alternaria alternata*, *Rhizobium leguminosarum* and *Fusarium* sp. modify the production of secondary metabolites, growth rate, motility, sporulation or resistance to ROS (Losi & Gärtner, 2021). The beneficial effect of *C. tofieldiae* on root growth under normal or Pi deficiency conditions is altered under different light intensities (Frerigmann *et al.*, 2021). This indicates that the role of light in bacterial and fungal interactions with plants should be considered under experimental conditions that limit the root illumination, since the root-microbes interactions occur mainly in darkness.

1.4. Root temperature effect on plant responses to high temperature, nutrition and microbiome

High temperature is an adverse condition that enormously affects plant growth and fitness (Bhattacharya, 2019). Nowadays, climate change is leading to more frequent extreme high temperatures, such as heat or freezing waves, that aggravate the negative impact on plant development and agricultural yield (Gray & Brady, 2016; Miller *et al.*, 2021). These extreme events are projected to increase in extension and intensity in the future (Ebi *et al.*, 2021). Heat stress causes important damage to proteins, disturbing their synthesis, folding, or changing the activity of enzymes, as well as harming membranes and cellular structures by massive oxidation (Hasanuzzaman *et al.*, 2013).

It also exerts different effects on physiological processes, such the reduction in photosynthetic efficiency, regulation of the respiration and transpiration rates and mineral nutrition (Giri *et al.*, 2017; Zhao *et al.*, 2020).

1.4.1. Plant responses to high temperature

To cope with heat-associated challenges and to ensure fitness, plants have evolved several adaptive strategies that involve, among others, gene expression changes, metabolic adjustments, and modification of morphological structures/organs (Zhao *et al.*, 2020). High temperature significantly alters plant gene expression and protein accumulation in *Glycine max* (soybean), *A. thaliana* or *Agrostis* grass species, among others (Xu *et al.*, 2008; Valdés-López *et al.*, 2016; Bellstaedt *et al.*, 2019; Gaillochet *et al.*, 2020; Sriden & Charoensawan, 2022). It also affects cell division and differentiation, reducing plant growth and development (Qi & Zhang, 2019; Liu *et al.*, 2022). Heat stress activates a transcriptional response mainly through the TF *HSFA2*, which is necessary to acquire tolerance to repeated exposure to high temperatures (Charng *et al.*, 2007; Liu *et al.*, 2011; Friedrich *et al.*, 2021). Heat also induces the expression of heat shock proteins (HSP), which are involved in preventing protein misfolding and aggregation (Al-Whaibi, 2011). HSPs function as molecular chaperones that prevent the denaturation and aggregation of proteins by correctly folding them (Al-Whaibi, 2011). HSPs are divided in five families according to their molecular weight, among which HSP70s and HSP90s have an important role protecting from heat associated damage (Al-Whaibi, 2011). The *HSFA* family of TFs, master regulators of the heat stress response, are repressed by HSP70-HSP90s. Once heat stress is produced, protein denaturation produces the recruitment of HSP70-HSP90s chaperones, thus releasing the HSFAs (Scharf *et al.*, 2012). Among the HSFAs, *HSFA2* is an essential regulator of the transcriptional response induced by heat and is necessary for the acquired thermotolerance memory (Charng *et al.*, 2007; Friedrich *et al.*, 2021).

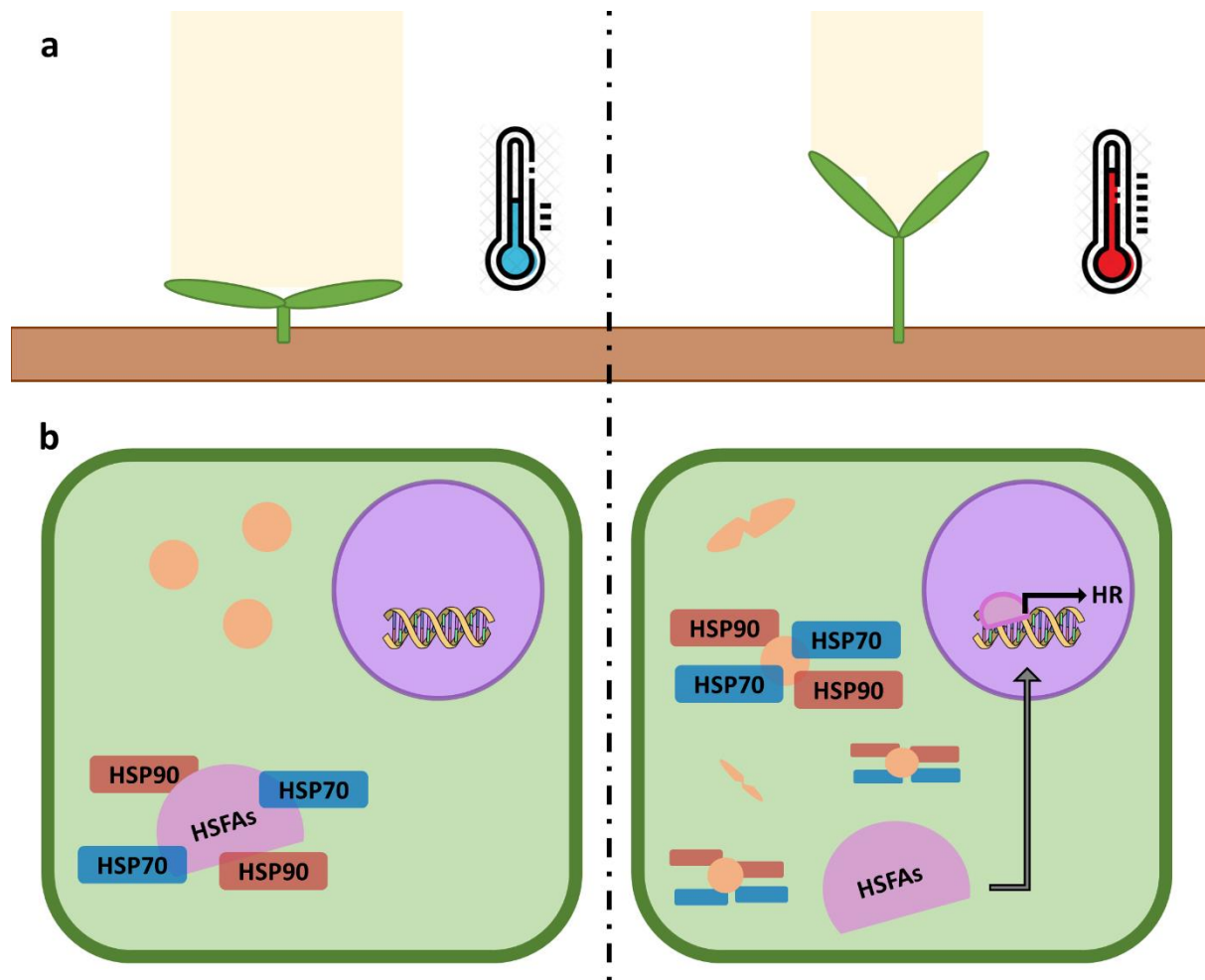


Fig. 8. Plant morphological and molecular responses to increase in temperature and heat stress.
a Morphological shoot responses of *A. thaliana* to temperature increase: hypocotyl elongation and petiole leaf angle increase. **b** Molecular responses to heat stress. Under normal conditions, HSFs TFs are repressed by HSP70-90s. When plant cells are heat stressed, HSP are recruited by proteins to maintain their folding or refold denatured proteins thus liberating the HSFs. Then HSFs move to the nucleus and produce the transcriptional Heat Response (HR). Notice that HSPs proteins are induced by heat stress.

Thermomorphogenesis is the developmental and differentiation process induced by the exposure to increased mild temperatures (Ding *et al.*, 2020). One of the best studied responses to warm temperatures is hypocotyl elongation, which increases linearly with temperatures until the optimum. If the temperature goes over, it begins to be detrimental (Gray *et al.*, 1998; Ding *et al.*, 2020). Hyponasty, the upward bend of petiole angles, is also induced by warm temperature and is also associated to reduce the surface expose to light irradiance in response to heat (Quint *et al.*, 2016; Ding *et al.*,

2020; Zanten *et al.*, 2021). Both responses are deployed to protect the tissues, separating them from soil heat irradiation and cooling them (Quint *et al.*, 2016).

In comparison to shoot, root responses to high temperatures have been understudied, and the majority of these studies have been focused on the thermomorphogenesis responses, rather than on heat stress responses (Nagel *et al.*, 2009; Bellstaedt *et al.*, 2019; Gaillochet *et al.*, 2020; Lee *et al.*, 2021). However, RSA plays an essential role in the absorption of water and nutrients and in communication with the microbiota, processes that are severely affected by soil temperature changes (Hatfield & Prueger, 2015; Giri *et al.*, 2017; Onwuka, 2018; Sabri *et al.*, 2018). Nevertheless, in the last years, there is an increasing interest in deciphering the role of roots in the plant adaptation to heat (Huang *et al.*, 2012; Calleja-Cabrera *et al.*, 2020; Tiwari *et al.*, 2022), driven primarily by the general concern on how climate change will affect crop production in agricultural systems (Fahad *et al.*, 2017). RSA changes in response to heat have genetic bases that might differ between species, which might have adapted to different optimum temperatures for root growth and LR production (Zhang *et al.*, 2015; Gray & Brady, 2016). For example, it has been shown that, depending on the plant species, a temperature increase promotes (Lahti *et al.*, 2005) or inhibits (Larkindale *et al.*, 2005; Takahashi *et al.*, 2019) root growth.

Many studies aimed at understanding how plants respond to heat or warming temperatures have been carried out (Quint *et al.*, 2016; Jagadish *et al.*, 2021; Kim *et al.*, 2022; Zhu *et al.*, 2022). However, the majority of these studies were done in *in vitro* or greenhouse conditions, where the entire plants (shoot and root) are cultivated at a homogeneous high temperature or, in some cases, with heated detached shoots and roots separately (Chen & Li, 2016; Valdés-López *et al.*, 2016; Estravis-Barcala *et al.*, 2021). However, waves of high atmospheric temperature have different effects on shoots and roots. Due to the soil geothermal properties, a decreasing temperature gradient from the topsoil to deeper layers is formed (Lynch *et al.*, 2012), preventing the overwarming of the root system, and therefore maintaining its functionality and growth (Michaletz *et al.*, 2015). In this thesis, to properly study the effect of high temperatures on plant growth, we have engineered a new device, called TGRooZ (Temperature

Gradient in the Root Zone), that generates a controlled temperature gradient in the root growth zone while keeping the aerial tissues exposed to the room temperature (RT). The TGRooZ generates a close-to-similar conditions to the ones found in natural ecosystems. This device is adaptable for *in vitro* analyses using agar-based systems on Petri dishes or soil-containing pots for greenhouse experiments.

2. OBJECTIVES

2. Objectives

The general objective of this Thesis is to investigate the genetic and environmental factors that affect root responses to plant nutrition, specifically to Pi starvation and heat stress, under closer to nature experimental conditions.

The specific objectives of this Thesis are:

1. To study the effect of root illumination in plant responses to Pi starvation.
2. To investigate the natural variation of *A. thaliana* from the Iberian Peninsula in Pi tolerance and RSA related traits to identify new genes involved in PSR.
3. To identify fungal endophytes with positive interactions with the plant under Pi starvation.
4. To analyze the contribution of root temperature to plant responses to high atmospheric temperatures.

3. MATERIAL AND METHODS

3. Material and methods

3.1. Characterization of *Arabidopsis thaliana* PSR using the D-Root system

3.1.1. Plant material and growing conditions

A. thaliana (Columbia-0 [Col-0] accession) seeds were surface sterilized using a 50% commercial household bleach solution (~1.5% sodium hypochlorite) for 3 minutes after gently shaking, followed by three washes with sterilized distilled water.

Sterile seeds were sown on squared plates containing 50 mL of half strength Murashige-Skoog medium ($\frac{1}{2}$ MS) supplemented with vitamins, 0.05% MES, 1% sucrose and 1% Difco agar at pH=5.7. Filter sterilized dihydrogen Pi potassium (KH_2PO_4) was added after autoclaving the media at the desired concentrations: 625 μM for $\frac{1}{2}$ MS and 5 μM for Pi deficiency. Seeds were stratified on horizontal plates for 3 days (4°C in completely darkness) and then these plates were transferred to a growth chamber (16/8h light:dark photoperiod, at 22°C) in a vertical position for the indicated days. The D-Root system was used to keep the roots in darkness as described previously (Silva-Navas *et al.*, 2015), unless specified.

For chemical hormonal treatments, seedlings were germinated in a $\frac{1}{2}$ MS medium and then transferred to $\frac{1}{2}$ MS supplemented with dimethyl sulfoxide (DMSO) as mock, 2.5 μM of tZ or 2.5 μM of cZ at the specified Pi concentration. All hormones were purchased from OlChemIm Ltd (Olomouc, Czechia).

All *A. thaliana* mutants used had Col-0 genetic background. The cytokinin (CK) receptor double mutant *ahk4-3 ahk3-4* (Franco-Zorrilla *et al.*, 2005) and the cZ synthesis single mutants *ipt2*, *ipt9* and the double mutant *ipt2 ipt9* (Miyawaki *et al.*, 2006) were used for genetic validation of cZ and tZ responses.

The reporter line pSKP2B::GUS was employed for LRP counting (Manzano *et al.*, 2012).

The *A. thaliana* Iberian Peninsula collection of natural ecotypes was used for the GWAS studies (Picó *et al.*, 2008; Méndez-Vigo *et al.*, 2011). Each ecotype is genetically unique and comes from a local geo-referenced natural population. Each population has been environmentally characterized, and its genetic structure has been resolved (Tabas-Madrid *et al.*, 2018). We phenotyped 174 accessions (Supplementary Table 1), which have been previously sequenced at the genomic level and genetically characterized (Méndez-Vigo *et al.*, 2011; 1001 Genomes Consortium., 2016).

3.1.2. Ionomics analysis

For ion content of *A. thaliana*, shoots and roots of 12-days-old seedlings were collected. The tissue was dried out at 100°C for 24-48h in an air-oven and digested with 3 mL of HNO₃ (Sigma-Aldrich, Trace Metal grade) at 100°C in a thermal bath for 4h. Each sample was diluted to 10 ml with 18 MW water. To determine the ion content, the samples were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) on a ThermoScientific iCAP 6500 using the following wavelengths: B, 208.9 nm; Ca, 184.0 nm; Ca, 317.9 nm; Cd, 228.8 nm; Cr, 206.1 nm; Cu, 324.7 nm; Fe, 240.4 nm; Fe, 259.9 nm; K, 766.4 nm; Mg, 279.0 nm; Mg, 285.2 nm; Mn, 293.9 nm; Mo, 203.8 nm; Na, 589.5 nm; Ni, 231.6 nm; P, 177.4 nm; P, 178.2 nm; S, 182.0 nm; Sr, 216.5 nm; Zn, 206.2 nm.

3.1.3 Hormonal profiling

Several plant hormones, including several CK tZ and cZ, dihydrozeatin (DHZ), tZ riboside (tZR) and cZ riboside (cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenosine, meta-topolin,

meta-topolin riboside, ortho-topolin and ortho-topolin riboside were quantified using MS based methods as described in Silva-Navas *et al.*, 2019.

3.1.4. Transcriptomic analysis

For the RNASeq studies, *A. thaliana* seedlings were grown *in vitro* as described before (Section 3.3.1.) in high Pi (625 μ M) or in Pi deficiency (5 μ M) for 8 days, and then treated with 2.5 μ M of cZ or tZ for 8h.

RNA library and sequencing were carried out by Beijing Genomics Institute (BGI-Zhenzhen, Shenzhen, China). 20 μ g of total RNA was subject to Poly-A RNA isolation by oligo-dT chromatography, followed by fragmentation. Fragmented RNA was converted into double-stranded cDNA using random-hexaprimers followed by end repair, 3' end adenylation and adapter ligation. cDNA fragments were selected by agarose gel extraction and enriched by PCR amplification. The library was loaded onto an Illumina HiSeq 2000 system for pair-end sequencing. Reads of 100 bp were generated as raw data.

For RNASeq analysis, FasQC (v 0.11.9) was used to assess quantity and quality (Andrews, 2010) and initial filtering (mean Q<30 and reads with unknown nucleotides), and adapter trimming of sequences was done by FASTX toolkit (Gordon & Hannon, 2017). The remaining reads were aligned using BWA (Li & Durbin, 2009) and distribution of the expression in the predicted unigene was determined with RSEM (Li & Dewey, 2011). Differential gene expression analysis was conducted with edgeR (Robinson *et al.*, 2010). To compare our RNASeq with the Moras-Macia data (Mora-Macías *et al.*, 2017), MeV software was utilized to load, annotate, analyze, visualize and compare the data (Howe *et al.*, 2011).

Gene ontology (GO) enrichment of differentially expressed genes (DEGs) (False discovery rate, FDR < 0.05) was performed with Genecodis 3 software (Tabas-Madrid *et*

al., 2012). Metabolic pathways enrichment was analyzed and visualized with Mapman (Thimm *et al.*, 2004).

3.1.5. Genome-wide association analysis

For the GWAs analysis, a total of 4 independent replicates with batches composed of randomized accessions each time were assayed. In each batch, control (625 μ M) and Pi deficiency (5 μ M) conditions were tested using the D-Root system. Around 11 ecotypes were assayed in each batch plus one Col-0 line that was employed as an internal control.

In each batch, seedlings of 12 days were scanned in their plates to analyze RSA parameters, and shoots and roots were collected separately. The tissue collected was weighted in an Eppendorf, to which 500 μ L of 1% acetic acid were added and flash frozen in liquid nitrogen to process afterwards. All samples were processed together for Pi extraction and quantification.

In each accession, shoot and root variables were processed independently. Five total variables were recorded for each accession (FW, Pi concentration, root length, number of LR and LR density). Before GWAs analysis, quality control was assessed through statistical analysis of the distribution and batch effect using SPSS v26 software.

GWAS analyses were performed in the GWA-Portal online platform (Seren, 2018) using the accelerated mixed model (AMM), which corrects for population structure. We evaluated for each parameter if data transformation was necessary. If data transformation was necessary, the Box Cox method was employed as indicated in each case. Single nucleotide polymorphisms (SNPs) selected had a minor allele frequency (MAF) of at least 3%. Manhattan plot representation was done using R package CMplot (Yin *et al.*, 2021). For a given SNPs to be considered significant we used the Bonferroni correction, setting the threshold at $\log(-pval) < 8$.

For inferring candidate genes of GWAS SNP associations, we selected a relaxed threshold of $-\log(P) > 4$ and a MAF of 3%. For each SNP, genes located at ± 3 kb from the genomic position of the SNP were selected.

Broad sense heritability was calculated as the percentage of variance corresponding to ecotype variability due to genetic differences. Trait correlations were calculated using Pearson's correlation coefficient using the corplot R package (Wei & Simko, 2021).

Soil phosphorous inference was done interpolating *A. thaliana* ecotypes population geolocation in "Heavy metals, organic matter and other parameters of farmlands and pasturelands of Spain" phosphorous levels map (Rodríguez Martín *et al.*, 2009). Due to the map being limited to Spain, soil phosphorous inference of Portugal populations could not be addressed.

3.2. Screening for fungal positive interactors under phosphate deprivation

3.2.1. Plant material

For screening and the following experiments, *A. thaliana* Col-0 wild-type plants were used. The following reporter lines (Col-0 genetic background) were employed: pSPX3::GUS, which is induced by PSR, and pDR5::LUC, the synthetic auxin response promoter reporter line used to check auxin signaling and the number of PBS.

For germination paper assays, *Hordeum vulgare* (barley) was employed. Seeds were placed in watered filter paper in petri dishes using 30 mL of sterile water and sealed with parafilm to keep high humidity. Then, plates were placed in a cold chamber to stratify for 3 days in darkness at 4°C. After stratification, the seeds were pregerminated

at 24°C in darkness for 5 days. Seeds were transferred to germination paper for the specified time in each experiment.

3.2.2. Fungal isolates

The fungal endophytes used in this work belong to the collection obtained by Dra. Soledad Sacristan (García *et al.*, 2013) from natural populations of *A. thaliana* from the center of the Iberian Peninsula. In brief, fungal isolates were recovered from surface sterilized plant fragments without apparent disease symptoms using potato dextrose agar (PDA) as growth media at RT.

3.2.3. Co-culture in vitro

To analyze plant-fungus interactions, *A. thaliana* seeds were stratified at 4°C for 3 days horizontally in darkness and grown in ½ MS without vitamins or sucrose on square plates after surface sterilizing as described before (Section 3.1.1.). Different Pi concentrations were added to the media as indicated before. Plants were grown in a growth chamber (16/8h light:darkness photoperiod, 22°C) for the indicated days. Afterwards, the seedlings were transferred to plates where the fungi had been growing with ½ MS without vitamins and sucrose. After transference, plants co-cultured with the fungus were cultivated for the indicated time. Roots of *A. thaliana* seedlings were protected from light using the D-Root system. Otherwise, if roots were illuminated is specified.

Fungus inoculation was done with fresh spores collected in sterile MiliRo water from isolates grown on PDA plates for 7-10 days. The inoculation was done by adding 3 µL of a spore suspension containing 10³ spores/mL at 3.2 cm from the sowing line in squared ½ MS plates without sucrose or vitamins. Autoclaved MiliRo water was used as mock treatment. Fungi were grown for 4 days in ½ MS medium using the D-Root

system. Afterwards, 4-day old *A. thaliana* seedlings were transferred to the plate to facilitate the interaction with the fungus using the same growth chamber that was used previously to germinate the seedlings (16/8h light:darkness photoperiod, 22°C).

3.2.4. Germination paper assays

Germination paper system is composed by a germination paper (20 cm x 30 cm, AHLSTROM MUNKSJÖ, BINZ2.383.200350) enclosed into a plastic zip bag. The germination paper was wetted with 80 mL of liquid $1/8$ MS without sucrose at pH 5.8. The pregerminated plantlets were placed on the top of the papers in openings made by cutting the zip bag, placing the aerial part outside and roots inside, in contact with the germination paper. The zip bags were set in an adapted D-Root system to keep roots in darkness. The adapted root system was placed in a growth chamber (16/8h light:darkness photoperiod, 24°C) and the germination paper was wetted on demand with $1/8$ MS. After 7 days of growing in this system, plants were scanned for further phenotyping using Fiji to measure the maximum root length and root coverage area (Schindelin *et al.*, 2012). Besides, shoots and roots were collected for FW quantification.

Fungus was inoculated by immersing roots in a fresh suspension of 10^5 spores/mL for approximately 30s. Inoculated plants were then transferred to the germination paper system.

3.2.5. Systemic colonization of endophytic fungi

To determine whether the fungal isolates were capable of shoot colonization, re-isolation of the fungus from *in vitro* co-cultured plants was performed. Isolation was done as described by Zhou *et al.*, 2018. Briefly, shoots of plants inoculated *in vitro* were cut and washed in sterile water. Afterwards, the tissue was surface sterilized by washing it in 75% ethanol for 1 min, 1.3% sodium hypochlorite for 3 min, 75% ethanol for 30 s and finally rinsing it with sterile MiliQ water. Proper surface sterilization was confirmed

by making imprints of the treated shoots on PDA plates. Finally, the surface sterilized tissue was cut into small pieces in a new PDA plate to release the endophytic fungi inside the leaf. PDA plates were left at RT over 10 days and then examined morphologically for recovered fungal growth of the same strain. Plants of mock treatment were also employed to confirm protocol efficiency.

3.2.6. Phosphate solubilization assay

All fungal strains were screened for calcium Pi solubilization using the Pikovskaya Agar medium (Pikovskaya, 1948). The medium composition was the following: 0.5 g/L yeast extract, 10 g/L dextrose, 5 g/L calcium phosphate, 0.5 g/L ammonium sulphate, 0.2 g/L potassium chloride, 0.1 g/L magnesium sulphate, 0.0001 g/L manganese sulphate, 0.0001 g/L ferrous sulphate and 15 g/L Bacterial agar (DIFCO).

The fungi were inoculated at the center of the petri dish, and they were grown during 14 days at RT. Solubilization of Pi was determined as the diameter of the clear halo formed around the fungal mycelia.

3.2.7. Total phosphorous quantification

Total phosphorous was quantified by Atomic Absorption Spectroscopy (AAS) using the ContrAA 800 (Analytik-Jena). For sample preparation, 14 days old *A. thaliana* shoots were collected in 2 mL tubes with screw cap and FW was recorded. The samples were dried over 2 days at 60°C and equilibrated at RT for one day, and dry weight was annotated. The dry samples were digested in 100 μ L 69% of nitric acid for 1 hour at 80°C, 30 min at 110°C using a thermoblock into the security hood and cooling at RT overnight. The samples were diluted at a final concentration of 2.76 % of HNO₃ before P measurements were done.

Elemental analysis of P was done in graphite furnace mode. Measurements were done in the P lines (193.696 nm) adding 5 μ L of 1.000 mg/L Pd(NO₃)₂ per measurement and using the following furnace program:

Step	Temp (°C)	Time (s)
Dry	80	10
Dry	90	10
Dry	110	10
Pyrolysis	350	20
Pyrolysis	1300	20
Gas adaption	1300	5
Atomize	2600	5
Cleaning	2700	4

A calibration stock solution of P (Inorganic Ventures; Chistiansburg, USA) was used to calculate the final concentration of the samples and a blank treatment was employed to certify the validity of the measurements.

3.3. Root temperature effect on plant fitness, nutrition and microbiome.

3.3.1. Plant material

For root temperature analysis, *A. thaliana* (Columbia-0 [Col-0] accession) seeds were employed. The mutants for heat responses (Col-0 genetic background) selected from our transcriptomics analysis were: *NAC085* TF (AT5G14490; SALK_208662), heat responsive *HSFA2* TF (AT2G26150; SALK_008978), heat shock response 70 kDa chaperon *HSP70.2* (AT5G02490; SALK_085076) and heat shock response 90 kDa chaperon *HSP90.1* (AT5G52640; SALK_075596).

The reporter line pSKP2B::GUS of LR founder cells was employed for LRP counting (Manzano *et al.*, 2012).

For microbiota and ionome analysis, tomato (*Lycopersicon esculentum* cv. MoneyMaker) was used.

3.3.2. Experimental system and growing conditions

To decouple shoot and root temperatures, we developed the TGRooZ system (patented as utility model U2002230407). TGRooZ allows to establish a decreasing temperature gradient between the top of the machine and the bottom part, where plates or pots can be inserted. This way, the portion of the plate/pot in which the aerial plant parts are growing is at the desired chamber temperature. Meanwhile, the segment inside the machine presents a top-bottom decreasing temperature gradient, that can be further regulated to different temperature amplitudes. Furthermore, this system allows roots to grow in darkness.

The machine is a metallic growth box that holds a cold-regulable bottom container which generates a vertical cooling gradient (Section 4.3.1, Fig. 31.). The gradient can be regulated, and it is measured by a digital thermometer (35 cm depth metallic probe inside the box). The gradient is generated by cooling down the base of the box using a water-based cooling system. The top part of the device is a replaceable lid, which can hold different cultivation supports. Here, we used three models: for squared petri dishes of 12 x 12 cm, for the germination paper system (15 x 30 cm) and for 3.5 L pots containing soil. To generate a temperature gradient, TGRooZ refrigerated liquid was cooled at 13°C for *A. thaliana* in 12 x 12 cm plates and at 10.5°C for tomato experiments in containing soil pots.

For *A. thaliana* *in vitro* experiments, seeds of Col-0 ecotype were surface sterilized as described previously (Section 3.1.1.). Sterile seeds were sown on squared plates containing ½ MS supplemented with vitamins, 0.05% MES, 1% sucrose and 1%

Difco agar at pH 5.7. Seeds were stratified on plates for 3 days horizontally (4°C darkness) and then transferred to a growth chamber (16/8h light:darkness photoperiod) with 22°C for 4 days. Then the seedlings were transferred to new fresh ½ MS plates and subjected to different temperature treatments for 6 additional days. There were three different temperature conditions: 22°C in root and shoot as standard conditions (22SR), 32°C as heat stress condition with all plate heated homogeneously in root and shoot (32SR) and 32°C as heat stress in aerial parts but establishing a temperature gradient in the roots using the TGRooZ (32TGRooZ). The root system was grown in darkness using either the D-Root system (Silva-Navas *et al.*, 2015) or TGRooZ.

For tomato (*Solanum lycopersicum* cv. Moneymaker) greenhouse experiments, plants were germinated at 22°C for 1 week in sterile vermiculite. Plants of similar size were selected and transferred to 3.5 L pots that were filled with a mix of natural soil (40.41° Latitude, -3.83° Longitude) and river sand in a 3:1 proportion. After transplanting, they were kept at 22°C for 2 more days and then transferred to different temperature treatments. Three different temperature conditions were used, similar to those described before: 22°C, 36°C (as heat stress) and 36°C with a gradient temperature in the root zone. In these conditions, plants were growing for 3 weeks, watering them twice a week with 150 mL of sterile water. Afterwards, plants were phenotyped and sampled.

3.3.4. Transcriptomic analyses

For RNA isolation and sequencing, total RNA was extracted from roots and shoots of *A. thaliana* of three different conditions (22SR, 32SR, TGRooZ) using the RNeasy kit (Qiagen). Library construction and RNA sequencing were carried out by Beijing Genomics Institute (BGI-Shenzhen, Shenzhen, China) using 100 nt paired-end reads at 30M reads of depth. Briefly, approximately 2 µg of total RNA was subject to Poly-A RNA isolation by oligo-dT chromatography, followed by fragmentation. Fragmented RNA was converted into double-stranded cDNA using random-

hexaprimers followed by end repair, 3' end adenylation and adapter ligation. cDNA fragments were selected by agarose gel extraction and enriched by PCR amplification. The library was loaded onto an Illumina HiSeq 2000 system for pair-end sequencing.

For RNASeq analysis, FasQC (v 0.11.9) and Trimmomatic (v 0.36) was used to assess quantity and quality and adapter trimming (Andrews, 2010; Bolger *et al.*, 2014). The remaining reads were aligned to the *A. thaliana* genome (TAIR 10 genome reference) using the Araport 11 annotation and the STAR aligner (v 2.5.3a) (Dobin *et al.*, 2013) with the following parameters:

Parameter	Value
--outFilterMultimapNmax	20
--alignSJoverhangMin	8
--alignSJDBoverhangMin	8
--outFilterMismatchNmax	8
--alignIntronMin	35
--alignMatesGapMax	10000
--alignIntronMax	20000

Reads counts per gene were generated using HTSeq (v 1.99.2) (Anders *et al.*, 2015) using the “union” mode. Differential gene expression analysis was conducted with DESeq2 Bioconductor package in R (Love *et al.*, 2014), using q -value > 0.05 and a fold-change $\log_2 > 0.8$ or $\log_2 < -0.8$ to consider statistical significance for a given DEG.

Heatmap visualization was done using the ComplexHeatmap package (Gu *et al.*, 2016b). GO analysis was done using Metascape (Zhou *et al.*, 2019) and ClusterProfiler (Yu *et al.*, 2012). The analysis was done using the following parameters: Minimum overlapping of 3, p -value cutoff of 0.05 and minimum enrichment of 1.5.

3.3.5. Stomata conductance and leaf temperature

A. thaliana seedlings were grown *in vitro* for 7 days and then transferred to 3.5 L pots filled with soil. The plants were grown for 3 weeks in the three temperature conditions described before (22SR, 32SR and 32TGRooZ). Water content was recorded using the Zentra Z6 datalogger through a Teros 10 probe (Meter group) every 8 h for the duration of the whole experiment.

For stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$), the leaf porometer SC-1 (Decagon Devices, Inc.; Pullman, USA) was used. Instrument calibration was done prior each experiment measurements, following the manufacturer's guidelines. Two leaves from the same position in each plant were measured for the three temperature conditions, using three plants as biological replicates.

Thermal pictures were taken with a FLIR-E96 camera (FLIR Systems; Boston, USA) and leaf temperature was quantified using the FLIR research studio suite. The quantification was done using the polymer tool to average the surface temperature of the whole leaf and soil measures were taken as control reference. For each treatment, 6 leaves of at least 5 different plants (each one in a different pot) were measured.

3.3.6. Microbiome analysis

For microbiome analysis, tomato plants were grown as described before. The natural soil was collected (40.41° Latitude, -3.83° Longitude) for the experiment and sampled before the treatments, in order to capture the initial microbiome before the treatments in the fraction that we called Soil or Inoculum. Samples of the three different temperature treatments were gathered for rhizosphere and roots at around the middle part of the root system depth. Rhizosphere fraction corresponds to soil close to the roots. To obtain root samples, roots were washed thoroughly with sterile water to detach all soil particles. Six replicates for each treatment were collected. All the samples

were frozen in liquid nitrogen and stored at -80°C. The information about mean temperature and precipitation of the collected soil area is displayed in Table 1.

Table 1. Average temperature and precipitation in each month for Pozuelo de Alarcon climatic station. Historical data from 1991-2021 by climate-data.org.

Temp (°C)	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Maximum	9.4	11.1	14.6	17.5	22.2	28.5	32	31.6	26.5	20	12.9	9.9
Average	4.6	5.8	9	11.9	16.4	22.4	25.7	25.3	20.6	14.8	8.5	5.4
Minimum	0.8	1.2	3.6	6.2	10.2	15.5	18.6	18.4	14.7	10.1	4.6	1.7
Rainfall (mm)	41	34	40	47	39	16	6	8	22	61	55	46

The samples were sent for 16S rRNA amplicon sequencing analysis of bacterial microbiome to Biome Maker laboratory (Sacramento, US). DNA extraction was performed with the DNAeasy PowerLyzer PowerSoil Kit (Qiagen). Libraries were prepared following the two-step PCR Illumina protocol using custom primers amplifying the 16S rRNA v4 region as described by Ortiz-Álvarez *et al.*, 2021. Sequencing was conducted in an Illumina MiSeq instrument using pair-end sequencing (2x300bp).

For the amplicon sequence analysis, raw amplicon sequences were quality filtered using fastp and then merged using PEAR with default parameters. The resulting high-quality filtered sequences were then denoised and collapsed into amplicon sequence variants (ASVs) using DADA2 v1.10.1 (Callahan *et al.*, 2016). Representative ASVs sequences were taxonomically classified with the mothur naïve bayes classifier trained on the SILVA B2 database (Quast *et al.*, 2013). ASVs that were assigned to chloroplast, mitochondria, oomycete, archaea or did not have a known kingdom assignment were filtered out. After filtering the low quality ASVs, rarefied and relative abundance tables were created using a threshold of depth of 10,000 reads per sample. The resulting abundance tables were processed and analyzed with functions from the ohchibi package (<https://github.com/isaig/ohchibi>).

To compare alpha diversity across conditions and fractions, the Shannon diversity and Richness indexes were calculated using the diversity function from the

vegan package v2.5-5 (Oksanen *et al.*, 2013). Analysis of variance (ANOVA) was used to test for differences in alpha diversity between conditions across fractions. Beta diversity analyses (Principal coordinate analysis, PCoA) were based on Bray-Curtis dissimilarity matrixes calculated from the abundance table. To compute the variance explained by the Fraction effect and Temperature treatments, permutational multivariate analysis of variance (PERMANOVA) were performed using the function `adonis` from the vegan package v2.5-5 (Oksanen *et al.*, 2013). Bar graphs showing raw and average relative abundance of phyla across conditions were computed using the `chibi.phylogram` function from the `ohchibi` R package. The R package DESeq2 v.1.24.0 (Love *et al.*, 2014) was used to compute the intra-fraction (Intra rhizosphere, intra root) specific enrichment profiles across the three temperature conditions. For each taxonomic unit of the following taxonomic levels, Phylum, Class, Order, Family, Genus, and ASV, their abundance differences in each of the three temperature conditions were estimated using a generalized linear model (GLM) with the following design:

$$\text{Abundance} \sim \text{Temperature condition.}$$

Within each fraction (rhizosphere and root), all vs all contrasts between temperature conditions (3 possible contrasts in total) were performed, keeping taxonomic units with a FDR p -value < 0.05. The results of the contrasts were visualized using a heatmap, in which the enrichment patterns (log₂ fold change) of all statistically significant taxonomic units across the three contrasts were shown using `ggplot2` v.3.2.1 R package (Wickham *et al.*, 2016).

3.3.7. Ionome Analysis

For the ionome analysis, tomato leaves were collected and washed three times with 18.2 MΩcm Milli-Q water (Merck Millipore) for measuring using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The samples were placed in weighted Pyrex digestion tubes and dried at 88°C for 20 h. After cooling, leaf samples were weighted on Mettler five-decimal analytical scale, and 1-3 mL (depending on the sample

dry weight) of the concentrated trace metal grade nitric acid Primar Plus (Fisher Chemicals) was added to each tube. Prior to the digestion, 20 µg/L of Indium (In) was added to the nitric acid as an internal standard to assess putative errors in the dilution process, variations in sample introduction and plasma stability in the ICP-MS instrument. The samples were then digested in DigiPREP MS dry block heaters (SCP Science; QMX Laboratories) for 4 h at 115°C. After cooling down, the digests were diluted to 10-30 mL (depending on the volume of the nitric acid added) with 18.2 MΩcm MilliQ Direct water, and elemental analysis was performed using an ICP-MS, PerkinElmer NexION 2000 equipped with Elemental Scientific Inc 4DXX FAST Dual Rinse autosampler, FAST valve and peristaltic pump. The instrument was fitted with a PFA-ST3 MicroFlow nebulizer, baffled cyclonic C3 high sensitivity glass spray chamber cooled to 2 °C with PC3X Peltier heated/cooled inlet system, 2.0 mm i.d. quartz injector torch and a set of nickel cones. Twenty-four elements were monitored including following stable isotopes: ⁷Li, ¹¹B, ²³Na, ²⁴Mg, ³¹P, ³⁴S, ³⁹K, ⁴³Ca, ⁴⁸Ti, ⁵²Cr, ⁵⁵Mn, ⁵⁶Fe, ⁵⁹Co, ⁶⁰Ni, ⁶³Cu, ⁶⁶Zn, ⁷⁵As, ⁸²Se, ⁸⁵Rb, ⁸⁸Sr, ⁹⁸Mo, ¹¹¹Cd, ²⁰⁸Pb and ¹¹⁵In. Helium was used as a collision gas in Kinetic Energy Discrimination mode (KED) at a flow rate of 4.5 mL/min while measuring Na, Mg, P, S, K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn, As, Se and Pb to exclude possible polyatomic interferences.

The remaining elements were measured in the standard mode. The instrument Syngistix™ software for ICP-MS v.2.3 (Perkin Elmer) automatically corrected any isobaric interferences. The ICP-MS measurements were performed in peak hopping scan mode with dwell times ranging from 25 to 50 ms depending on the element, 20 sweeps per reading and three replicates. The ICP-MS conditions were as follow: RF power – 1600 Watts, auxiliary gas flow rate 1.20 L/min. Torch alignment, nebuliser gas flow and quadrupole ion deflector (QID) voltages (in standard and KED mode) were optimized before analysis for highest intensities and lowest interferences (oxides and doubly charged ions levels lower than 2.5 %) with NexION Setup Solution containing 1 µg/L of Be, Ce, Fe, In, Li, Mg, Pb and U in 1 % nitric acid using a standard built-in software procedure. To correct for variation between and within ICP-MS analysis runs, liquid reference material was prepared using pooled digested samples and run after the instrument calibration and then after every nine samples in all ICP-MS sample sets.

Equipment calibration was performed at the beginning of each analytical run using seven multi-element calibration standards (containing 2 µg/L In internal standard) prepared by diluting 1000 mg/L single element standards solutions (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd) with 10 % nitric acid. As a calibration blank, 10 % nitric acid containing 2 µg/L In internal standard was used, and it was run throughout the analysis. Sample concentrations were calculated using the external calibration method within the instrument software. Further data processing, including calculation of final elements concentrations, was performed in Microsoft Excel.

For the data analysis of ions, a matrix (samples x ion) was created in which each cell was filled with the calculated element concentration in a given sample. Afterwards, z-score transformation of each individual ion across the samples was applied in the matrix. The concentration of each ion across the three temperature treatments was applied by a GLM design as follows:

Ion Concentration ~ Temperature Condition.

After fitting the model, statistical significant differences between conditions were determined by performing pairwise comparisons and visualizing the results of the comparisons using the compact letter display (CLD) as implemented in the *multcomp* v.1.4-12 R package. The abundance profiles of each ion across temperature conditions were visualized using a heatmap created by the *ggplot2* v.3.2.1 R package (Wickham *et al.*, 2016) using the standardized (z-score) ion abundance.

3.4. Root system architecture quantification and GUS straining

For root measurements, each plate was scanned at high resolution (>300 dpi, Epson 600V scanner). Root length and the number of LRs were later quantified using FIJI (Schindelin *et al.*, 2012). LR density was calculated as the number of LRs per cm of main root. For LRP determination, the reporter line pSKP2B::GUS was used (Manzano

et al., 2012). The counting and imaging of LRP was done using a Leica stereomicroscope MZ9.5 with a DCF280 camera.

Root hairs were imaged using a Leica M205FA adapted with Hamamatsu EMCCD X2 camera.

For GUS reporter staining, whole seedlings in plate with the media agar were stained using the X-gluc substrate (PhytoTechnology Laboratories). The X-gluc was diluted in N,N-Dimethylformamide in a 1000x solution (50 mg in 300 μ L) and then diluted to a 1x in 50 mM Pi buffer at 7 pH with 0.2% of acetone and 0.2% triton X-100. The solution was extended over the plate and roots and incubated at RT for 16h in darkness.

3.5. Free inorganic phosphate quantifications

Free inorganic Pi was quantified using the colorimetric method described by Ames (Ames, 1966). Briefly, shoots and roots of *A. thaliana* seedlings were collected and weighted separately. The soluble Pi was extracted from plant material using 1% acetic acid buffer and flash freezing cycles. The amount of Pi was determined using the colorimetric reduction reaction of free Pi with ammonium molybdate absorbing at 660 nm.

3.6. Statistical Analysis

Most of the data were analyzed using either Prism5 software or R statistical environment (R Core Team, 2020; RStudio Team, 2020), using the tidyverse (Wickham *et al.*, 2019) package, unless specified otherwise. The statistical analysis and the number of replicates is indicated in each comparison. In general, comparisons between two groups were done with t-test and comparisons of more than two groups were done by

one-way ANOVA and Tukey as a post-hoc analysis. Statistical significance was set at p -values < 0.05 .

4. RESULTS

4. Results

4.1. Characterization of *Arabidopsis thaliana* PSR using the D-Root system

4.1.1. Light limits root growth during Pi starvation

Roots, as underground organs, should be growing in a dark environment. For this reason, we decided to analyze the PSR using the D-Root device (Silva-Navas *et al.*, 2015) and compare it with the effect on light grown roots (LGRs). In DGRs seedlings, inorganic Pi deficiency (5Pi) slightly inhibited root growth compared with roots grown in Pi-supplemented medium (+Pi). However, root growth was significantly inhibited in Pi-starved LGRs seedlings (Fig. 9a-c). Pi starvation reduced root meristem size in LGRs by almost 50%, whereas this reduction was only 8% in DGR seedlings (Fig. 9f). Pi-starved LGRs increased LR density, mainly as a result of the significant reduction in PRL (Fig. 9c-d). By contrast, DGRs showed no difference in LR density when media contained a low amount (5 μ M) of Pi (Fig. 9d). Nevertheless, we found a higher total number eLR in Pi-starved DGR than in LGR seedlings (Table 2). We also found that root illumination accelerated root hair development in response to Pi starvation, which started to develop closer to the root meristem, and reduced root hair length (Fig 9e). Next, we quantified the free Pi and total phosphorous concentration and found a significant reduction in Pi-starved plants (Fig. 9g, Fig. 10). However, we did not find significant differences in total or free Pi concentrations between LGR or DGR seedlings (Fig. 9g, Fig. 10). It has been reported that high levels of iron may contribute to root growth inhibition during Pi starvation (Ward *et al.*, 2008). Ionomics analyses showed that Pi deficiency decreased iron concentrations in roots of both LGR and DGR seedlings, but iron concentration was almost three-fold higher in LGR than in DGR seedlings (Fig. 10). In addition, the concentrations of several other elements were also

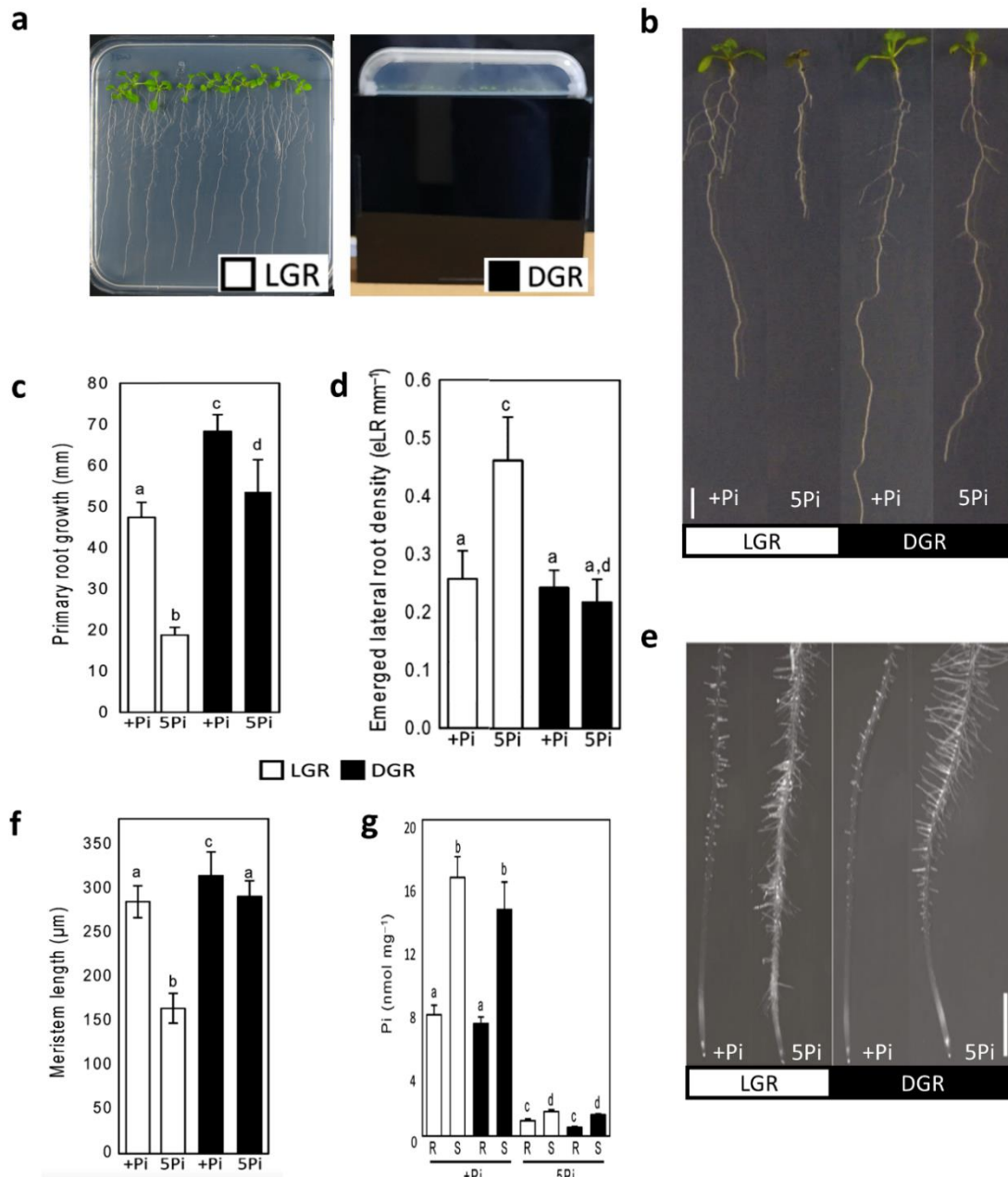


Fig. 9: Root PSR response is altered by root illumination. **a** *In vitro* squared petri dish with roots exposed to light (left, LGR) and D-Root system device adapted to squared plates to shield the roots from light (right, DGR). Note that the aerial part is above the box, to allow complete illumination. **b** Representative picture of 12 days old *A. thaliana* seedlings grown in 625 μM (+Pi) or 5 μM (5Pi) Pi with roots exposed to light (LGR) or grown in darkness (DGR). Bar equals 0.5 cm. **c** Root length of *A. thaliana* grown as in **a-b**. **d** Density of eLR of seedlings grown as in **a-b**. **e** Representative pictures of root hairs in the root tip of 8 days old LGR or DGR seedlings grown in ½ MS containing different concentrations of Pi. Bar, 500 μm. **f** Root meristem length of plants grown as in **e** (n = 14). **g** Free Pi concentration in roots (R) or shoots (S) of 10 days old LGR or DGR *A. thaliana* seedlings grown in ½ MS containing +Pi or 5Pi (n = 10). Significance was analyzed by ANOVA and Tukey honestly significant difference post-test. The same lower-case letters above the standard deviation (SD) indicate no significant differences (*p*-value < 0.01).

Table 2. Mean $\pm$ SD number of eLR of 12 days old *A. thaliana* seedlings grown in 625 μ M (+Pi) or 5 μ M (5Pi) Pi with light-grown roots (LGR) or dark-grown roots (DGR) as in Fig. 9a-b. Significance was analyzed by ANOVA and Tukey HSD post-hoc test. The same lower-case letters above the SD indicate no significant differences (p -value < 0.01).

Pi condition	LGR	DGR
+P	12.3 $\pm$ 2.6 ^b	16.7 $\pm$ 2.76 ^a
5P	8.6 $\pm$ 1.35 ^c	11.6 $\pm$ 2.57 ^b

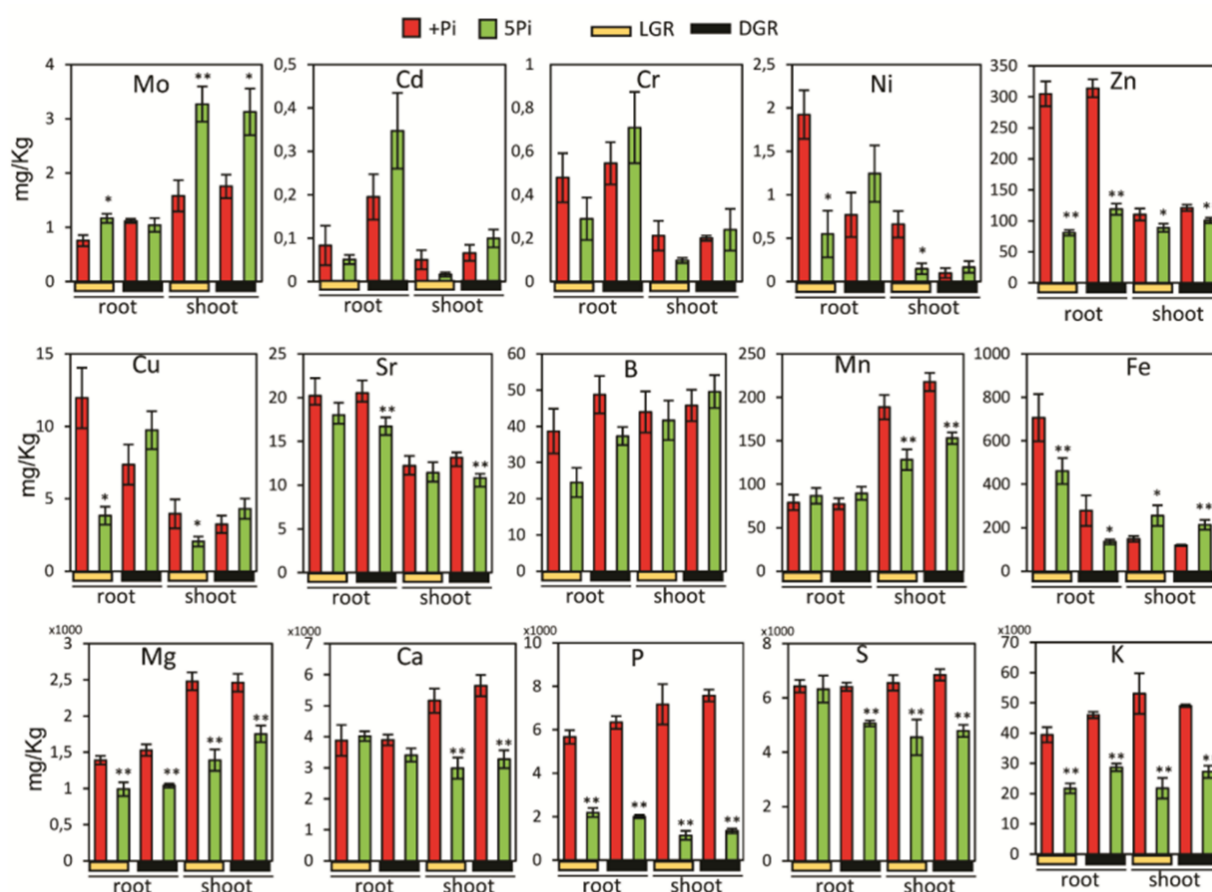


Fig. 10: Root illumination alters plant ionic profile. Ion accumulation of shoots and roots of dark-grown roots (DGR, dark horizontal bars) or light-grown roots (LGR, yellow horizontal bars) of *A. thaliana* seedlings grown for 12 days in $\frac{1}{2}$ MS in 625 μ M (+Pi, green bars) or 5 μ M (5Pi, red bars) Pi. Values correspond to the average of 4 samples $\pm$ SD. Statistical differences between +Pi and 5P in at the same organ and illumination regimen are indicated by * p -value < 0.05 or ** p -value < 0.01 by a t-Test analysis.

altered by both Pi starvation and / or light (Fig. 10), suggesting that both factors unbalance plant nutrition. Taken together, these data indicated that light influences the physiological and morphological response to Pi starvation in roots. Therefore, unless indicated, all the subsequent analyses have been done in DGR seedlings.

4.1.2. Hormonal profiling reveals a cZ:tZ ratio alteration in response to Pi deficiency

As hormonal signaling modulates plant adaptation to different environments, we decided to profile plant hormones of roots and shoots of DGR seedlings. Pi deficiency increased the concentration of IAA, ABA and jasmonic acid (JA) in shoots (Fig. 11a). Interestingly, the defense hormones in roots were altered, JA levels decreased while salicylic acid (SA) concentration increased (Fig. 11a). However, the most striking change was in the concentration of different types of CKs. In Pi-starved plants, we found that in both roots and shoots the concentration of tZ decreased significantly whereas that of cZ increased significantly (Fig. 11b). A similar effect was also observed for cZR and tZR. However, this trend was not observed for free or riboside conjugated isopentenyladenine (iP or iPR). *IPT3* and *IPT5*, which are involved in tZ biosynthesis, were downregulated by Pi starvation (Fig. 11d). Conversely, the level of *IPT2* and *IPT9*, involved in cZ synthesis, did not change (Fig. 11d). We found that the expression of *LONELY GUY Cytokinin-Activating 2* and *5* (*LOG2* and *LOG5*, respectively), involved in downstream steps of zeatin biosynthesis (Kurakawa *et al.*, 2007), were upregulated by Pi starvation (Fig. 11d). These data suggest that Pi-starved plants increase cZ:tZ ratio by repressing tZ synthetic genes and by activating downstream steps of the biosynthesis of cZ. However, we cannot rule out a posttranslational regulation of enzymes involved in cZ biosynthesis.

4.1.3. Zeatins control root growth and cell proliferation under low-Pi conditions

Due to the differential accumulation of cZ and tZ, we decided to evaluate the role of these isomers during the PSR. First, we found that tZ-treated plants showed more root growth inhibition than cZ, irrespective of the amount of Pi in the medium (Fig. 12 a, b). It is known that CK treatment reduces the size of the root meristem (Dello Ioio *et al.*, 2007). We found that both, cZ and tZ, reduced the meristem size, but the reduction was more severe in tZ-treated than in cZ-treated roots (Fig. 12b). Furthermore, in a medium containing Pi, cell elongation in the root transition zone was reduced by tZ, but not by cZ (Fig 12d). In Pi-starved plants, the cell length in the root transition zone was significantly reduced. Remarkably, cZ treatment, but not tZ, reversed this cell length reduction facilitating root growth (Fig 12d).

To analyze the effect of these zeatin isomers on root and LR development, SKP2B::GUS, a LR marker (Manzano *et al.*, 2012), was used. As shown in Fig. 12e, tZ and cZ inhibited the root growth (both primary and LRs) and the number of LR primordia (LRP) and eLRs. However, this inhibition was more pronounced for tZ- than for cZ-treated plants, especially in Pi-starved roots (Fig. 12e). We also found that, compared with primary root growth, LR growth was more sensitive to a low concentration of both cZ and tZ during Pi starvation (Fig. 12e). It is noticeable that, even though tZ was more effective in inhibiting LRP specification, both cZ and tZ have additive functions in this process, preferentially during Pi starvation (Fig. 12e).

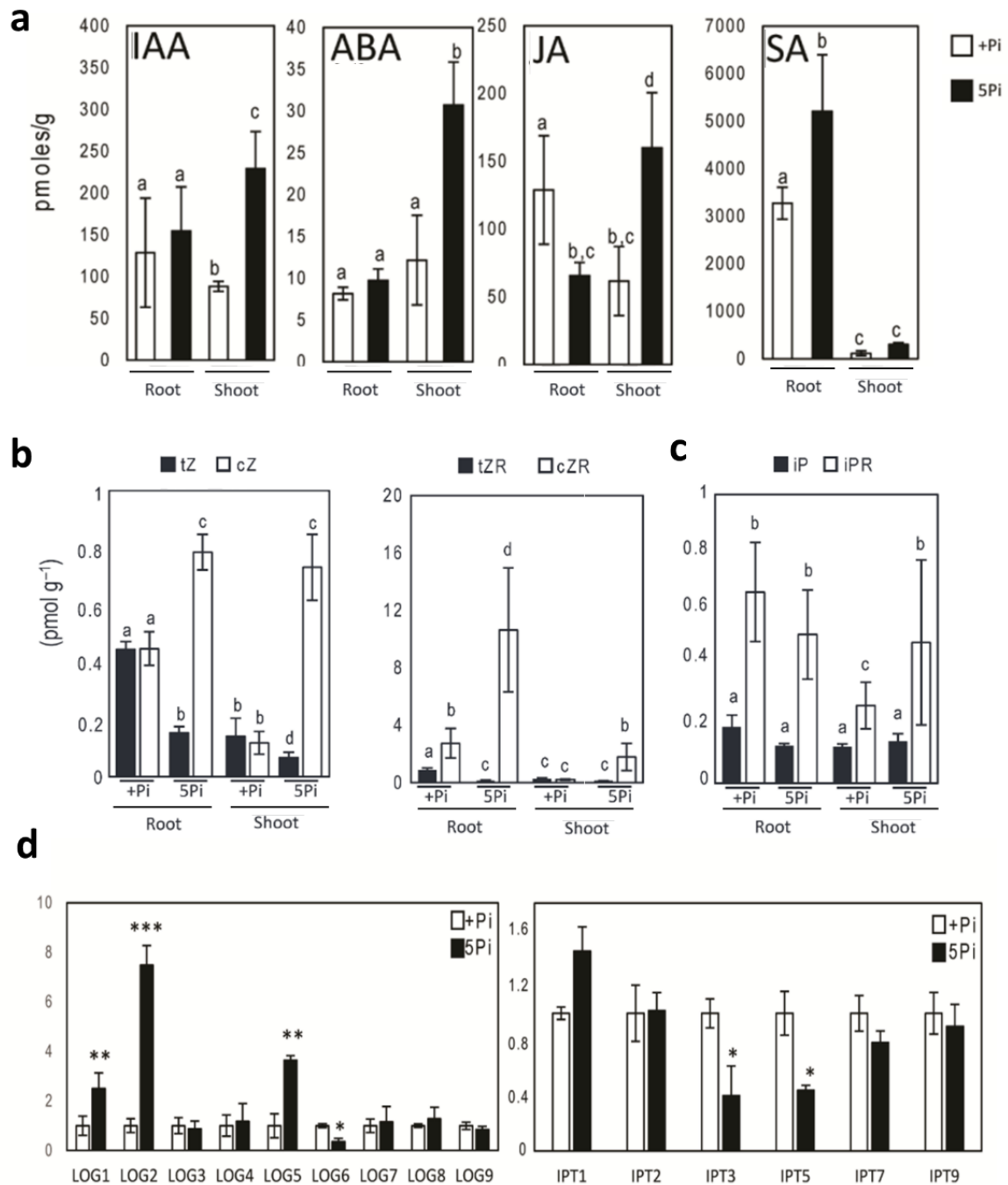


Fig. II: Hormonal concentrations under phosphate deprivation using the D-Root. **a** Concentration of Indole-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) of 10 days old *A. thaliana* seedlings grown in $\frac{1}{2}$ MS containing 625 μ M (+Pi), or 5 μ M (5Pi) Pi. **b** Concentrations of CK Zeatin isomers: cis-zeatin (cZ) and trans-zeatin (tZ) and its conjugated forms: cis-zeatin-riboside (cZR) and trans-zeatin-riboside (tZR); in roots (R) or shoots (S) of *A. thaliana* grown as in **a**. **c** Concentration of CK isopentenyladenine (iP) and its conjugated form isopentenyladenine-riboside (iPR) of *A. thaliana* grown as in **a**. Values shown correspond to the means of four replicates $\pm$ SD. Significance was analyzed by ANOVA and Tukey HSD post-test. The same lower-case letters above the SD indicate no significant

difference (p -value < 0.01). **d** Gene expression of *LOG* (*LONELY GUY* family) or *IPT* (*ISOPENTENYLTRANSFERASES*) genes in *A. thaliana* roots. Bars correspond to normalized FPKM expression of three experimentally independent RNAseq experiments carried out containing 3, 3 or 2 biological replicates each. Values are related to gene expression in the control seedlings. Significance was analyzed by t-test; * p -value < 0.001; *** p -value < 0.001.

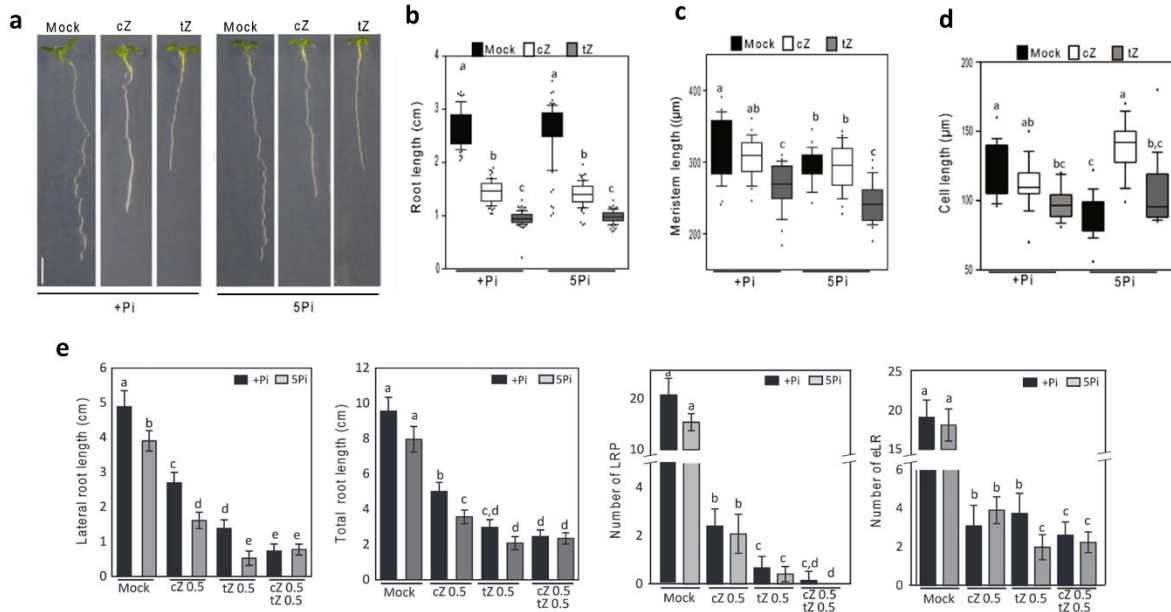


Fig. 12: cisZeatin and transZeatin differentially modulates root growth. **a** Representative pictures of *A. thaliana* seedlings grown in $\frac{1}{2}$ MS with high Pi (625 μ M; +Pi) or low Pi (5 μ M; 5Pi) for 5 days and then treated for extra 5 days with 2.5 μ M cZ or trans-zeatin (tZ). Bar, 0.5 cm. **b** Root length of seedlings grown as described in **a** ($n = 30$). **c** Root meristem size of *A. thaliana* seedlings grown in +Pi or 5Pi media for 7 days and then treated with 2.5 μ M cZ or tZ for 24 h ($n = 12$). **d** Cell length of the first four cells after the last root meristematic cell of seedlings grown as in **c** ($n = 14$). **e** Lateral root length, total root length, number of lateral root primordia (LRP) and number of emerged lateral roots (eRL) of plants grown as in **a** ($n \geq 30$). Significance was analyzed by ANOVA and Tukey honestly significant difference post-test. The same lower-case letters above the SD indicate no significant differences (p -value < 0.01). Error bars are the 95% confidence interval.

4.1.4. cZ and tZ have a differential effect on gene expression

We showed that Pi starvation changed the cZ:tZ balance. To further explore the role of both isomers at the molecular level, we compared cZ- and tZ-treated root transcriptomes. In high-Pi conditions, we found that cZ induced 986 and repressed 780

genes, while tZ induced 1442 and repressed 1471 genes. Among the induced ones, 569 genes were common between cZ and tZ, but there were > 400 and 800 genes that were specifically induced by cZ and tZ, respectively (Fig. 13a; Supplementary File 1 – Table e). It is remarkable that out of the genes specifically upregulated by cZ, 30.2% were also upregulated by Pi starvation and only 5% were downregulated (Fig. 14a). Conversely, out of the specifically upregulated genes by tZ, only 6.7% were upregulated by Pi starvation, and a higher number (21.2%) were downregulated. Interestingly, the opposite trend was observed in the genes specifically downregulated by cZ or tZ (Fig. 13b). Taken together, these findings suggest that tZ acts somewhat as antagonist of PSR whereas cZ seems to act as agonist. Among the transcripts specifically induced by cZ, we found genes involved in cell wall loosening and organization and multidimensional and unidimensional cell growth (Fig. 13c) that might be involved in root growth. It is noticeable that, among the tZ-repressed transcripts, several genes involved in cell division and DNA replication were identified (Fig. 13d), what might explain the stronger effect on root growth inhibition executed by this isomer (Fig. 12a-d). In addition, we found a large number of genes that were up- or downregulated by both cZ and tZ, but to a different extent, indicating that cZ and tZ modulate common pathways, although with different intensity, but also specific genes.

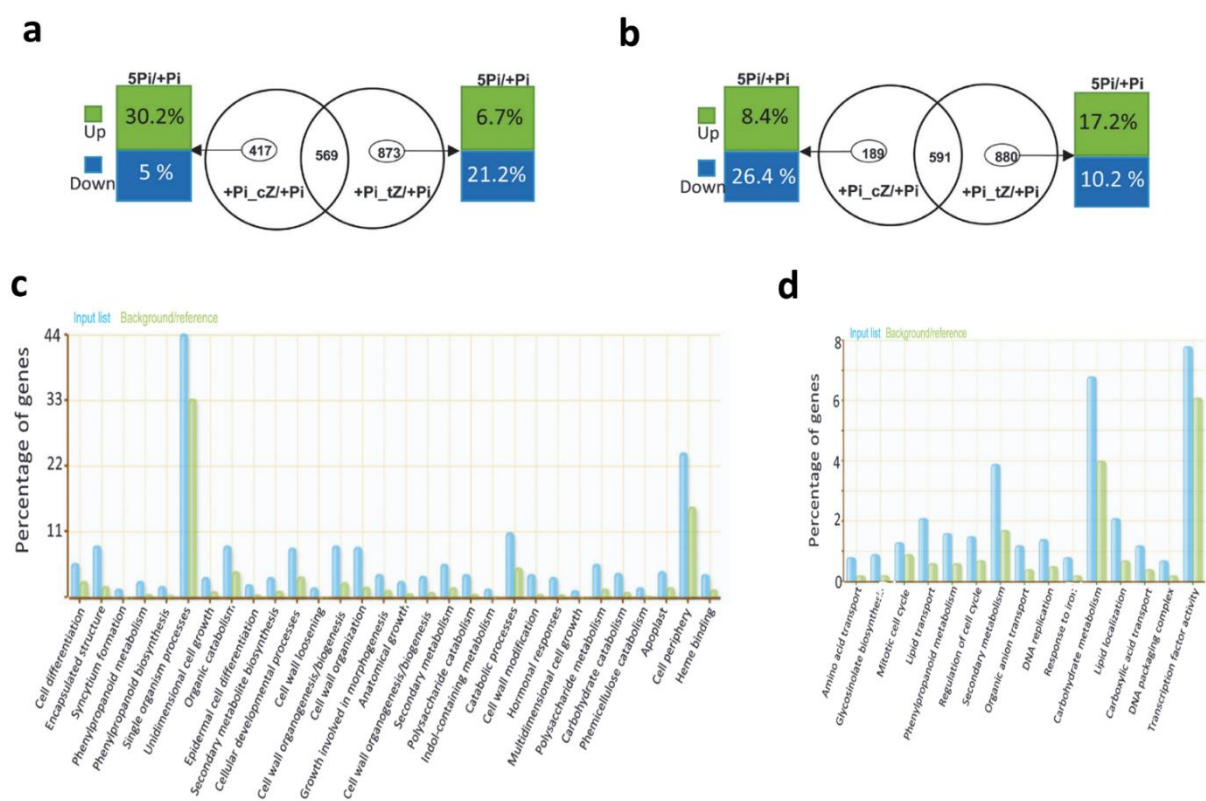


Fig. 13: cisZeatin and transZeatin regulate common and specific genes. **a-b** Venn's diagrams of upregulated **a** or downregulated **b** genes by cZ or tZ treatment. Percentages in boxes indicate the amount of cZ- or tZ-specific genes that are induced (green) or repressed (blue) and that are also deregulated by phosphate starvation (5Pi/+Pi). **c** Gene ontology (GO) analysis of specific genes induced by cZ in a Pi-containing medium (417 genes). **d** Gene ontology (GO) analysis of specific genes repressed by tZ in a Pi-containing medium (880 genes). Input list corresponds to our gene list and background reference to the expected frequency of that GO category in the genome.

Next, we analyzed the effect of cZ and tZ during the PSR (Supplementary File 1). We found that during Pi starvation, cZ and tZ affected the expression of a number of common genes, but also some specific ones (Fig. 14a-b). Among the specific genes, cZ upregulated the expression of flavonol biosynthetic genes, cell wall, stress responses or auxin response genes (Fig. 14c). In this regard, we found that Pi starvation increased the concentrations of two flavonols, quercetin and kaempferol, which are involved in root growth control (Silva-Navas *et al.*, 2016). Genes specifically upregulated by tZ during Pi starvation were mainly related to auxin transport and lipid metabolism or regulation of

hormonal concentrations (Fig. 14d). Regarding the effect of cZ and tZ on the expression of genes down regulated by Pi starvation, we found a similar trend, that is, genes specifically regulated by each isomer and genes commonly regulated by both isomers. Furthermore, cZ repressed genes involved in stress responses and primary metabolism, whereas tZ reduced the level of genes that function in glucosinolate biosynthesis, iron binding or oxide-reductase activities (Fig. 14e-f).

In addition to the specific gene regulation, we identified transcripts, either induced or repressed by Pi starvation, whose expression level was differentially modulated (by at least $\geq 20\%$) by cZ or tZ (Fig. 15). The expression level of Pi starvation upregulated genes was, in general, more reduced by tZ than by cZ, suggesting that tZ exerts a stronger repression on PSR genes (Fig. 15). In the case of Pi starvation-downregulated genes, we found that tZ, in general, exacerbated the repression of these genes, while cZ's effects were more moderate (Fig. 15). Taken together, these data suggest that tZ reduces the transcriptional PSR.

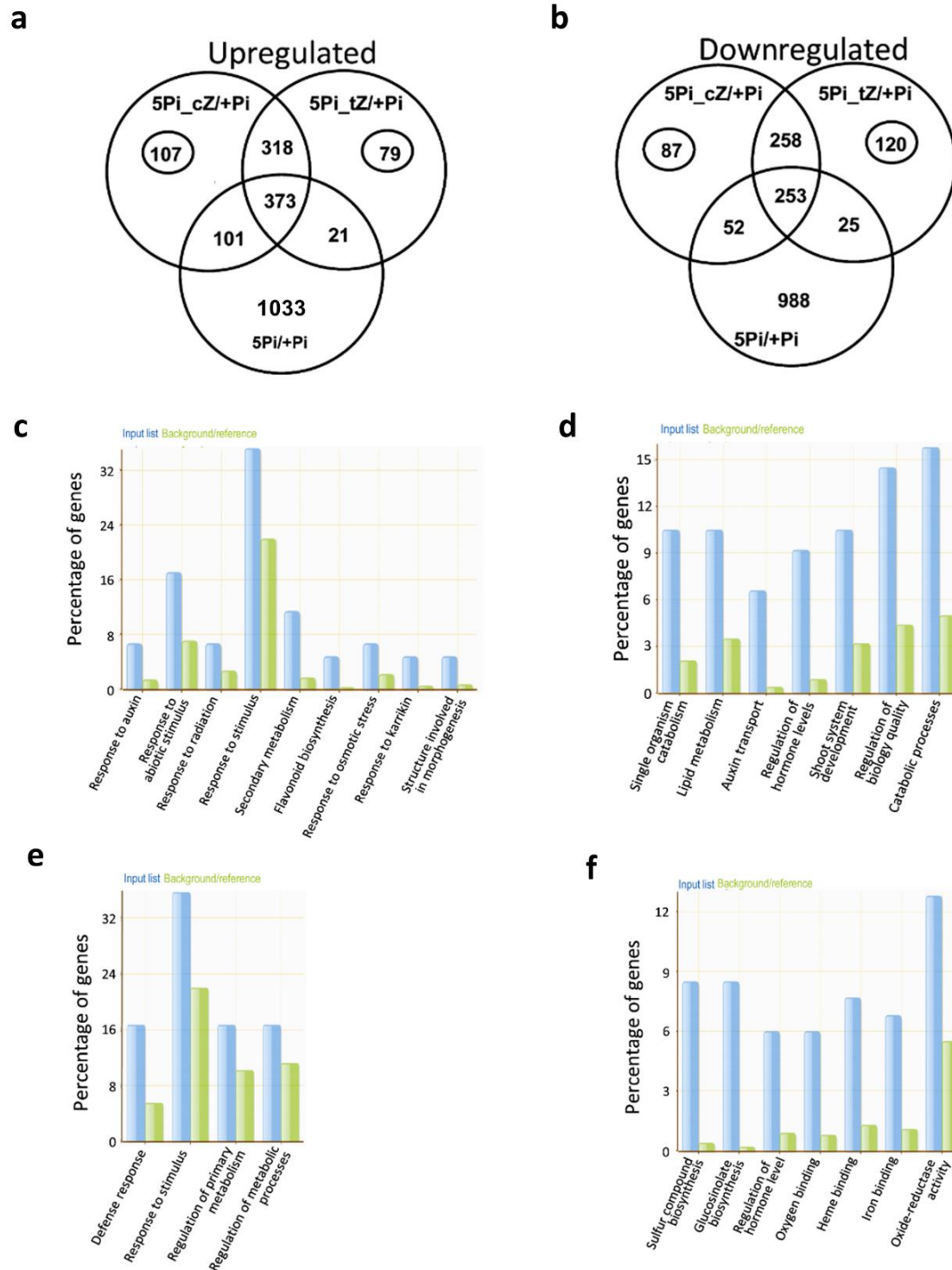


Fig. 14: Effect of cisZeatin (cZ) and transZeatin (tZ) on the expression of PSR genes. a-b Venn's diagrams showing phosphate (Pi) starvation upregulated **a** or downregulated **b** genes that are specifically controlled by cZ or tZ. c-f Bar charts show the most significant gene ontology categories of the Pi starvation-upregulated genes that are specifically regulated by cZ **c** or tZ **d** or Pi-downregulated genes that are specifically regulated by cZ **e** or tZ **f**. Input list corresponds to our gene list and background reference to the expected frequency of that GO category in the genome.

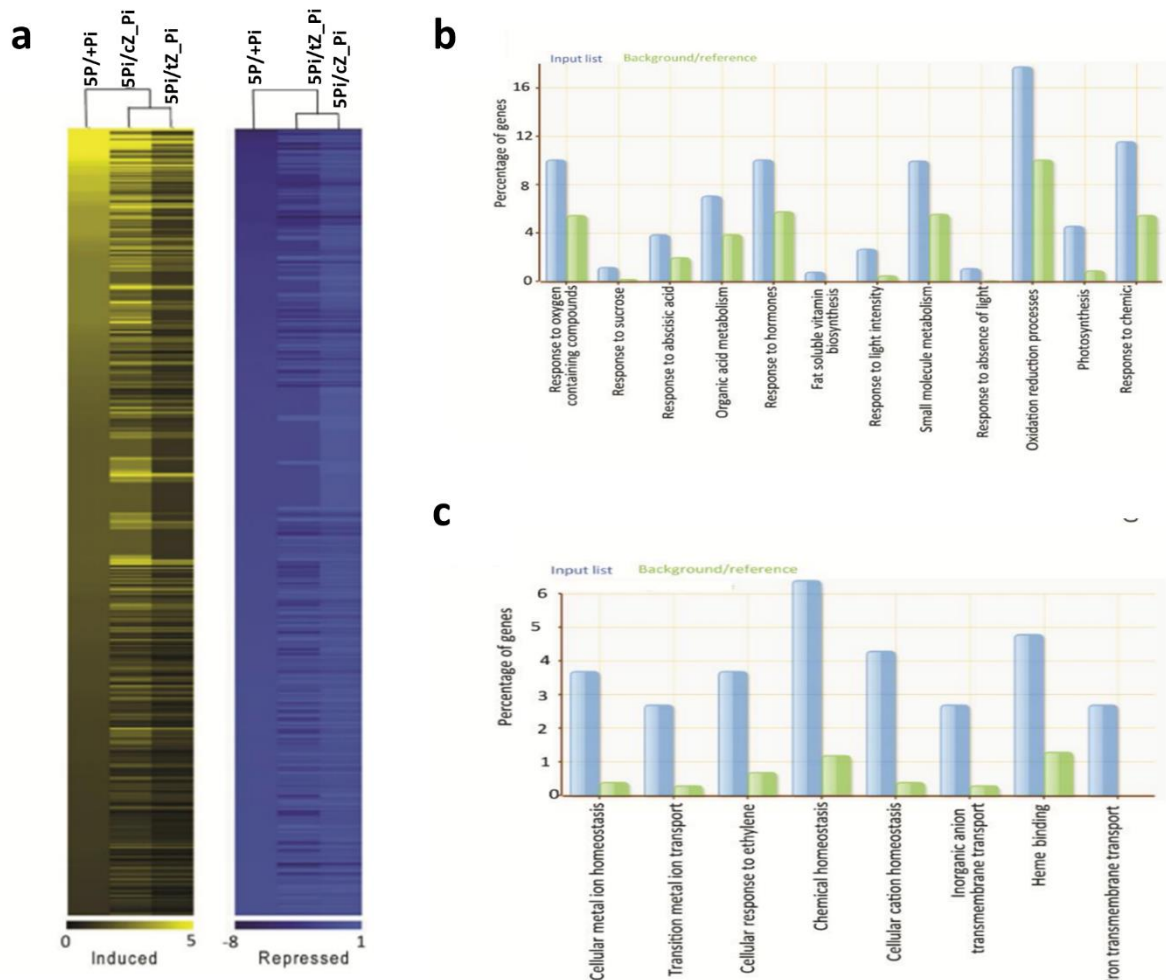


Fig. 15: cZ and tZ regulate common and specific genes. **a** Hierarchical clustering of Pi induced or repressed genes which expression level is differentially modulated by cZ or tZ during PSR. **b** Gene ontology (GO) analysis of genes specifically repressed by cZ in high Pi media. **c** Gene ontology (GO) analysis of genes specifically repressed by tZ in high Pi media.

4.1.5. cZ regulates root PSR responses

Pi starvation increases FW root:shoot ratio and alters Pi allocation (Raghothama, 1999). We found that cZ treatment of DGR seedlings significantly increased the root:shoot ratio in high- and low-Pi medium, by 1.7 and 1.4-fold, respectively (Fig. 16a). By contrast, this ratio was not increased by tZ treatment in either high- or low-Pi medium (Fig. 16a), suggesting a specific role of cZ in this differential growth. Furthermore, treatment with both cZ or tZ increased Pi content in roots and decreased

it in shoots, leading to a significant increase in the Pi root:shoot ratio (Fig. 16b). This response is dependent on CK signaling, as the double mutant for CK receptor *ahk3 ahk4* did not show a change in the Pi root:shoot ratio (Fig. 16b). We found that Pi allocation in cZ-treated seedlings was significantly higher than in tZ-treated under Pi sufficient conditions (Fig. 16b). However, under Pi deficiency we did not find differences between cZ- or tZ-treated seedlings, but it always tended to be higher in the cZ-treated, although no significant (Fig. 16b). These data seem to indicate that zeatins function in growth control and Pi allocation in *A. thaliana* plants through the AHK receptor signaling.

Pi deprivation increases the number and size of root hair to enlarge root system absorbing surface (Péret *et al.*, 2011). As Pi starvation increases the cZ:tZ ratio, we analyzed the effect of both isomers on root hair elongation in control plants as well as in the double CK receptor mutant *ahk3 ahk4*. In control seedlings, both cZ and tZ increased root hair elongation in the mature zone of the roots (Fig. 16c,d). This elongation was significantly higher in cZ-treated seedlings grown with or without Pi (Fig. 16d). In Pi starved plants, cZ treatment increased, to a greater extent than tZ, root hair elongation in an AHK3-AHK4-dependent manner (Fig. 16c,d). These data suggest the role of cZ mediating Pi induced root hair elongation, which depends, at least partially, on the CK AHK-receptors.

In *A. thaliana*, cZ synthesis is mediated by IPT2 and IPT9 enzymes (Miyawaki *et al.*, 2006). The *ipt2 ipt9* double mutant, which shows a reduction in root growth and LR, has no detectable cZ-type CKs, except a residual cZOG (Köllmer *et al.*, 2014). We found that root hair elongation diminished in the *ipt9* and *ipt2 ipt9* mutants, but not in the *ipt2* mutant (Fig. 16e). When we analyzed the effect in Pi allocation in control conditions, we found that *ipt2* and *ipt9* did not modify the root:shoot Pi ratio, only the double mutant showed a reduction in the Pi root/shoot ratio (Fig. 16f). During Pi deficiency, the Pi root/shoot ratio increased in wild type and *ipt2* seedlings, while in *ipt9* or *ipt2 ipt9* double mutant the ratio did not increase and was similar to control plants in +Pi condition (Fig. 16f). These experiments suggest that cZ mediates root hair and Pi allocation responses in PSR mainly through *IPT9*.

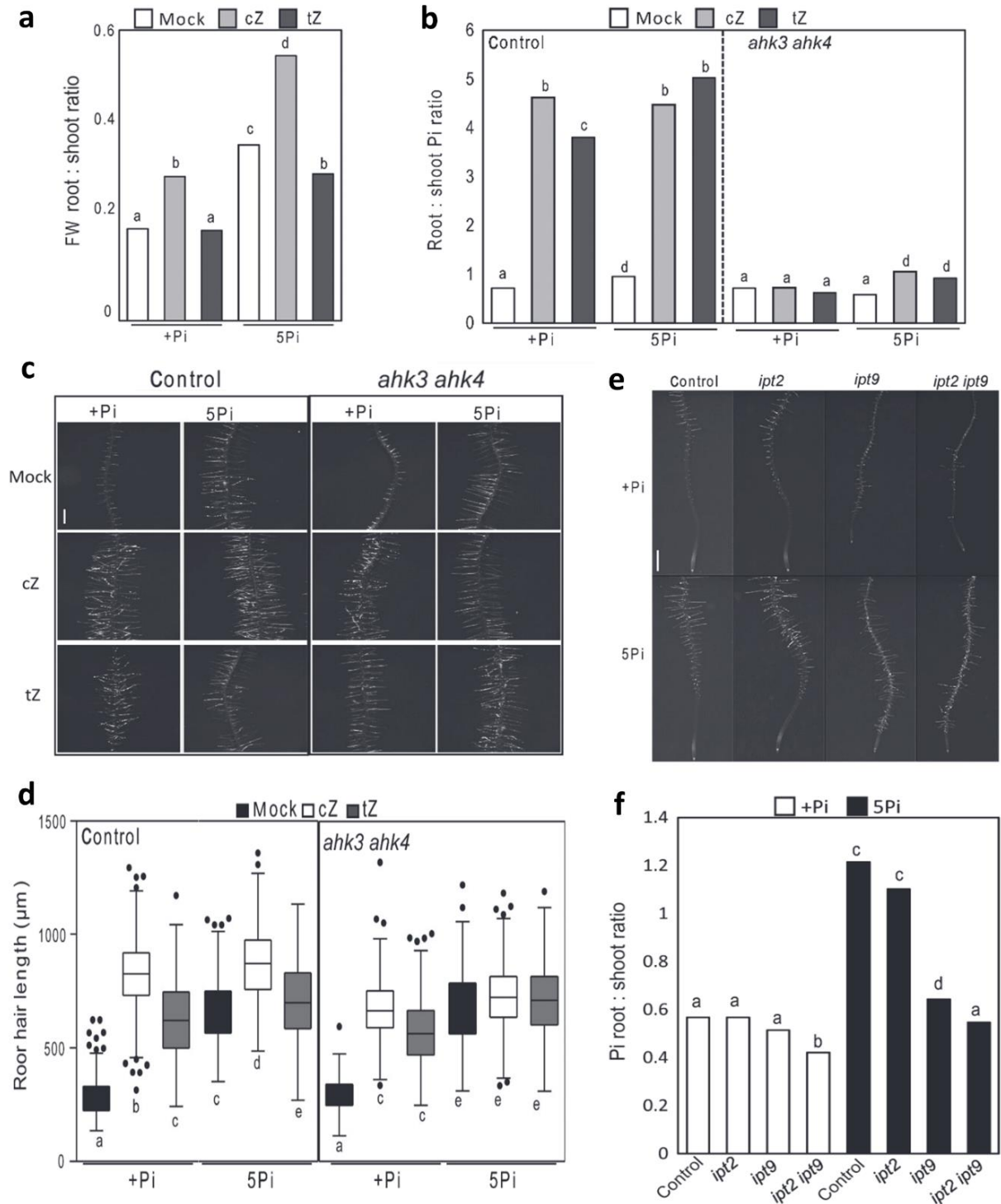


Fig. 16: cisZeatin regulates PSR responses specifically. **a** Fresh weight (FW) root:shoot ratio calculated of *A. thaliana* seedlings grown in $\frac{1}{2}$ MS with 625 μ M (+Pi) or 5 μ M (5Pi) phosphate for 5 days and then treated with 1 μ M of cZ or tZ for extra 5 days. Values shown means of five replicates $\pm$ SD. **b** Root:shoot Pi content of control or *ahk3 ahk4* double mutant seedlings grown as in **a**. **c** Representative pictures of root hairs from the mature area of control and double mutant *ahk3 ahk4* grown in medium containing +Pi or 5Pi for 6 days and then transferred to a similar medium containing mock or 2.5 μ M cZ

or tZ for extra 2 days. Pictures were taken in the zone of transference. Bar, 500 μm . **d** Root hair length quantification of seedlings grown as in **c**. $n = 10$ different roots; total number of root hairs ≥ 450 . **e** Representative pictures of root hairs close to the root tip of *ipt2*, *ipt9* or *ipt2 ipt9* seedlings grown +Pi or 5Pi for 10 days. Bar, 500 μm . **f** Root:shoot Pi content ratio in of *A. thaliana* control, *ipt2*, *ipt9* or *ipt2 ipt9* mutants grown as in **e**. All plants were grown using the D-Root system keeping roots in darkness. Values shown are means of at least four replicates $\pm$ SD. Significance was analyzed by ANOVA and Tukey HSD post-test. The same lower-case letters above the SD indicate no significance difference (p -value < 0.05).

4.1.6. Natural variation of shoot and root traits under high and low Pi availability

We showed before that the exposure of roots to light alters PSR, especially in the root system (Section 4.1.1., Fig. 9). Thus, part of the PSR could be masked if plants are responding to the combination of light and Pi starvation stresses rather than specifically to Pi starvation. Thus, we wanted to use the D-root system to identify the genetic bases of PSR and new loci that could be involved in the response without the influence of the root illumination. We used the natural variation of *A. thaliana* to explore the genetic architecture of different traits related to Pi starvation through GWA studies. We selected four traits related to plant adaptation to Pi deficiency: weight and Pi concentration for shoots and PRL and number of LR for roots.

We phenotyped 173 accessions of the Iberian Peninsula natural collection of *A. thaliana* (Fig. 17a), which has been successfully used in other studies (Tabas-Madrid *et al.*, 2018; Delgado *et al.*, 2019; Montes *et al.*, 2021; Arteaga *et al.*, 2022). We cultivated the different accessions on randomized batches, with Col-0 as an internal phenotypic control in all of them, using media containing high Pi (625 μM) and low Pi (5 μM) concentrations. Four independent replicas for each concentration were grown simultaneously for 12 days (Fig. 17b). Due to problems in seed germination, irregular growth, or high individual variability, we selected 158 accessions for further GWAs analysis (Supplementary Table 1 and Supplementary File 2 – Table a).

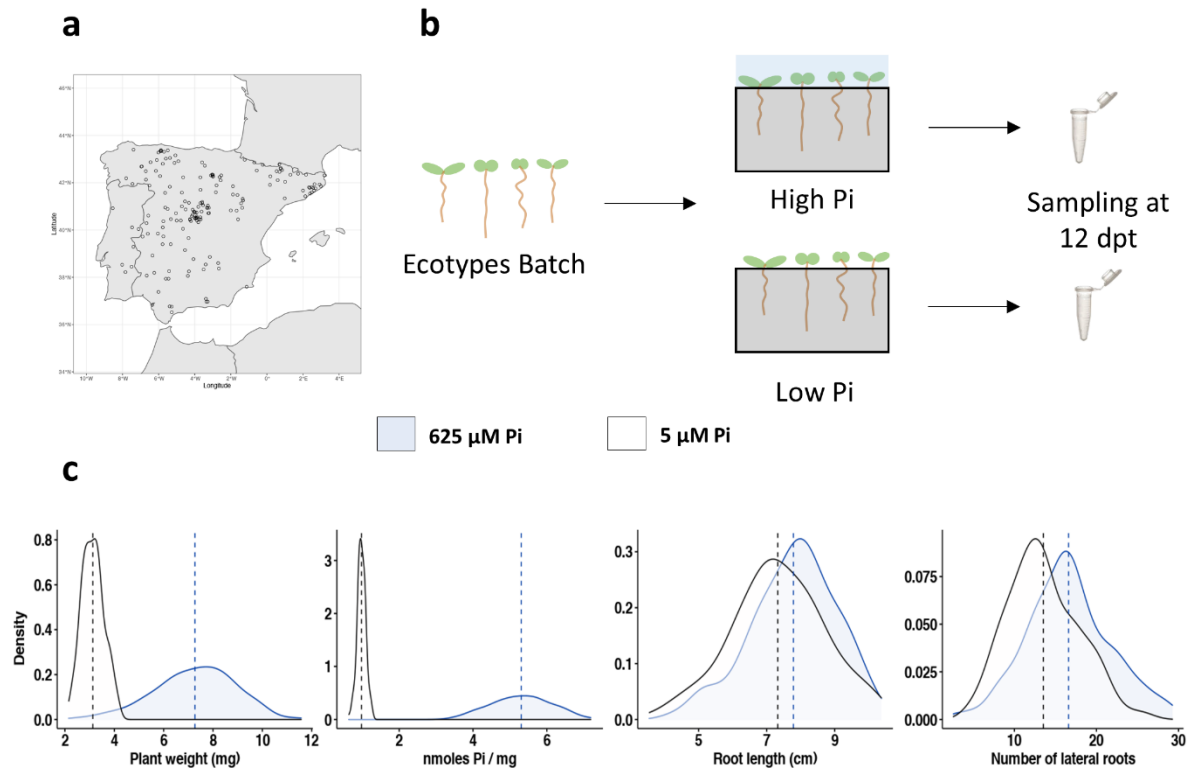


Fig. 17: *A. thaliana* Iberian collection: geography, GWAS design and trait distribution. **a** Geographical distribution of the collection in the Iberian Peninsula. Each population is represented by a grey circle. **b** Graphic of the experimental design. Each batch of ecotypes was grown on $\frac{1}{2}$ MS high (625 μ M) or low (5 μ M) of phosphate simultaneously for 12 days. Afterwards, plants were sampled for the traits showed below. **c** Mean distribution for a given trait in the collection. Shoot weight, Pi concentration, root length and the number of lateral roots are represented. Data distribution is displayed in blue for high phosphate conditions (control), and in white for phosphate deficiency. The dashed line points to the mean of the distribution for all ecotypes in the specified trait and condition.

Shoot traits showed more variation under high Pi, while PRL and number of LRs presented less variation in these conditions compared to Pi deficiency (Fig. 17c, Table 3). Pi deficiency reduced the mean value of all traits, having higher impact on shoot weight and Pi concentration (Fig. 17c). Heritability varied for the different characters, ranging from 18% to 38% and being the lowest for Pi concentration, intermediate for weight and higher for PRL and number of LRs (Table 3). The highest heritability for root attributes suggests a strong genetic component for these traits (Table 3). The means of the different traits were normally distributed (Table 3), except for Pi concentration, which was Boxcox transformed for further GWA analysis.

Table 3. Summary of the characteristics for the observed traits of Iberian Peninsula accessions. Weight: Aerial part weight. Pi: Pi concentration in shoots. PRL: principal root length. nLRs: number of lateral roots. Each trait was evaluated under High Pi (625 μ M) and Low Pi (5 μ M). CV%: coefficient of variation. H²: broad sense heritability. Transformation: Type of transformation used for the trait in GWAs analysis. SW test: Shapiro-Wilk test for normality, if p -value < 0.05 the data does not have a normal distribution.

	Trait	Condition	CV%	H ²	Transformation	SW test
Shoot	Weight	High Pi	21.73	32.12	Not transformed	0.551
		Low Pi	13.99	28.42	Not transformed	0.157
	Pi	High Pi	14.72	18.06	Boxcox	0.536
		Low Pi	11.74	22.62	Boxcox	0.906
Root	PRL	High Pi	15.92	33.91	Not transformed	0.050
		Low Pi	18.24	36.84	Not transformed	0.232
	nLRs	High Pi	30.63	35.16	Not transformed	0.434
		Low Pi	31.59	38.43	Not transformed	0.085

To understand how traits are related between each other, we made a correlation matrix analysis using Pearson's statistic (Supplementary Table 3, Fig. 18). Under high Pi, correlations between the analyzed traits were mainly positive (Fig. 18a). Interestingly, the highest correlation was observed between weight and the number of LRs, suggesting the importance of the root system for shoot growth. Pi concentration was only significantly correlated with root length. However, under Pi starvation Pi concentration negatively correlated with weight and the number of LRs (Fig. 18b). The other parameters remained positively correlated, indicating common regulation under Pi sufficient and starvation of these characteristics. We inferred soil levels of Pi through geolocation of the accession population, however we only found a significant correlation under soil Pi levels and Pi concentration of plants grown under high Pi (Fig. 18a-b).

Next, we wanted to examine the relationship of each trait under the two Pi conditions (Fig. 18c). Weight and PRL displayed high significant correlations ($R = 0.56$, $p < 0.001$ for weight; $R = 0.58$, $p < 0.001$ for PRL) and the number of LRs was the highest ($R = 0.79$, $p < 0.001$). These data suggest that the genetic programs for those parameters

are partly independent of Pi availability. For Pi concentration there was no significant correlation between high and low media Pi, suggesting that the shoot Pi concentration varies differently in each accession in response to Pi availability. Thus, the genetic component that regulates shoot Pi concentration in response to Pi availability differs between accessions.

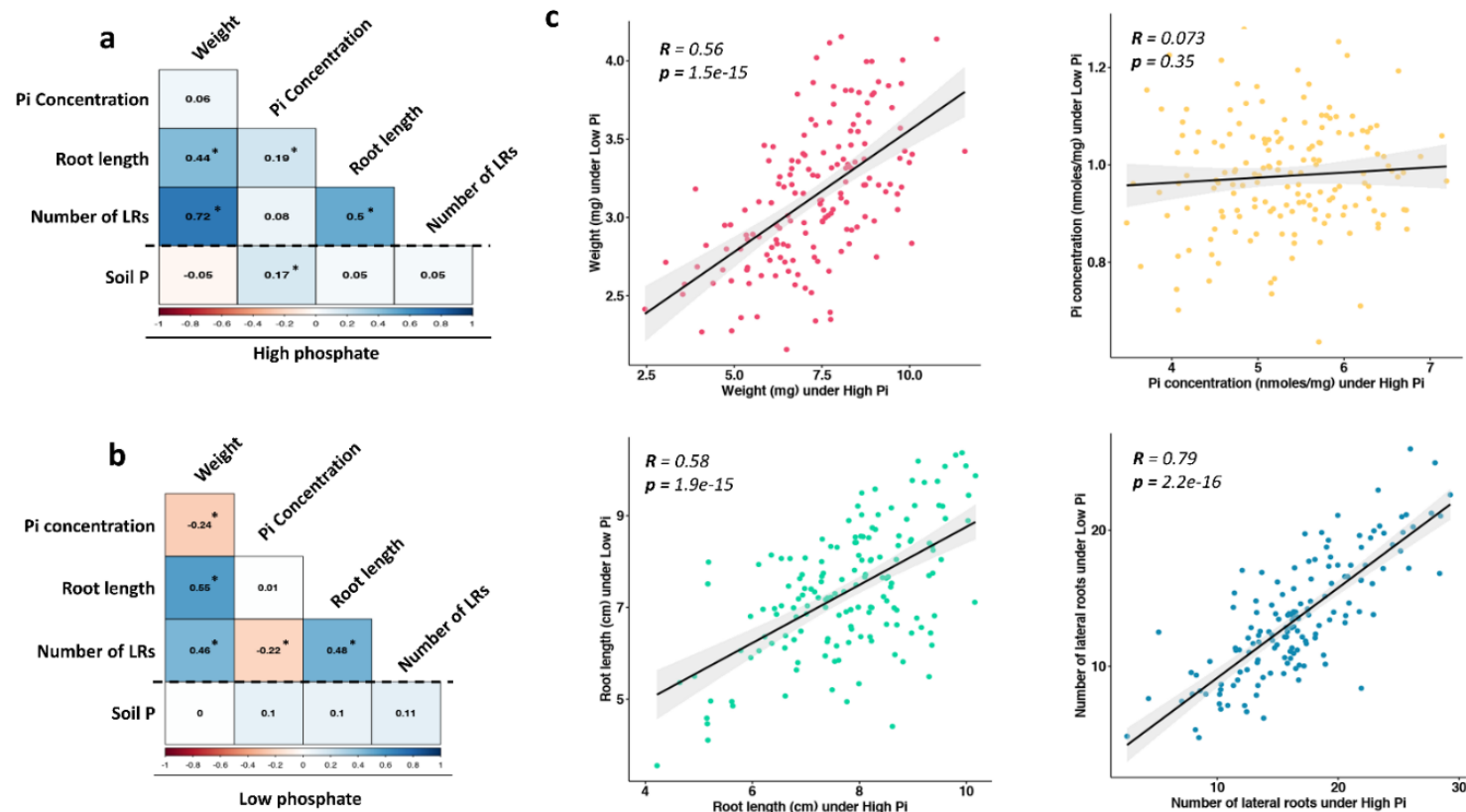


Fig. 18: Trait correlations in the *A. thaliana* Iberian collection. **a,b** Heatmap of the correlation matrix for the measured traits (shoot weight, shoot Pi concentration, root length and number of lateral roots) and the inferred level of soil phosphate in high phosphate (625 μ M) **a** or low phosphate (5 μ M) **b** respectively. The number in each square indicates the Pearson's correlation. The color scale represents correlation coefficient in each square visually. Statistical significance correlations are marked with an asterisk (p -value < 0.05). **c** Scatter plot of the correlation for the phenotypes in low phosphate compared with high phosphate. Pearson's correlation and significance are annotated for each trait in its plot. The black line represents the linear regression and the grey area indicates the 95% confidence interval.

4.1.7. Genome-wide associations for PSR traits

To understand the genetic architecture involved in natural variation of our PSR traits, we performed GWA analysis in our phenotypes under high and low Pi growth media (Fig. 19). Overall, only one SNPs for PRL under high Pi reached statistical significance for Bonferroni's correction threshold ($-\log(P) > 8$). To obtain an insight on which loci are contributing to the observed phenotypes, we selected a less conservative threshold of $-\log(P) > 4$ and $MAF > 3\%$ to select SNPs associated for each trait and inferred their associated genes (Table 4; Supplementary File 2 – Table b-I; Supplementary File 3).

Table 4. Number of SNPs and inferred associated genes for each trait and phosphate condition.

	Trait	Condition	Number of SNPs	Number of Genes
Shoot	Weight	High Pi	146	97
		Low Pi	174	93
	Pi	High Pi	180	88
		Low Pi	112	123
Root	PRL	High Pi	298	130
		Low Pi	157	102
	nLRs	High Pi	141	120
		Low Pi	522	104

For shoot weight, there were 97 and 93 significantly associated genes corresponding to high and low Pi respectively (Fig. 19a, Supplementary File 2 – Table b-c). Seven of these genes were common for both conditions and were located at two different genomic locations, one on chromosome 4 with 3 associated genes and one on chromosome 5 with 4 associated genes. The common genes corresponded to a protein phosphatase and putative lipoproteins (Supplementary File 2 – Table I). In low Pi, 11 genes corresponded to TFs. Among them, we found LRL1, which is described to regulate root hair development (Karas *et al.*, 2009).

For shoot Pi concentration, the GWAs analyses assigned 88 genes and 123 in high Pi or low Pi medium respectively (Supplementary File 2 – Table d). Surprisingly, the association in $-\log(P)$ of these peaks was higher in high Pi than in low Pi conditions (Fig. 19b). In this case, there were no common genes between high and low Pi, suggesting that, as the correlation analyses, plants use different genetic components for Pi acquisition/usage in high or low Pi conditions. Interestingly, the low Pi gene list was enriched for the GO term for “cellular response to phosphate starvation” (5.86-fold enrichment, $p < 0.05$, Hypergeometric test) (Supplementary File 2 – Table e,j). Three of these genes are known to have important functions under Pi deprivation: *MIPS2* (myo-inositol 1-phosphate synthase 2), which is involved in myo-inositol synthesis (Donahue *et al.*, 2010); *PS2*, which encodes for an inorganic pyrophosphatase induced under Pi starvation and involved in root hair elongation (Chandrika *et al.*, 2013); and *LPRI*, a key gene that participates in adjusting root growth to Pi availability (Müller *et al.*, 2015). Furthermore, the synthesis route of the InsP metabolism is also enriched in our associations (8.5-fold enrichment, $p < 0.01$, Hypergeometric test; Supplementary File 2 – Table k), corresponding to the following genes: the *ITPK4*, *PHOSPHATIDYLINOSITOL 4-PHOSPHATE 5-KINASE 8* (*PIP5K8*) and, previously commented, *MIPS2*. These three enzymes act in the synthesis of InsP₈, the cellular Pi sensor (Dong *et al.*, 2019). In addition, there are 9 TFs, such as *CYTOKININ-RESPONSE FACTOR* (*CRF7*) and an *ETHYLENE-RESPONSE FACTOR 15* (*ERF15*), both responsive to hormones that control the PSR (Zhang *et al.*, 2014a). These results suggest that our data, despite the low SNPs significance, might uncover Pi-responsive loci.

For the PRL phenotype under Pi sufficient conditions, we have identified 130 putative genes that could be potentially involved in this trait (Supplementary File 2 – Table f). Moreover, it has the most significant SNP associated located at chromosome 1 (Fig. 19c). Further inspection of this genomic region revealed that the SNPs clustered around the promoter and the first exon of an ankyrin repeat protein (ANK) (Fig. 20a-b). The SNPs located at the first exon are predominantly non-synonymous substitutions (Fig. 20b) with a missense mutation, being these the most associated SNPs (Fig. 20b-c). However, this genetic locus was not present in the Pi deprivation GWA (Supplementary File 2 – Table g). The common associated genomic areas in Pi sufficient

and starvation were of small significance. Nevertheless, two TFs were common, *WRK4* and *GATA14*, suggesting that they might be involved in PRL.

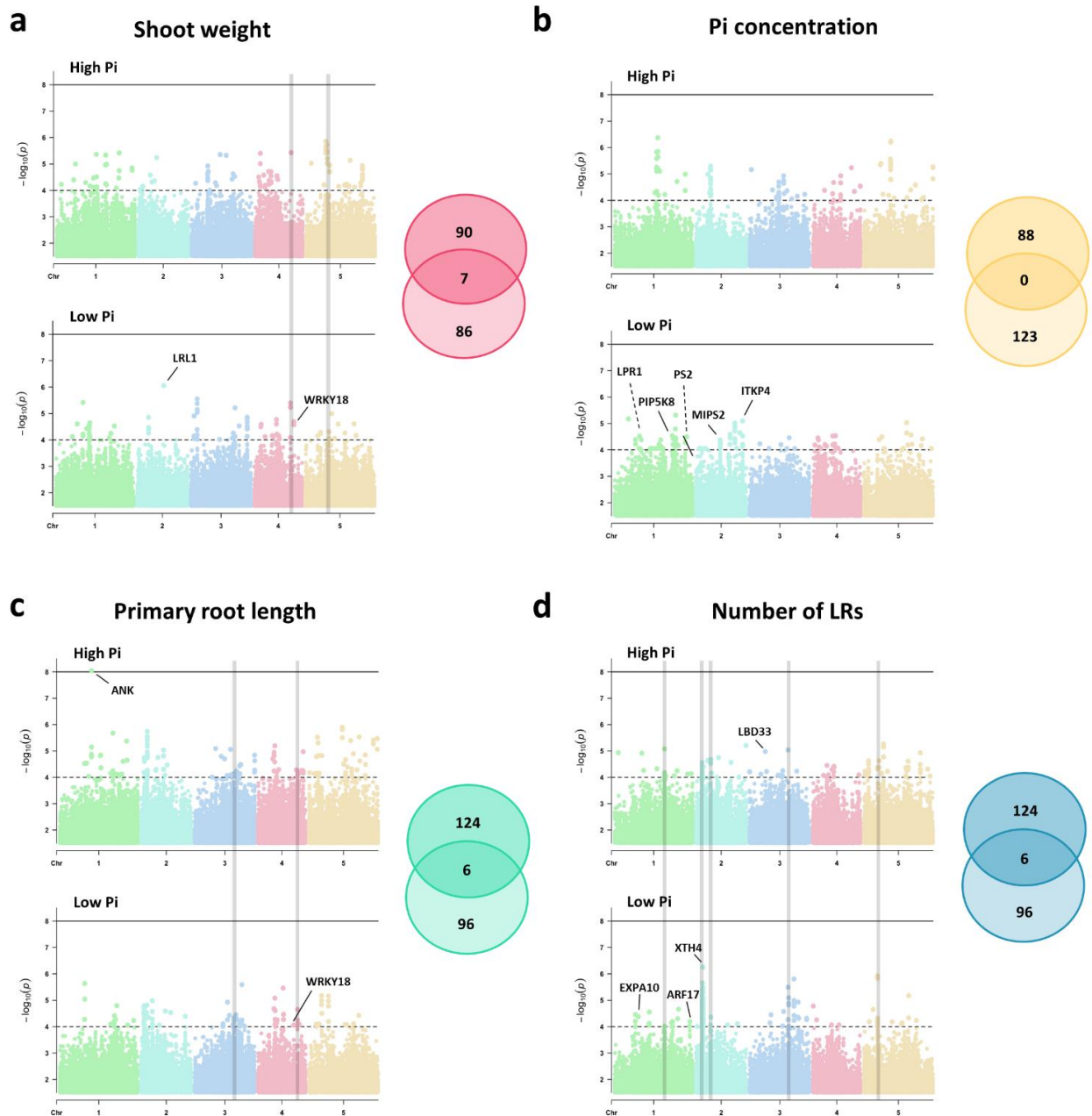


Fig. 19: Genome Wide Association (GWA) analysis of traits under high and low phosphate. a-d Manhattan plots for shoot weight **a**, shoot Pi concentration **b**, principal root length **c** and number of lateral roots **d** under high phosphate (625 μ M) and low phosphate (5 μ M). Each chromosome is represented with a different color. The continuous horizontal line represents the corrected Bonferroni's significance threshold ($-\log(P) = 8$). The dashed line corresponds to threshold $-\log(P) = 4$. The grey vertical lines connect common SNPs associated with a given trait under high and low phosphate

conditions at $-\log(P) > 4$. Venn diagrams represent the number of genes significantly associated with a given trait for high phosphate (upper) and low phosphate (lower) conditions (red: shoot weight, yellow: Pi concentration, green: PRL, blue: nLRs). The intersection represents the number of common genes significantly associated with the analyzed traits between the two Pi conditions. The genes labeled in the Manhattan plots correspond to putative candidates associated to selected SNPs.

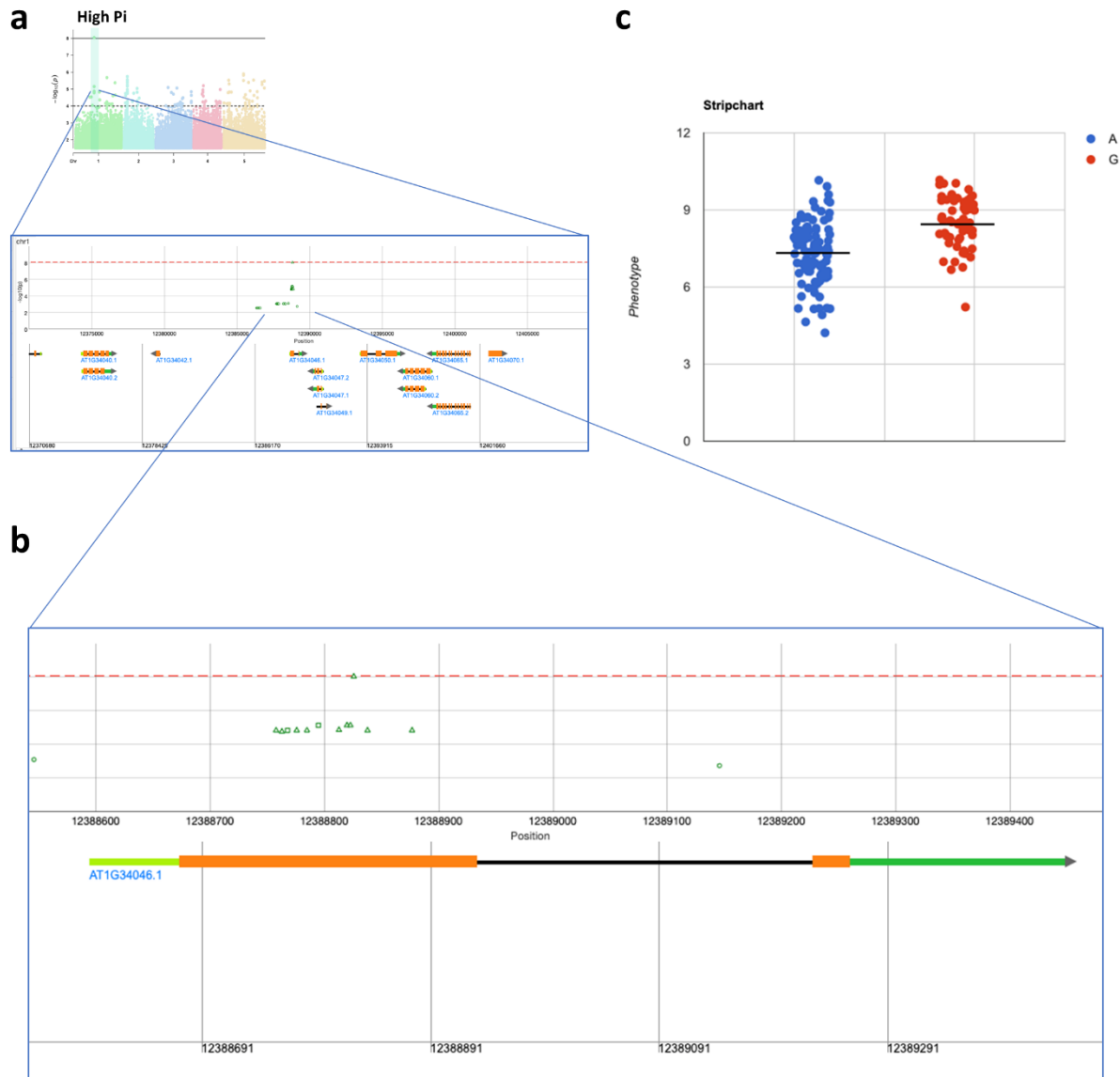


Fig. 20: PRL GWAS significant peak under phosphate sufficiency correspond to an Ankyrin repeat protein. **a** Augment of $\pm 10\text{kb}$ from the highest and significant peak in our PRL GWAs. Dots indicate SNPs associated with their corresponding $-\log(P)$ value. Boxes represent synonym mutations and triangles miss-sense mutations. **b** Zoom to the genomic region of AT1G34046, an ANK protein. **c** Stripchart of the most significant SNP accessions values. The SNP corresponds to a missense mutation

on the first exon of AT1G34046. Blue color represents the SNP corresponding to an Adenine (A) and red corresponds to Guanine (G).

For the number of LR phenotype in high Pi medium, we have identified 120 associated genes (Supplementary File 2 – Table h). *LBD33*, a known player in LR morphogenesis (Berckmans *et al.*, 2011), has the highest association for this trait in chromosome 5. In low Pi, this trait was associated with 104 genes (Supplementary File 2 – Table i), and, one of them, *PIP5K2*, is involved in LR development (Mei *et al.*, 2012). Interestingly, among the candidate genes in high and low Pi, we identified 6 common genes in 5 different genomic areas. Overall, these 6 common genes showed a higher significance under Pi starvation. The highest peak, located in chromosome 2, covered a wide genomic area of 50kb.

To explore if there were genomic regions commonly associated with different traits under Pi deprivation, we compared all genes for all traits through a venn diagram (Fig. 21a). There were common genes for some of the traits:

- (i) Pi concentration and PRL had in common an RNA helicase.
- (ii) PRL and number of LR shared a protein with a domain of unknown function.
- (iii) Shoot weight and PRL shared 4 genes in total; *WRKY18*, *AT2G11200* (an F-box protein), *POLAR* (a scaffold protein associated with asymmetry of meristems) and *AT4G31810* (an ATP-dependent caseinolytic protease).

Since TFs control transcriptional reprogramming in response to stimuli, we selected the TF *WRKY18* for additional exploration. We visualized its interaction network to gain insight of what processes could be regulating. This TF interacts with genes that play a known role in PSR, such as *WRKY42* or *PIF4* (Su *et al.*, 2015; Sakuraba *et al.*, 2018) (Fig. 21b). These results suggest that *WRKY18* might have a function in Pi starvation responses.

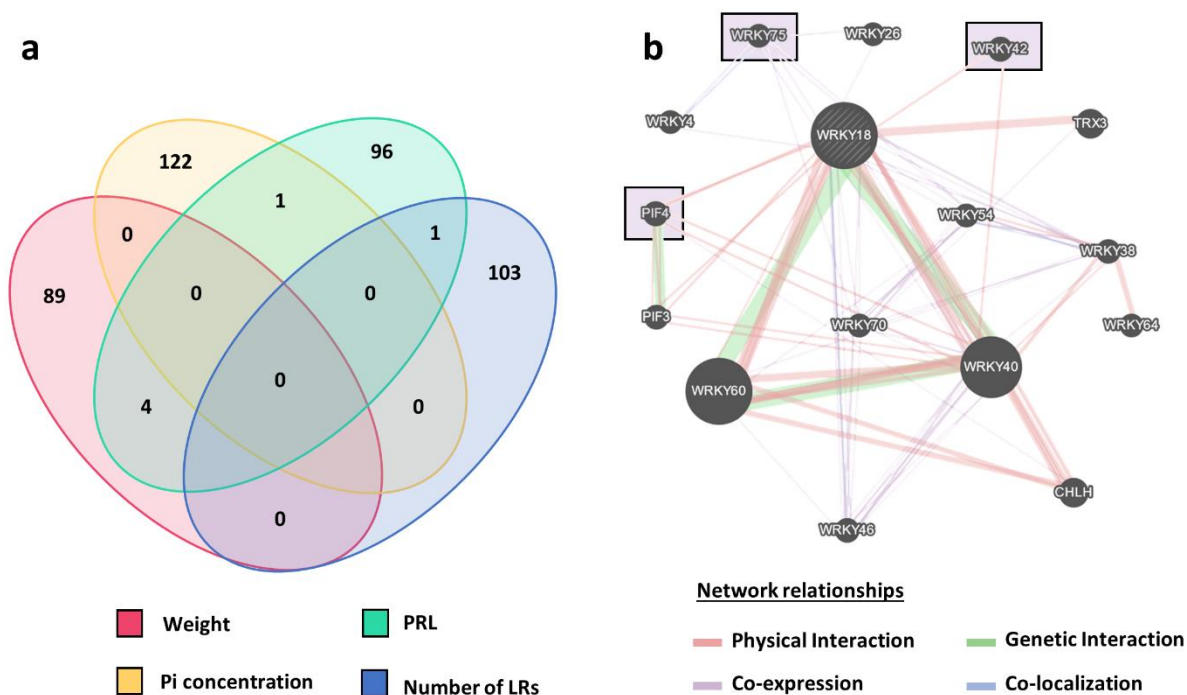


Fig. 2l: Common associated genes in all traits under phosphate deficiency. **a** Venn diagram of shoot weight, phosphate concentration, root length and number of lateral roots genes associated through GWA analysis under phosphate deficiency. **b** Network of interaction of WRKY18 through Genemania. In purple boxes, those interacting genes with known functions under phosphate starvation.

4.2. Screening for fungal positive interactors under phosphate deprivation

4.2.1. Screening natural fungal endophytes for Pi deficiency alleviation

In this objective, we wanted to identify fungal endophytes that promote plant growth under Pi deficiency. To do this, we screened the collection of fungal isolates from natural populations of *A. thaliana* generated by Dr. Soledad Sacristan (García *et al.*, 2013). This collection is composed of fungi isolated from different internal compartments of the plants, from the roots to the siliques, so they are therefore putative endophytes. We started by filtering the collection for high viability after long-term storage (glycerol stocks in -80°C freezers), rapid growth and high sporulation (data not

shown), and we selected 24 fungal isolates, for further analysis (Fig. 22a). We barcoded each isolate in 10 different categories according to their morphology and growth, and then numbered them if more than one isolate corresponded to the same classification (i.e., for Co category: Co-1, Co-2, etc).

Plant-fungus interactions can vary depending on environmental factors (Balestrini, 2021). *In vitro* co-cultivation of plant and microorganisms in MS growth medium containing sucrose and vitamins may lead to the overgrowth of the microorganisms, which can behave as pathogens as it has been observed with the beneficial fungus *Colletotrichum tofieldiae* (data not shown). Therefore, to set up the screen for positive interactors, we decided to cultivate plants and fungus without sucrose or vitamins. We then observed that root illumination dramatically altered root growth when no sucrose was present in the growth media (Fig. 22b). It was previously reported that higher illumination intensity (including roots) increases PGP effect of *C. tofieldiae* under Pi deprivation (Frerigmann *et al.*, 2021). In fact, our previous results (Section 4.1.1., Fig. 9) showed that root illumination and Pi deprivation have an additive effect on plant stress. Therefore, the combination of both stresses could amplify the observation of a PGP effect of the fungus. However, we also noticed that standard Pi concentration in the ½ MS medium inhibited root growth when plants were transferred from D-root system to root illumination (Fig. 22c). This effect was reduced as the Pi concentration of the media decreased, with an optimum in 312 µM Pi (Fig. 22c). Thus, we capitalized on these results to design our screening. We grew the seedlings for 7 days in optimal standard conditions (312 µM Pi) without sucrose or vitamins using the D-Root system to improve root and shoot growth. In parallel, we grew fungal isolates for 4 days using the D-Root, as illumination might produce an effect on fungal growth (Idnurm & Heitman, 2005; Cabrera *et al.*, 2022). These 7 days-old seedlings were transferred to the plates where the different fungi had been previously grown in low Pi conditions and with root illumination to increase the “stress” in the plant and the possible PGP produced by the fungus (Fig. 22d). After 7 days of co-cultivation, plants were sampled to screen for positive phenotypes: shoot fresh weight (FW), primary root length (PRL) and number of LRs as parameters (Fig. 22d).

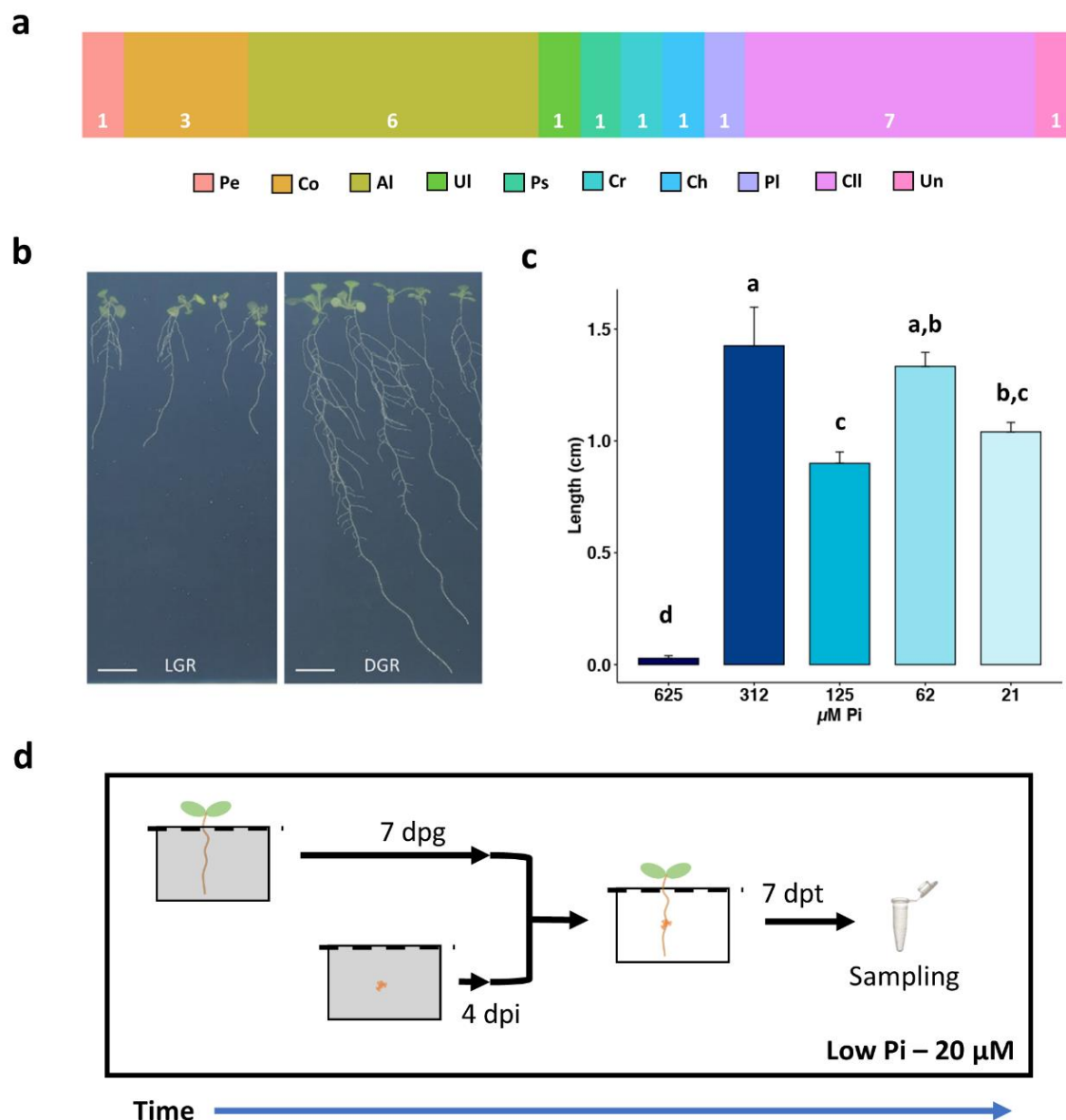


Fig. 22: Designing the screening conditions for fungal isolates. **a** Barcode assigned composition of the 24 selected fungal isolates from *A. thaliana*. The number inside the bar indicates the number of isolates corresponding to that category. **b** *A. thaliana* seedlings grown in $\frac{1}{2}$ MS without sucrose for 12 days, maintaining the roots exposed to light (LGR) or in the dark (DGR) using the D-Root system. Photosynthetic photon flux density: $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Bar, 1 cm. **c** PRL growth of *A. thaliana* (cm) light exposed roots at different Pi concentrations at 48h post transference from $625 \mu\text{M}$ of Pi plates that had previously been maintained in D-Root for 7 days ($n \geq 5$). **d** Graphic of the experimental procedure for the screening. Seedlings of *A. thaliana* are grown for 7 days and fungal isolates for 4 days in a different plate, then plants are transferred and co-cultivated with the fungus for 7 additional days to screen the interaction. Dpg: days post germination, dpi: days post infection, dpt: days post transference.

As Pi immobilization by mineralization is a limiting factor for plant growth (Péret *et al.*, 2011), we started our screening by assaying the Pi solubilization capability of the isolates. However, we did not find any isolate in our selection capable of Pi solubilization (Fig. 23). Then, we tested the plant-fungus interaction using the screening system explained above (Fig. 22d). Initially, to quickly select those isolates with positive effects in our experimental set up, we started analysing only one replicate. We relativized the results to the mock condition to easily visualize the growth promoting effect (Fig. 24a-c). We observed a continuum of interactions from positive to negative in all selected parameters. This continuum was also observed within a given category of morphological similar fungi (Fig. 24a-c). For SW parameter, we observed that Co-1 and Ps-1 isolates produced the greatest promotion of shoot growth, although other isolates had slightly positive effects as well. Different isolates were also able to increase relative PRL and/or relative number of LR. To summarise the results and have an overall vision on the effects, we summed all the three relativized parameters to give a performance score of each fungus (Fig. 24d). We observed that the highest net positive effect corresponded to Ps-1 strain, followed by two Coll type isolates. Overall, these results suggest that Ps-1 is the fungal isolate with highest growth promoting potential under low Pi in our experimental conditions.

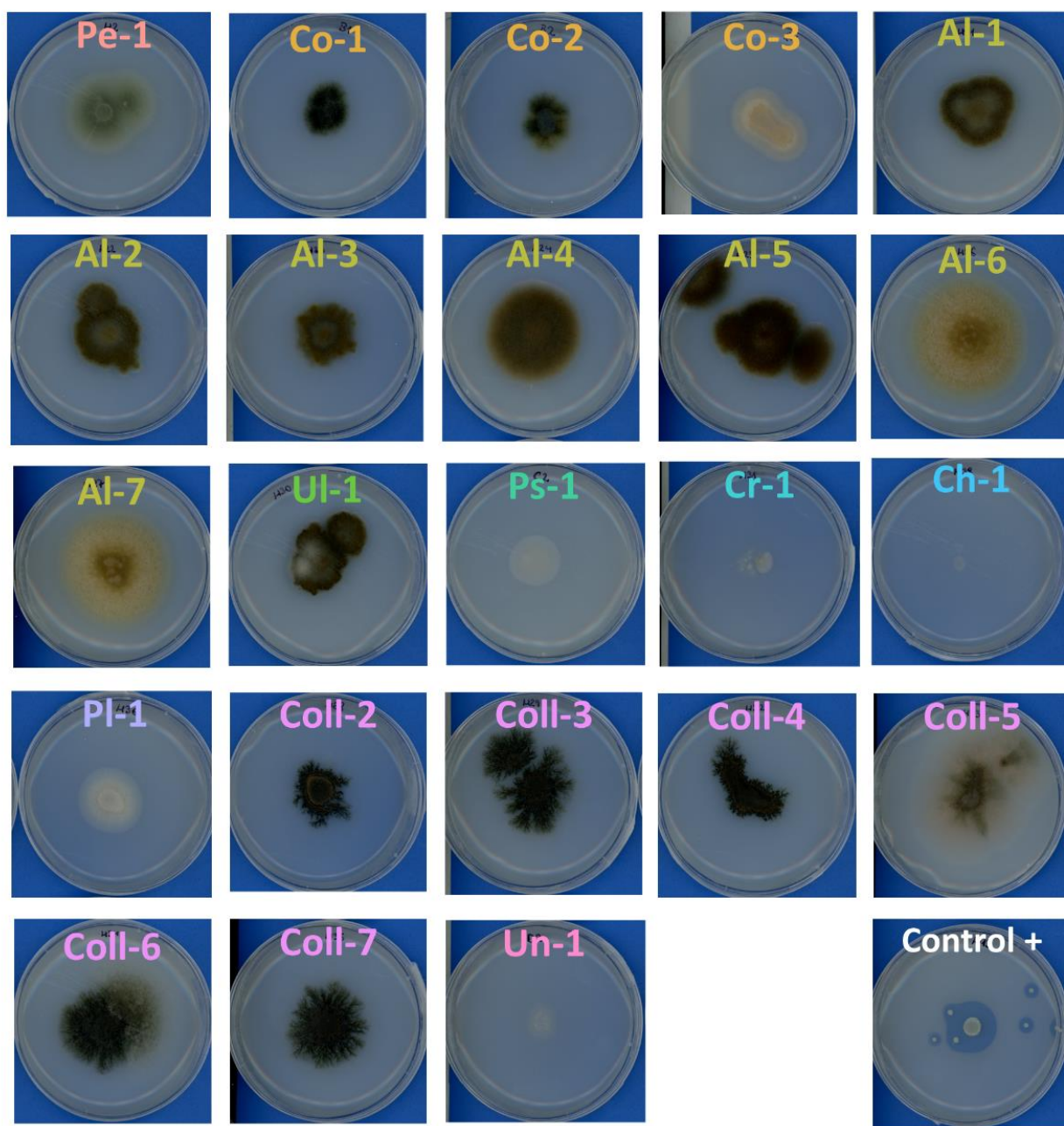


Fig. 23: Pi solubilization assay on fungal isolates. All the fungal strains were inoculated in Pikovskaya agar plates to test for phosphate solubilization (calcium Pi). Solubilization is measured as the diameter of the clear halo formed around the fungus. Notice none of our fungal isolates solubilizes calcium Pi.

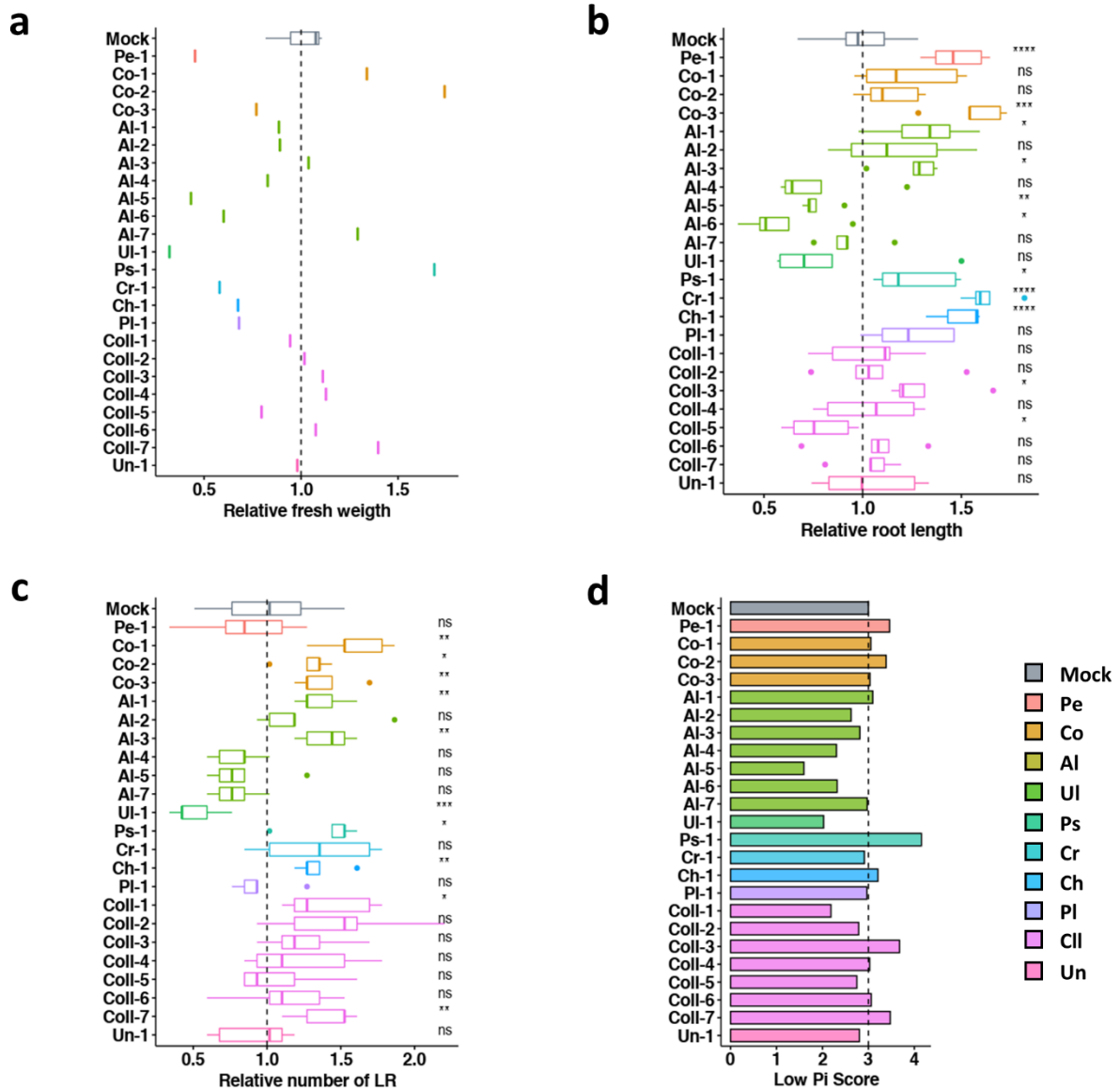


Fig. 24: Fungal isolates effect on plant growth. **a-c** *A. thaliana* seedlings of 7 days and fungal isolates previously grown for 4 days were co-cultivated for 7 additional days in low (20 μ M) phosphate. Fresh weight **a**, main root length **b** and lateral root (LR) number **c** were quantified. All the parameters were relativized to mock plants. The colors indicate the genus of the isolate. The vertical dashed line represents the average for the mock. The screening was one experiment with $n > 5$ plants. Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 . **d** Scores for each fungal strain evaluated in the screening. The score represents the sum of all relativized parameters showed in **a** to **c** (fresh weight, root length and number of lateral roots) in low phosphate (20 μ M).

4.2.2. *Ps-1* produces plant growth promoting effects under Pi starvation

Next, we wanted to further investigate the effect of *Ps-1* on plant growth. First, we evaluated how this isolate behaves in a Pi replete condition, as nutritional status can modify plant-fungus interactions (Hiruma *et al.*, 2016). We used the same experimental design shown in Fig. 22.d and quantified again our parameters of interest in high (300 μ M) or low (20 μ M) Pi. We corroborated our screening results, as *Ps-1* enhances plant growth under Pi deficiency, increasing shoot weight, root length and the number of LR (Fig. 25). However, we did not observe any promoting effect in high Pi (Fig. 25), suggesting that *Ps-1* growth enhancement might be, at least in part, related to Pi nutrition.

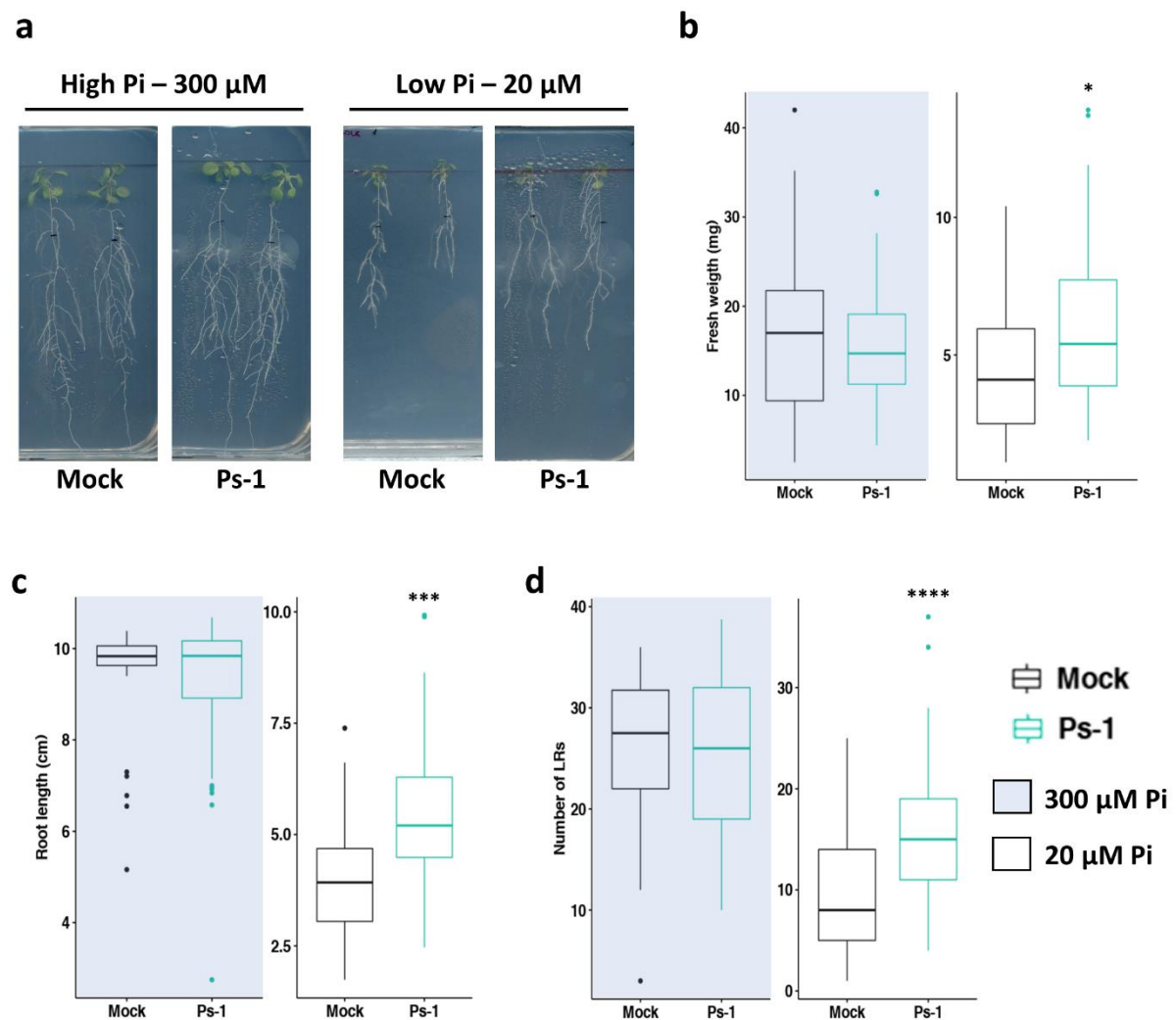


Fig. 25: *Ps-1* enhances plant growth in low Pi conditions. **a** Representative picture of *A. thaliana* seedlings grown for 7 days in D-Root and co-cultivated with mock or *Ps-1* for 7 additional days with roots

exposed to light in high (300 μ M) or low (20 μ M) Pi media. **b-d** Fresh weight **b**, root length **c** and number of lateral roots (LR) **d** of plants grown as in **a** in high (300 μ M, left graphs with blue background) or low (20 μ M, right graphs with white background) Pi media. The boxplots correspond to three independent replicates with $n \geq 3$ plants. Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.001, **** p -value < 0.0001.

To test Ps-1 involvement in plant Pi nutrition, we first quantified the Pi of inoculated plants. We found that Ps-1 co-cultivated plants did not accumulate more Pi in either condition (Fig. 26a). Since Pi accumulation was unaltered but plants showed an increase in biomass (Fig. 25a-b), we wondered if Ps-1 co-cultivated plants converted Pi in organic forms. To investigate this, we quantified total P (organic plus inorganic Pi) in Ps-1 co-cultivated plants growing under Pi deficiency. We determined that total P was higher in treated plants (Fig. 26b). Taken together, these results suggest that Ps-1 mediated PGP effect under Pi deficiency might involve P homeostasis.

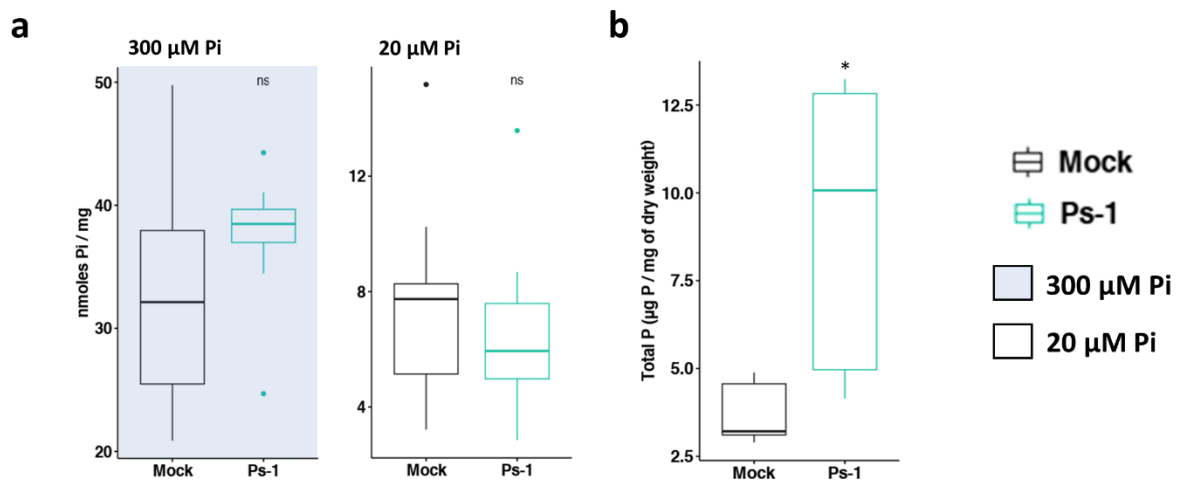


Fig. 26: Ps-1 alters phosphate accumulation. Pi accumulation of free cellular inorganic phosphate (Pi) **a** or total phosphorous (P) **b** in *A. thaliana* seedlings grown for 7 days in D-Root and co-cultivated with mock or Ps-1 for 7 additional days with roots exposed to light in high (300 μ M, left graphs with blue background) or low (20 μ M, right graphs with white background). Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.001, **** p -value < 0.0001.

4.2.3. Root meristem and PBS formation is modified by Ps-1 under Pi deficiency

As Ps-1 had a strong effect in roots under low Pi availability, we evaluated if there was an alteration on LR specification. The LR clock is an oscillating mechanism that primes cells to form LR PBS that acquire the competence to establish a new LR (Moreno-Risueno *et al.*, 2010). To minimize differences in root growth, we co-cultivated the plants with Ps-1 for 2 days in the D-Root, as light could influence root development (Silva-Navas *et al.*, 2016). We used the *pDR5::LUC* line to visualize the PBS as a maximum of auxin signalling along the main root (Fig. 6a). Root length after the point of transference was slightly but significantly larger in Ps-1 co-cultivated plants, as well as the number of PBS in a medium containing low Pi (Fig. 27b). However, the density of PBS was not significantly different between the two conditions. As before, the growth promoting effect was not observed in high Pi conditions (Fig. 28). These results suggest that Ps-1 might be affecting the LR clock under Pi deficiency.

The root meristem supports the root growth and therefore determines its growth potential (Perilli *et al.*, 2012). Longer roots can be the consequence of more cell division or longer cells. To analyse these possibilities, we evaluated Ps-1 effect on root meristem by using the *pWOX5::GFP* marker line to visualize the QC cells, and propidium staining (Fig. 27c). We observed that *A. thaliana* plants treated with Ps-1 had longer meristems with a significant higher number of cortical cells (Fig. 27d), suggesting that Ps-1 mediated increase in root growth occurs through an increase in cell division rather than the elongation of the cells.

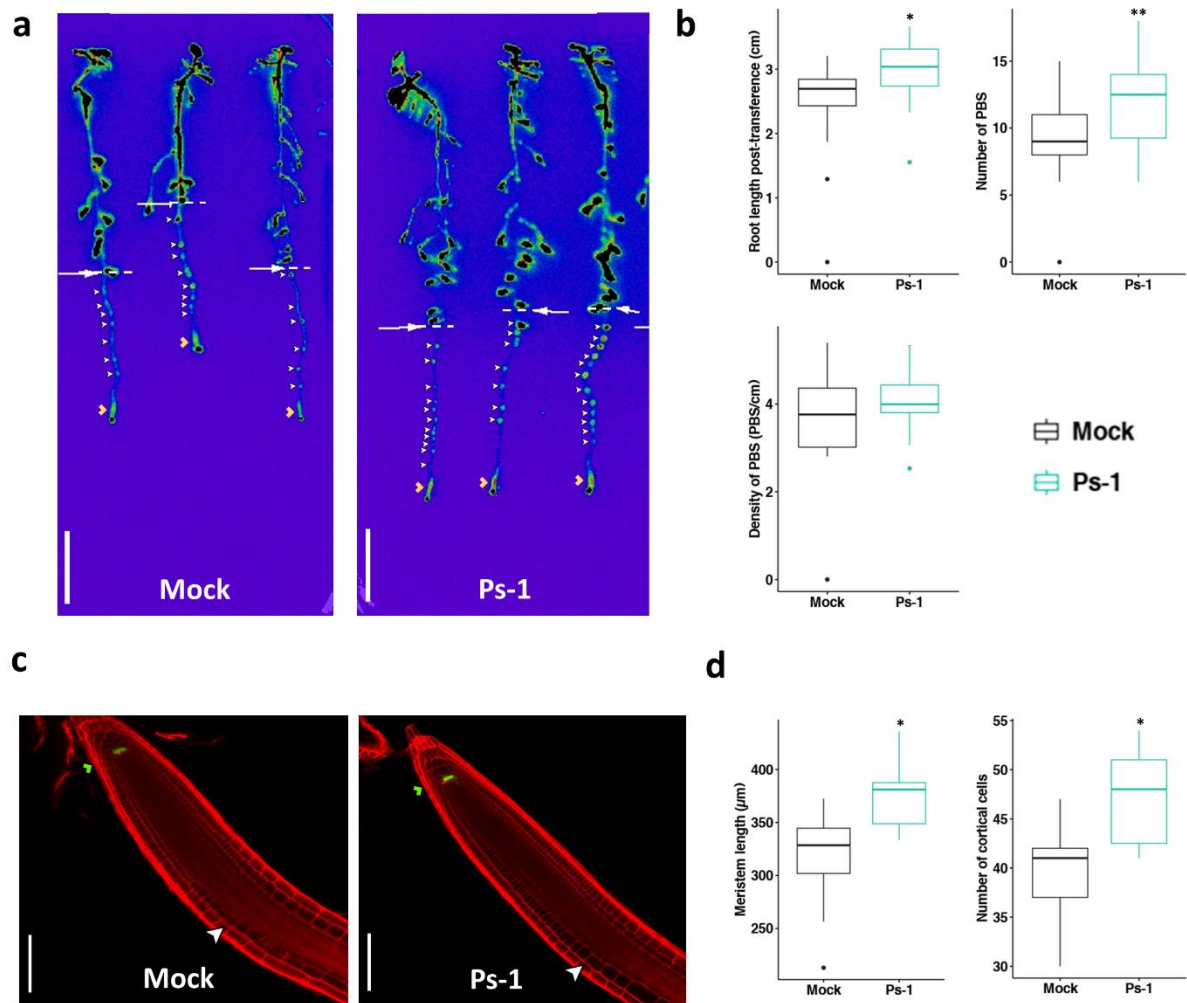


Fig. 27: Effect of Ps-1 on *A. thaliana* root PBS formation and root meristem under Pi starvation.

a Image of luciferase expression of *pDR5::LUC* seedlings grown in $\frac{1}{2}$ MS without sucrose or vitamins in a medium with low Pi concentration (20 μ M Pi) for 7 days and then co-cultivated for 2 days with Ps-1 previously grown for 4 days. Roots were kept in darkness during the whole experiment. The large arrow and dashed white line indicate the root length at transference. The white arrowheads point to pre-branching sites (PBS), marked as the spatially restricted stronger luciferase signaling. The yellow arrowheads indicate the oscillation zone (OZ). White bar equals 1cm. **b** Quantification of root length, number of PBS and density of PBS at post-transference of plants grown as in **a**. The boxplots correspond to three replicates with $n \geq 3$ plants. Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05, ** p -value < 0.01. **c** Representative confocal microscopy pictures of *pWOX5::GFP* seedlings grown as in **a**. Scale bar equals 100 μ m. The green arrowheads indicate the quiescent center (QC) marked by *pWOX5::GFP* and the white arrowheads the end of the meristem. **d** Root meristem length and number of cortical cells of root meristems of *pWOX5::GFP* plants grown as in **a**. $n \geq 6$ plants. Significance was analyzed by t-test compared with Mock condition as the reference group (* p -value < 0.05).

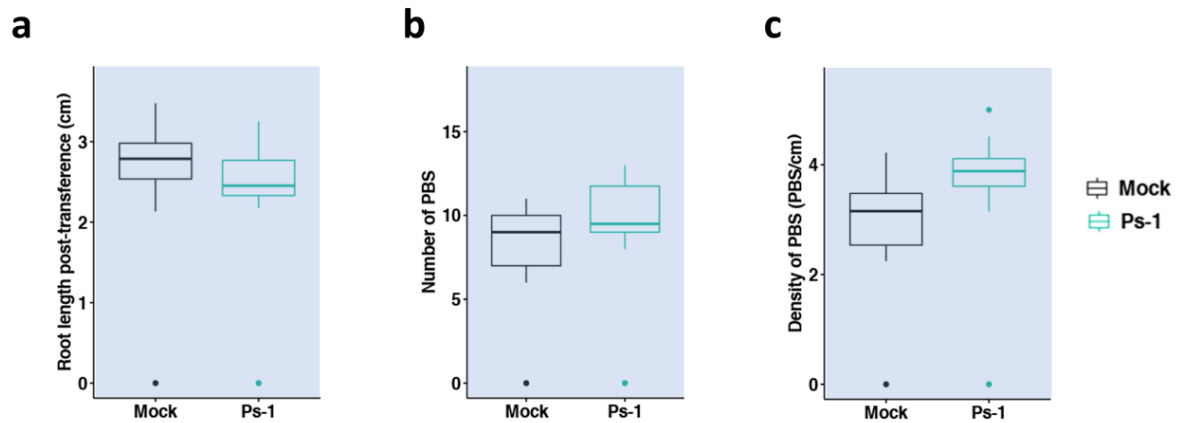


Fig. 28: Effect of Ps-1 on PBS formation under high Pi media. **a** Root length growth after transference of *A. thaliana* *pDR5::LUC* seedlings grown in $\frac{1}{2}$ MS without sucrose or vitamins with 300 μ M Pi for 7 days and then co-cultivated for 2 days with Ps-1 previously grown for 4 days. Roots were kept in darkness during the whole experiment. **b** Number of pre-branching sites (PBS) in the post-transference root of *A. thaliana* grown as in **a**. **c** Density of PBS per cm of root length post-transferences of **a-b** plants. The boxplots correspond to three replicates with $n \geq 3$ plants. Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05, ** p -value < 0.01, *** p -value < 0.001.

4.2.4. *Arabidopsis thaliana* aerial parts are not colonized by Ps-1

We evaluated whether Ps-1 is capable of colonizing *A. thaliana* aerial parts. We grew plants *in vitro* and inoculated them as stated before, and then attempted to recover Ps-1 growth in PDA plates from *A. thaliana* leaves. First, we surface sterilized the leaves to discard any superficial contamination and then we disrupted the tissue to allow the internal compartments to be released. We could not observe any growth of Ps-1 from *A. thaliana* leaf extracts (Fig. 29). As a control, we analysed the colonization of other fungus, which was indeed recovered from leaves (data not shown). These data suggest that this fungus does not colonize aerial parts of plants and that interaction occurs mainly through roots.

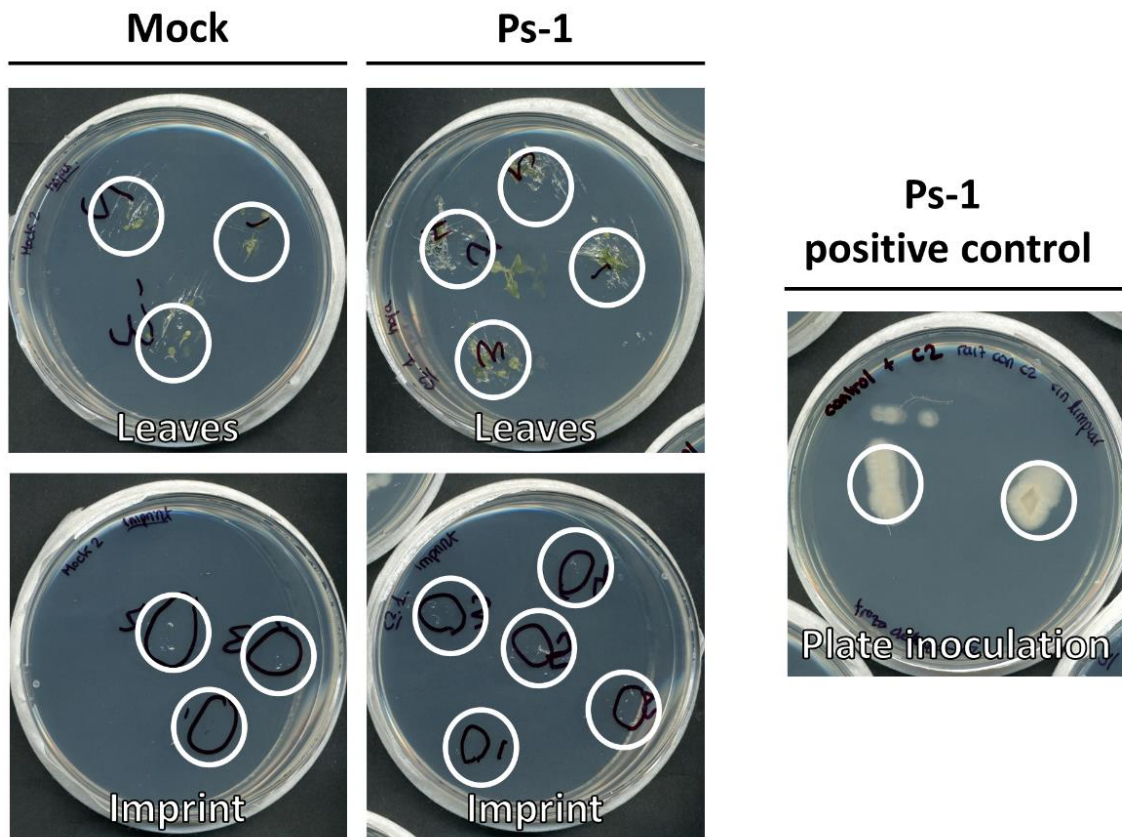


Fig. 29: Ps-1 is not-isolated from *A. thaliana* leaves after surface sterilization. Plants were co-cultivated for 7 days with Ps-1 with roots exposed to light. Before the interaction, plants were growing for 7 days and Ps-1 was growing for 4 days. Notice that no fungal growth is observed in leaves or imprints of the leaves, circled in white, compared with the positive control plate where roots co-cultivated with Ps-1 without sterilization were placed.

4.2.5. *Ps-1* induces plant growth on *Hordeum vulgare*

We next examined whether Ps-1 isolate was capable of promoting growth of a crop. We grew barley (*Hordeum vulgare*) plants in germination papers irrigated with a $\frac{1}{8}$ diluted MS in an adapted D-Root system for crops (Fig. 30a). With this system, we were able to record root growth while keeping the root in darkness. We germinated the seedlings for 4 days under darkness and then treated them by dipping the roots in a Ps-1 spore suspension. After 7 days, we observed an increase in shoot biomass, as in *A. thaliana* plants (Fig. 30b). As LR is complex to track in *H. vulgare* roots, we decided to

use root area as similar parameter for root growth. We could not find differences between mock and treated plants in terms of root area (Fig. 30c). However, maximum root length of Ps-1 treated plants was higher than mock-treated (Fig. 30d). Taken together, these results suggest that the Ps1-growth promotion effect is conserved in *H. vulgare*.

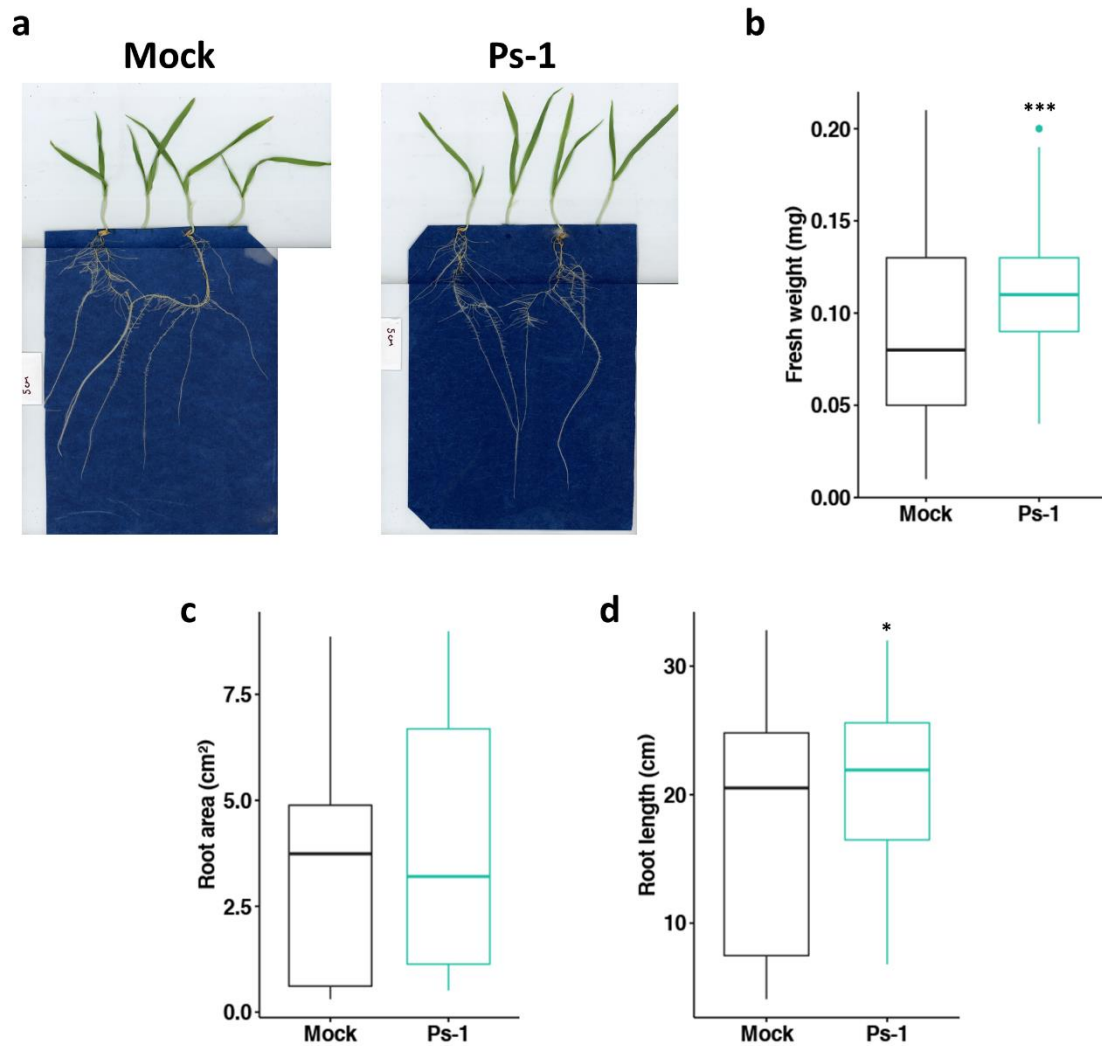


Fig. 30: Plant-growth promoting effect of Ps-1 on *H. vulgare*. **a** Representative scans of *H. vulgare* shoots and roots of mock and Ps-1 root treated plants. Seeds were germinated for 4 days in darkness, then treated by dipping the roots on a Ps-1 spore suspension and kept growing for 7 additional days. **b-d** Fresh weight **b**, root area **c** and root length **d** of plants grown as in **a**. Data comes from three independent experiments, $n > 20$. Significance was analyzed by t-test compared with Mock condition as the reference group. * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 .

4.3 Root temperature effect on plant responses to high temperature, nutrition and microbiome

4.3.1. *Roots are sensitive to high temperatures*

We wanted to analyse the effect of high temperatures on plant growth and responses, focusing on root responses. While designing our experiments, we realized that in previous heat experiments, the shoot and the root were heated to a similar temperature. However, this condition is rarely found in nature as, due to the geothermal properties of the soil, the temperature in the root growth zone should be different, and more buffered, than in the aerial part. To corroborate this, we measured soil temperature in the root growing area at a natural site using digital thermometers. We noticed that this temperature was substantially lower than the atmospheric temperature affecting aerial parts of the plant. Furthermore, a decreasing temperature gradient was formed in the soils, from topsoil to deeper zones (Table 5). In addition, we analysed the temperature variation during day and night for a period of 4 days in June 2022 (Table 6). We observed that, despite the high variance in the atmospheric and topsoil temperature during a day-night cycle, the temperature variations at 5 or 15 cm depth during a day-night cycle were small (Table 7). Thus, soil buffers extreme changes in temperature in the root zone and almost absorbs the day-night temperature changes, forming a stable temperature gradient that likely protects the root system from excessive high temperatures.

Taking in to account these observations and with the aim of analysing the effect of heat stress on plant nutrition, we engineered a device called TGRooZ to generate a temperature gradient in the roots growing zone for *in vitro*, using square plates, or pots containing soil. The device is a metallic box with a cold-regulable bottom container that generates a gradient through the temperature differences between the bottom and the top of the device (Fig. 31a-b). The gradient can be adjusted by regulating the temperature of the bottom. TGRooZ has a replaceable lid in the top part that can hold

different seedling containers (*in vitro* squared plates of 12x12 cm or germination papers) (Fig. 31a, c). Besides, this TGRooZ was also adapted for pots, to cultivate plants in the greenhouse (Fig. 31b,d).

Table 5. Temperature of the soil (40.41° Latitude, -3.83° Longitude) in May 2017 and June 2019 using a digital thermometer containing a metal probe. Measurements were taken every 2 cm and the temperatures correspond to the average of 3 different measurements.

	<u>Temperature (°C)</u>	
	May, 2017	July, 2019
Atmospheric	30	39.6
Top soil	29.4	38.7
2 cm	28.9	37.4
4 cm	27.7	34.3
6 cm	26.8	35.7
8 cm	25.9	34.1
10 cm	23.8	32.2
12 cm	22.1	29.6
14 cm	19.6	28.2

Table 6. Maximum (Max temp) and minimum (Min temp) temperatures during 4 days in a natural soil (40.41° Latitude, -3.83° Longitude) between June 27th and 30th, 2022. The temperatures were probed at surface (top soil), 5 and 15 cm depth into the soil.

	<u>Max temp (°C)</u>				<u>Min temp (°C)</u>			
	27th June	28th June	29th June	30th June	27th June	28th June	29th June	30th June
Top soil	56	58	57	58.7	10.9	11.6	12.8	14.7
5 cm	36.5	36.3	36.9	36.5	31	30.9	31.5	31.4
15 cm	33.4	32.9	34.1	33.8	29	29.3	29.4	29.8

Table 7. Temperature range (maximum - minimum temperature in °C) of data from **Table 6**.

	<u>Temperature range (°C)</u>				Average	SD
	27th June	28th June	29th June	30th June		
Top soil	45.1	46.4	44.2	44	44.9	1.1
5 cm	5.5	5.4	5.4	5.1	5.4	0.2
15 cm	4.4	3.6	4.7	4	4.2	0.5

We used this system to test whether roots were sensitive to high temperature. We germinated *A. thaliana* seeds at 22°C for four days to avoid temperature effects on germination (Zanten *et al.*, 2021), and then seedlings were transferred to three different temperature conditions: homogeneous 22°C (shoot and root at 22°C: 22SR seedlings), homogeneous high temperature of 32°C (shoot and root at 32°C: 32SR seedlings), or 32°C in the shoot and a temperature gradient in the root zone (32TGRooZ seedlings) for 6 more days (Fig. 31c). The gradient in roots was kept from 32°C in the top of the plate to 24°C at the bottom (Fig. 31c). The roots were kept in darkness through the experiments using the D-Root device (Silva-Navas *et al.*, 2015). In 32°C treatments, night temperature was lowered to 28°C in order to mimic day-night temperature cycles in the shoot, while the gradient in the root was constant, similarly to natural soil condition as indicated above.

We found that high-temperature treatment (32SR) significantly reduced root growth (Fig. 32a-b), as well as the number of LR and LRP (Fig. 32c-d). However, when roots were cultivated in a temperature gradient using the TGRooZ device, all pernicious phenotypes derived from high temperature were reverted or even improved as the shoot fresh weight (Fig. 32a-d). Taken together, these results suggest that high temperature in the root severely affects its development, and that the use of the TGRooZ offers an advantage to study the plant and root responses to heat stress in a controlled environment in a closer-to-soil condition.

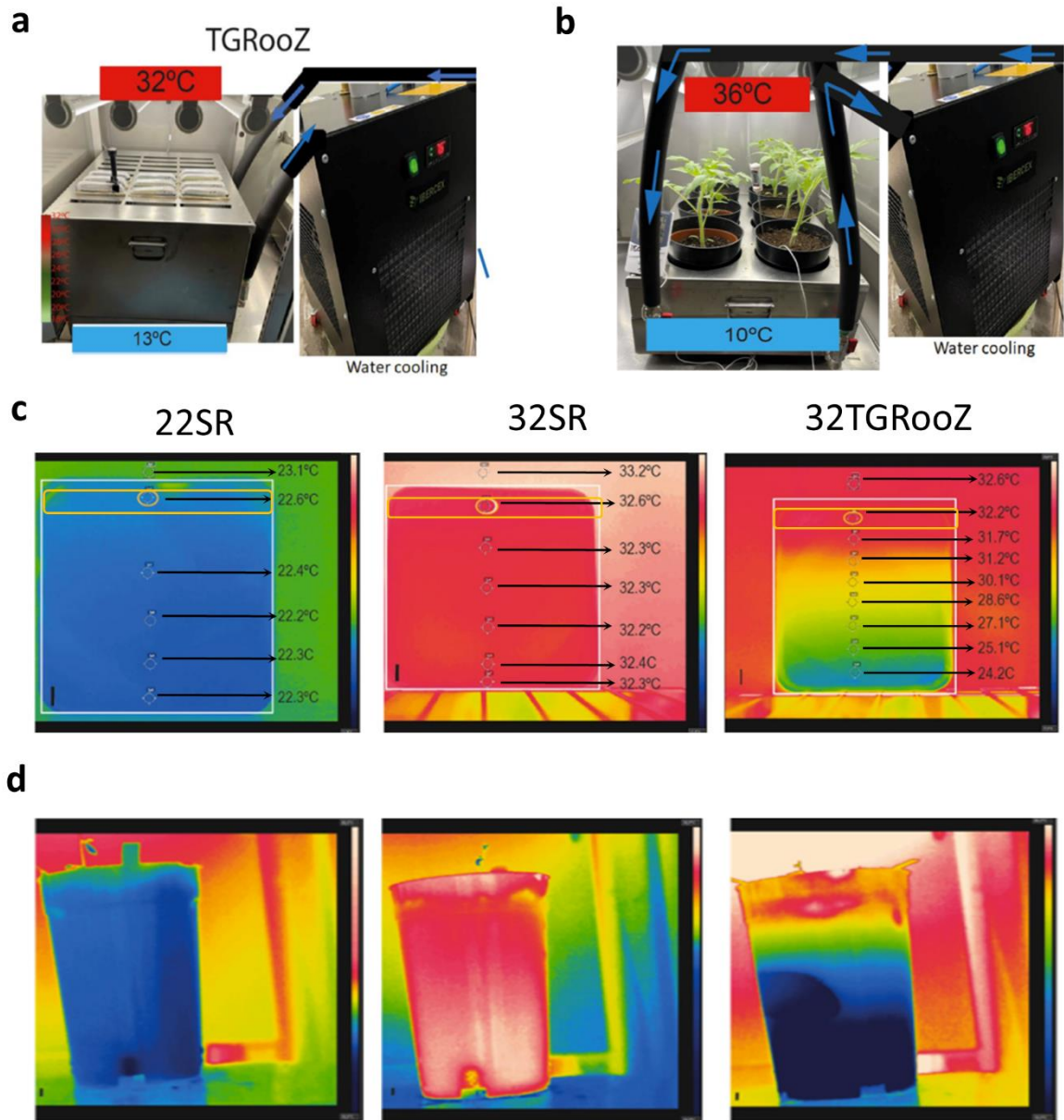


Fig. 3I: TGRooZ device. **a-b** TGRooZ device to cultivate seedlings in 12x12 cm petri dishes containing agar-based medium **a** or pots with soil **b**. To generate a temperature gradient in the root zone, the base of the device is refrigerated by circulating cold water at 10-13 °C, depending on the desired gradient, using a water-cooling machine. **c** Thermal pictures of 22SR, 32SR or 32TGRooZ 12x12 petri plates with agar medium. The arrows indicate the temperature registered in the circle at that point, calculated using the FLIR studio software. The yellow box indicates where shoots of *A. thaliana* are located. Notice that the temperature in gradient formed in the 32TGRooZ, ranged from 32°C at the top of the plate to 24°C at the bottom. **d** Thermal pictures of pots with soil cultivated in a modified TGRooZ. Similarly, the base of the device was refrigerated by circulating cold water using a water-cooling machine at 10°C as in **b**. From left to right, pots cultivated at 22SR, 32SR or 32TGRooZ.

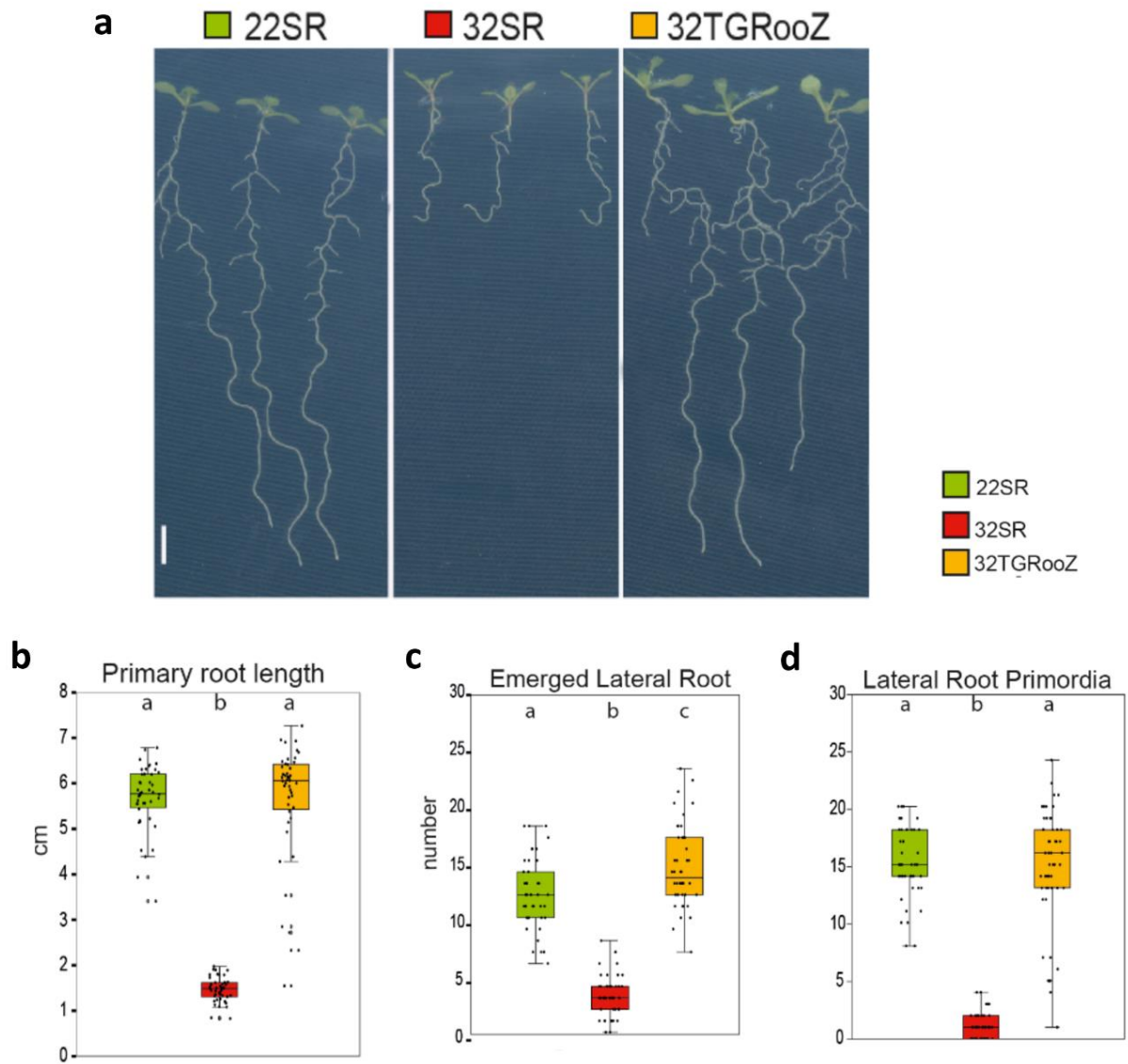


Fig 32: Effect of homogeneous high temperature or gradient on root development. a Representative pictures of *A. thaliana* seedlings grown at homogeneous 22°C (22SR), 32°C (32SR) or 32°C in the shoot and roots at TGRooZ (32TGRooZ). Scale bar: 0.5 cm. **b** Quantification of PRL. **c** Quantification of the number of eLR. **d** Quantification of the number of lateral root primordia (LRP) prior emergence. (**b-d**) $n \geq 25$. Error bars correspond to SD. Significance was analyzed by ANOVA and Tukey HSD post-test (p -value < 0.05). Letters compared the effect of temperatures.

4.3.2. Shoot responses to prolonged heat stress partially depend on root temperature

Our experimental set up provided evidence that TGRooZ protects roots from excessive heat and allow a proper development. We next examined the effect of the different temperatures on shoot development. We found that heat stressed 32SR plants produced less shoot biomass than 22SR (Fig. 33a-b). However, when roots are grown in a temperature gradient (TGRooZ), shoot biomass improved compared to both conditions (Fig. 33a-b), suggesting that a proper root growth and development has an important role in plant performance.

Then, we wondered whether other shoot responses associated with increased temperature are altered by root temperature. We quantified the elongation of the hypocotyl, which is a developmental response to warmer temperatures (Gray *et al.*, 1998; Samach & Wigge, 2005). The hypocotyl length of 32SR seedlings increased by more than 80% compared to 22SR, while the increase in 32TGRooZ seedlings was slightly smaller (Fig. 33c). We also quantified the cotyledonary petiole angle, a rapid response to high temperatures (van Zanten *et al.*, 2009; Quint *et al.*, 2016). The cotyledonary petiole angle in 22SR or 32TGRooZ seedlings was similar, and both significantly smaller than in 32SR seedlings (Fig. 33d). These results demonstrate that root temperature affects shoot thermomorphogenetic responses in different ways, being the hyponastyc angle more connected to root-soil temperature than hypocotyl elongation.

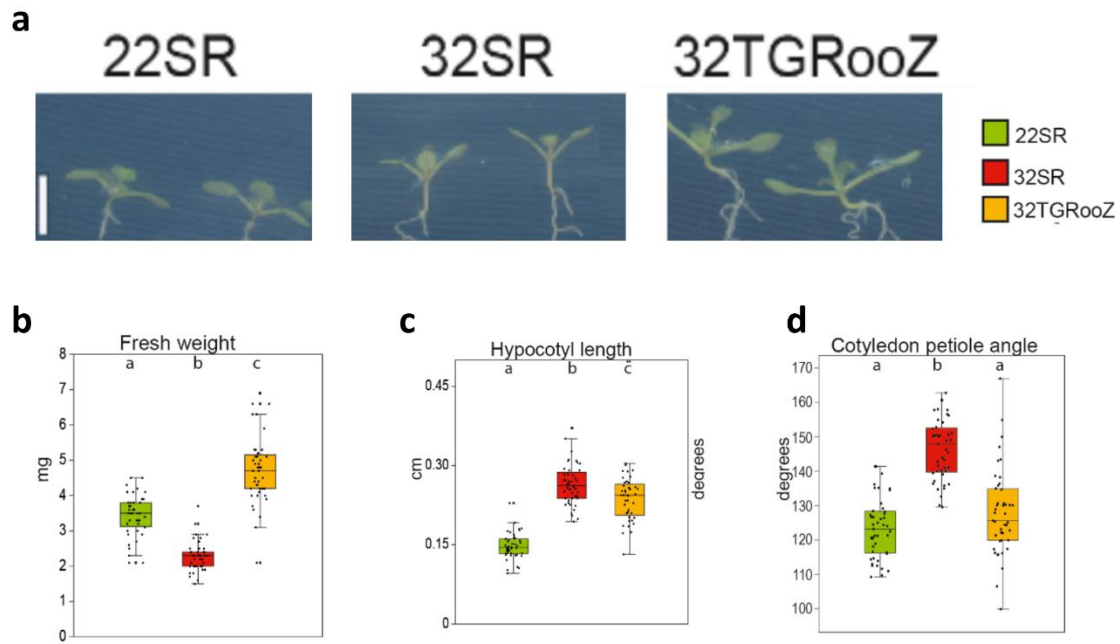


Fig 33: Effect on shoot responses of high temperature or temperature gradient applied to the root zone. **a** Representative shoot pictures of *A. thaliana* seedling grown at homogeneous 22°C (22SR), 32°C (32SR) or 32°C in the shoot and roots at TGRooZ (32TGRooZ). Scale bar: 0.5 cm. **b** Quantification of fresh weight per seedling. **c** Hypocotyl length from root-shoot junction to the base of the meristem. **d** Cotyledon petiole angle from the hypocotyl. Error bars correspond to SD. Significance was analyzed by ANOVA and Tukey HSD post-test (p -value < 0.05). Letters compared the effect of temperatures.

Next, we wanted to investigate the effect of the TGRooZ on plants growing in soil. To do this, we adapted the TGRooZ to hold soil-containing pots (Fig. 31b) and generated a temperature gradient along the soil layers (Fig. 31b,d). We tested whether *A. thaliana* plants exposed to high temperature in the root showed differences in physiological leaf parameters influenced by heat, such as leaf transpiration or temperature. We measured leaf transpiration and observed that 32SR plants have a reduced stomatal conductance compared to 22SR control plants. In 32TGRooZ, stomatal conductance was restored to control levels (Fig. 34a). This effect is likely driven by root temperature since soil water content was maintained at similar levels across all treatments (Fig. 34b). In addition, we recorded leaf temperature, which is also influenced by transpiration. We found that leaves from 32SR plants showed a significant higher temperature than leaves from 22SR or 32TGRooZ plants (Fig. 34c-d). 32TGRooZ leaves were cooler than 32SR plants, what may be derived from to the increase in transpiration rate (Fig. 34a-c), suggesting a more functional phenotype under heat

stress conditions. These results indicate that leaf ability to cope with high air temperature is influenced by root temperature and/or root function.

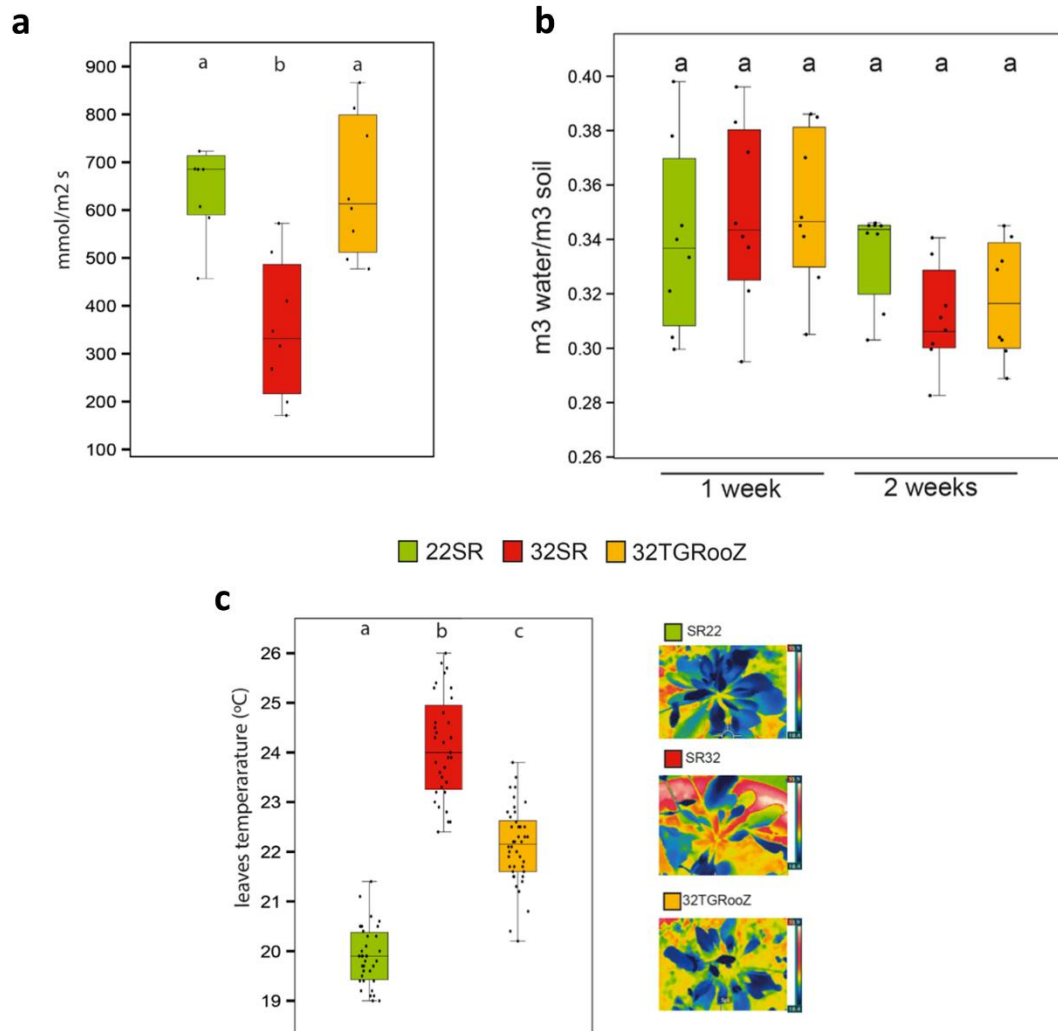


Fig 34: Root temperature in soil affects leaves transpiration and temperature. **a** Conductance (mol m⁻² s⁻¹) of leaves of *A. thaliana* plants grown in pots containing soil at 22°C (22SR), 32°C (32SR) or the modified 32TGRooZ for 2 weeks (n = 8). **b** Soil water content in all treatments, measured with the Teros 10 probe (Meter group) and Zentra Z6 datalogger. The graph represents the measurements taken by different probes at 1 week or 2 weeks after the seedlings were transplanted in soil. **c** Leaves temperature of plants grown as in **a** (n≥33) and their representative thermal pictures. Error bars correspond to SD. Significance was analyzed by ANOVA and Tukey HSD post-test (p-value < 0.05). Letters compared the effect of temperatures.

4.3.3. Root temperature drives transcriptional response to heat stress in plant

Based on our observations, we wanted to analyse the effect of high temperature on gene expression in plants growing with the root system at standard or high temperatures or in a temperature gradient. We performed an RNA-Seq analysis of roots and shoots of wild type *A. thaliana* seedlings grown at 22°C for four days and then transferred at 22SR, 32SR and 32TGRooZ during six days. We identified a higher number of DEGs when comparing roots of 32SR seedlings versus 22SR (~7800 DEGs) than in the 32TGRooZ versus 22SR seedlings (~2600 DEGs) (Supplementary File 4 – Table a-c). In fact, each temperature condition showed a distinct transcriptomic profile of the genes showing highest expression differences in roots (Fig. 35a). We next carried out GO analysis of root DEGs in the different temperature conditions. We compared the high temperature and gradient conditions with 22°C and found that both transcriptomes were enriched in the same categories for downregulated genes (Fig. 35c). However, for the upregulated genes, shared categories only corresponded to heat and protein folding, while the others differed (Fig. 35c). Compared to 32SR, root upregulated genes in the temperature gradient were enriched in categories related to secondary metabolic processes and responses to hypoxia and oxidative stress. For downregulated genes, the categories were related to response to heat and protein folding (Fig. 35e), suggesting a downregulation of heat stress response compared with 32SR. Taken together, these results indicate that TGRooZ expression profile is specific, sharing both common and differential responses with 32SR.

In shoots, we found that 32SR and 32TGRooZ plants had a quite similar expression profile, which significantly differed from 22SR (Fig. 35b, Supplementary File 4 – Table d-f). In fact, GO enrichment analysis of 32SR and 32TGRooZ DEGs compared with 22SR showed the same enriched categories (Fig. 35d), reinforcing the similarity between both treatments that could be due to the fact that shoots were maintained at the same temperature in both conditions. However, almost 700 genes showed a significant different expression level in the shoot when 32SR and 32TGRooZ treatments were compared (Supplementary File 4 – Table f). GO term enrichment analysis of these genes revealed that the response to auxin was enriched in TGRooZ, while processes of

response to heat and protein folding were downregulated (Fig. 35e), similarly to root. These results indicate that a temperature gradient in the root and/or root functionality influences the expression of genes in the shoots and support the idea that shoot responses to high temperatures are partially influenced by soil-root temperature.

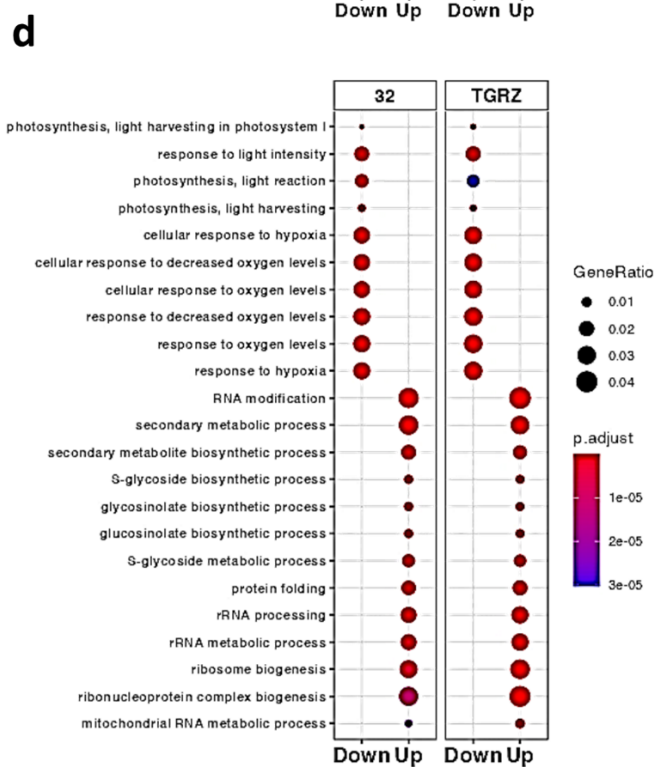
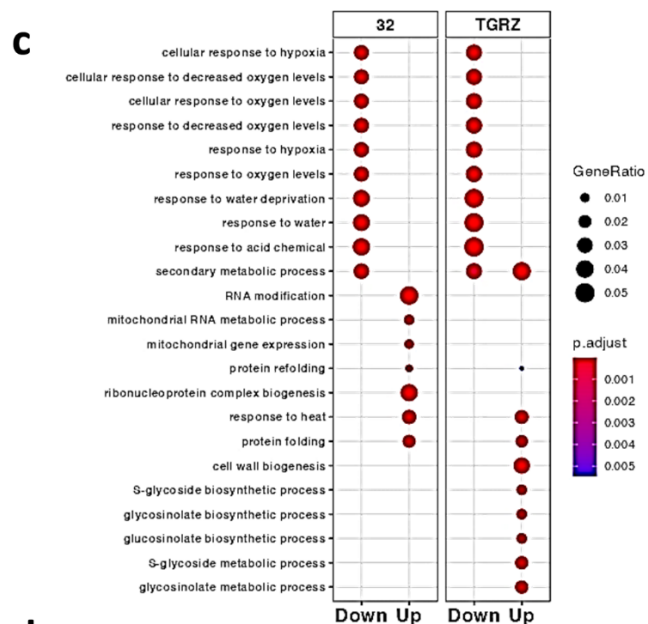
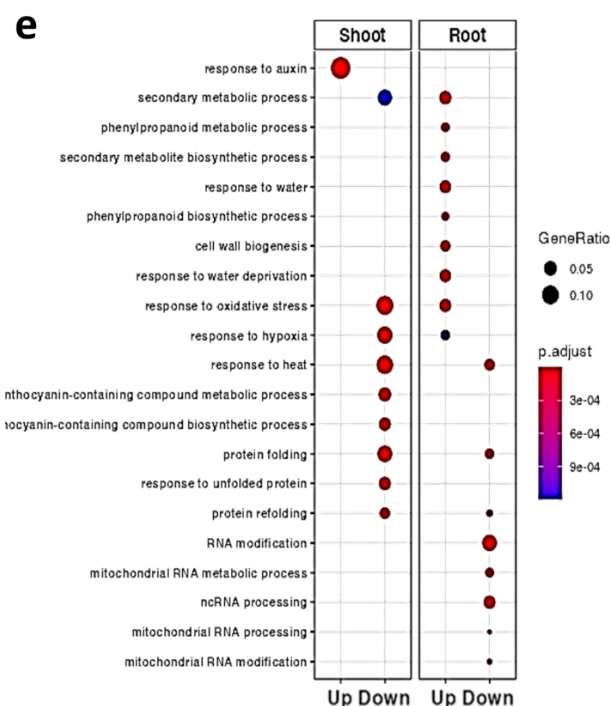
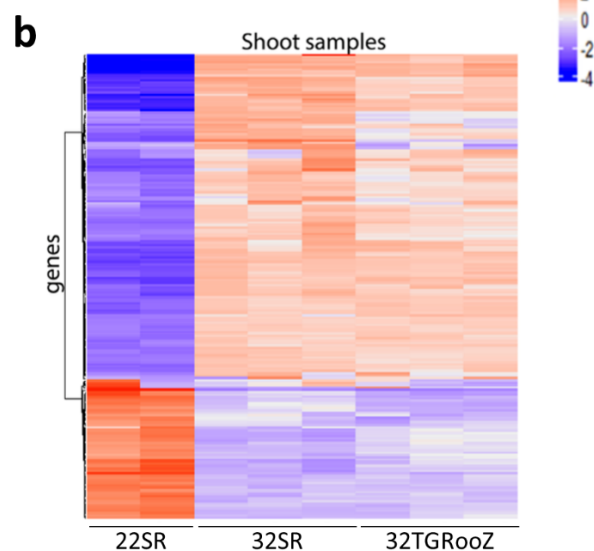
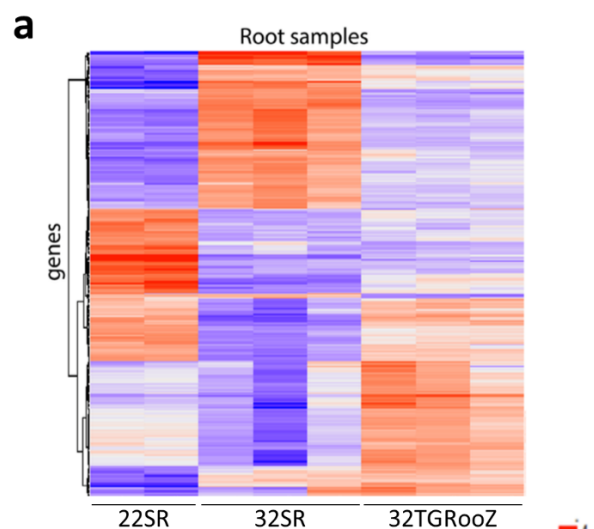


Fig 3.5: Root zone temperature modifies gene expression in shoots and roots under heat stress. **a,b** Clustering of the genes showing the highest variance in root **a** or shoot **b** of seedlings grown a 22RS, 32SR or 32TGRooZ. **c,d** Gene ontology enrichment of roots **c** or shoots **d** differentially expressed genes (DEG) from the 32SR vs 22 and 32TGRooZ vs 22 comparisons. Down and up indicates downregulated and upregulated genes respectively. **e** Gene ontology analysis of 32TGRooZ vs 32SR of shoots and roots DEGs.

4.3.4. Heat-responsive genes affect root development under TGRooZ

As transcriptomic comparisons provided evidence that expression profile is altered by root temperature, and TGRooZ induced a different root phenotype than high temperatures, we next wanted to examine whether genes involved in heat responses affect root and shoot responses in TGRooZ. Using the transcriptomic comparisons, we identified a set of genes involved in the response to extreme temperature that were induced in both roots and shoots in all the comparisons (32SR vs 22SR; 32SR vs 32TGRooZ and 32TGRooZ vs 22SR) (Fig. 36a-b, Supplementary File 4 – Table g). A GO analysis of this gene set identified heat response and protein folding as a common functional category (Fig. 36c-d). In fact, around 60% of the common DEGs encode for heat shock proteins and chaperones (Supplementary File 4 – Table g). Among the induced root and shoot common genes, we selected the *HSFA2* TF, since it is critical for thermotolerance and thermomemory (Schramm *et al.*, 2006; Friedrich *et al.*, 2021; Haider *et al.*, 2022). We also chose the *NAC085* TF, which is involved in G2 arrest in response to heat stress in roots (Takahashi *et al.*, 2019). Additionally, we picked two heat shock proteins that are chaperones critical for an adequate thermotolerance response, *HSP70.2* and *HSP90.1* (Yamada *et al.*, 2007; Leng *et al.*, 2017).

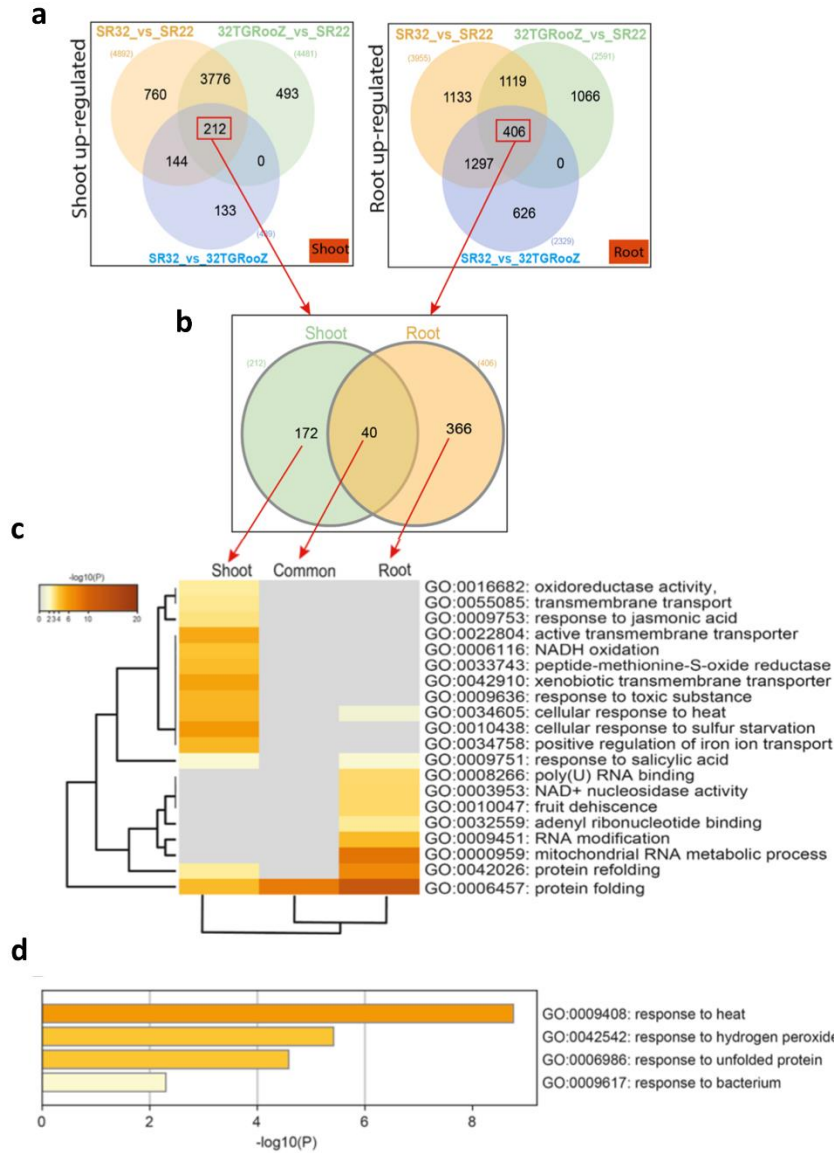


Fig 36: Identification of genes regulated by high temperature. **A** Venn diagrams of genes up-regulated by the effect of temperature in root and shoot in all the treatments (32SR vs 22SR, 32SR vs 32TGRooZ and 32TGRooZ vs 22SR). **b** Venn diagram of common up-regulated genes identified in **a**. **c** Gene ontology (GO) heat map of DEG analyzed in **b** (shoot specific, root specific or common). **d** GO term enrichment of common DEG identified in **b**.

We tested the selected genes using T-DNA mutant lines in the previously observed root traits. We found that there was no difference in root length between all the genotypes at 22°C, but this trait was severely compromised at 32°C (Fig. 37a). However, root growth in TGRooZ *nac85* and *hsp90* seedlings was recovered to wild-type plants level, while that of *hsf2a* and *hsp70* remained significantly smaller (Fig. 37a). When we compared the total root length (primary root plus LR length), we observed

that all lines recovered less than the wild-type in TGRooZ, suggesting a defect in LR growth (Fig. 37b). Indeed, the LR number was significantly reduced by the effect of high temperature in all genotypes (Fig. 37c), but this parameter was fully recovered in the mutants when grown at 32TGRooZ, except for *hsp70* (Fig. 37c) indicating a role for this chaperon in LR development under heat stress. These results indicate that genes involved in heat responses are affected to different extent by root temperature and highlights the importance of TGRooZ as system to study the effects to prolonged high temperature treatments in root and shoot development.

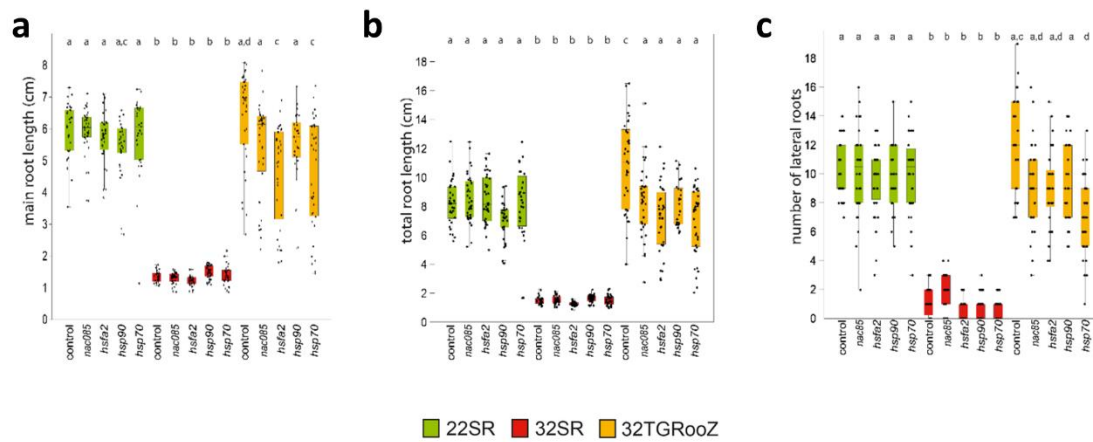


Fig 37: Effect of root temperature on roots of heat response mutants. a Root length, b total root length (principal plus lateral roots length) and c number of emerged lateral roots of wild-type and mutants in heat responses grown a 22SR, 32SR or 32TGRooZ ($n \geq 24$). Significance was analyzed by ANOVA and Tukey HSD post-test (p -value < 0.05).

Next, we evaluated the effect of the different lines on shoot thermogenic responses. We did not find any significant differences in hypocotyl length (Fig. 38a) or cotyledonary petiole angle (Fig. 38b) between wild type and mutants. Taken together, these results suggest that genes involved in responses to extreme heat are more susceptible in root than in shoot development.

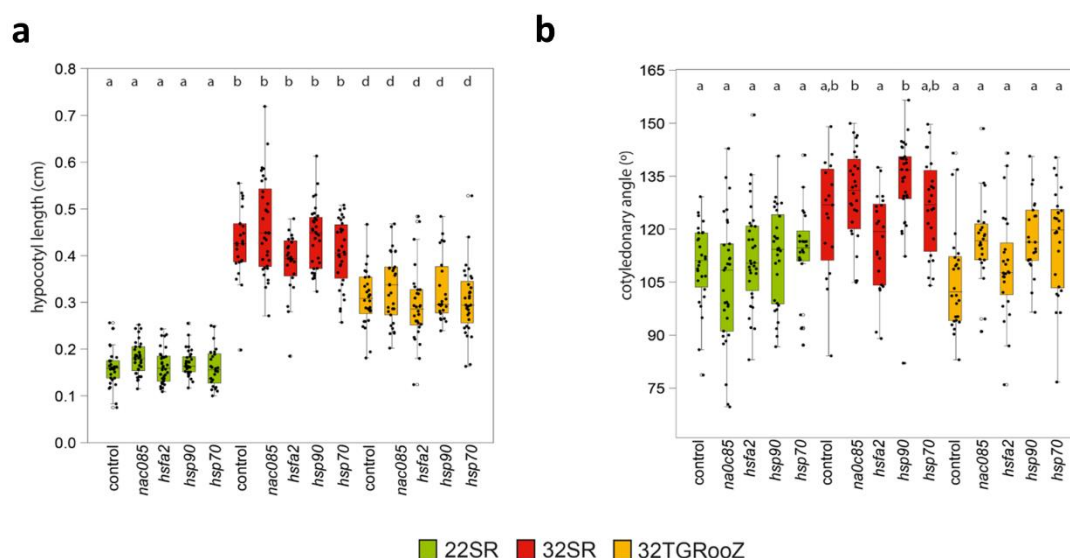


Fig. 38: Effect of root temperature on shoots of heat response mutants. **a** Hypocotyl length of 22SR, 32SR or 32TGRooZ on wild-type and mutant seedlings. **b** Cotyledonary petiole angle of 22SR, 32SR or 32TGRooZ wild-type and mutant seedlings ($n \geq 24$). Significance was analyzed by ANOVA and Tukey HSD post-test (p -value < 0.05).

4.3.5. Root temperature modifies plant nutrition

As root development is affected by root temperature, we next wanted to investigate whether root functionality is also altered by temperature treatments. Several studies have reported that plant nutrition is altered during heat events (Ihsan *et al.*, 2019; Hassan *et al.*, 2021). In fact, when we looked at GO categories downregulated exclusively in 32SR versus 22SR, response to metal ion was identified at the top of them (Fig. 39a-b). Conversely, TGRooZ exclusively upregulated DEGs were enriched in response to nutrient levels (Fig. 39a,c). To determine whether root temperature influences other ions homeostasis, we leveraged again our transcriptomic data and the known ionome gene (KIG) list. The KIG list is a collection of curated genes experimentally proven to be involved in the uptake, accumulation or distribution of mineral nutrients in plants (Whitt *et al.*, 2020). We have overlapped this KIG list with our temperature deregulated genes, and we found a significant overlap (Supplementary File 5 – Table a). In roots, 57% of the curated list were deregulated by at least one

temperature condition (Supplementary File 5 – Table a), suggesting that plant nutrition is altered through different temperature treatments.

One of the primary macronutrients with major changes was phosphate. Remarkably, *PHO2* and *PHR1*, central regulators of the Pi response, were down-regulated in response to heat in almost all comparisons, while several Pi transporters were up-regulated in roots or shoots in response to heat in most of the comparisons (Supplementary File 5 – Table a, Fig. 39d). Thus, to validate this expression changes, we analysed whether Pi accumulation on *A. thaliana* seedlings was altered by different temperature treatments. We found that shoot Pi levels in 32SR seedlings were significantly lower than in 22SR seedlings (Fig. 40a-b). Interestingly, in 32TGRooZ seedlings these levels were similar to 22SR seedlings (Fig. 40a), although not fully recovered at longer times (Fig. 40b), suggesting that temperature influences Pi homeostasis. Together, these results suggest that TGRooZ is needed to maintain a correct Pi nutrition under heat stress conditions.

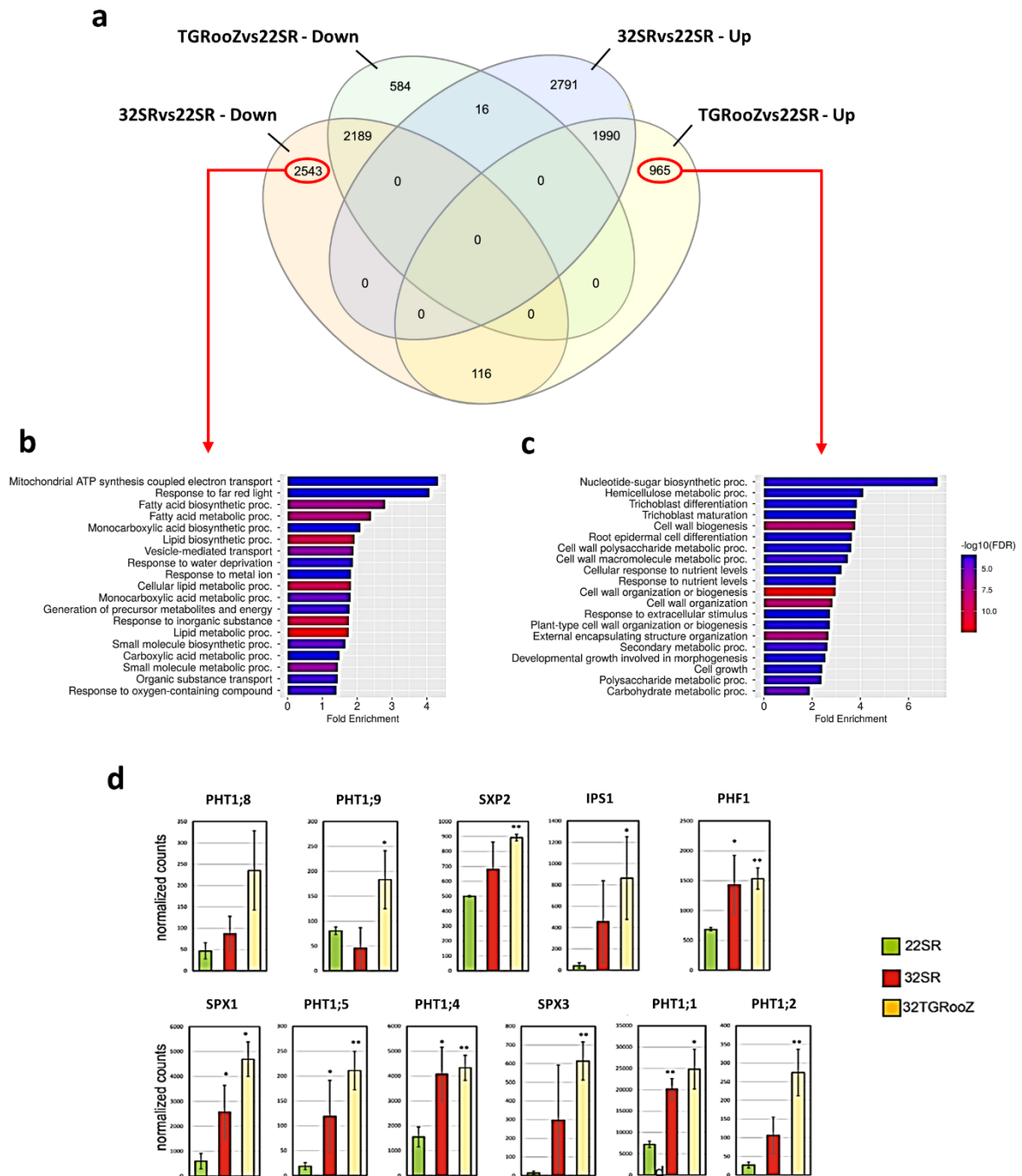


Fig. 39: Transcriptomic changes indicate alteration in plant nutrition and phosphate homeostasis. A Venn diagrams of DEGs between 32SR vs 22SR and TGRooZ vs 22SR for up- and down-regulated genes. **b-c** Enrichment of GO terms for exclusively downregulated genes in 32SR **b** or upregulated genes in TGRooZ **c** compared with 22SR. **d** Changes in gene expression of phosphate transporters (PHTs), PHT transporter facilitation (PHF1) and signaling (SPXs and IPS1) genes. Values correspond to normalized counts from our RNAseq experiments. *, p -value < 0.05; **, p -value < 0.01 by T-test.

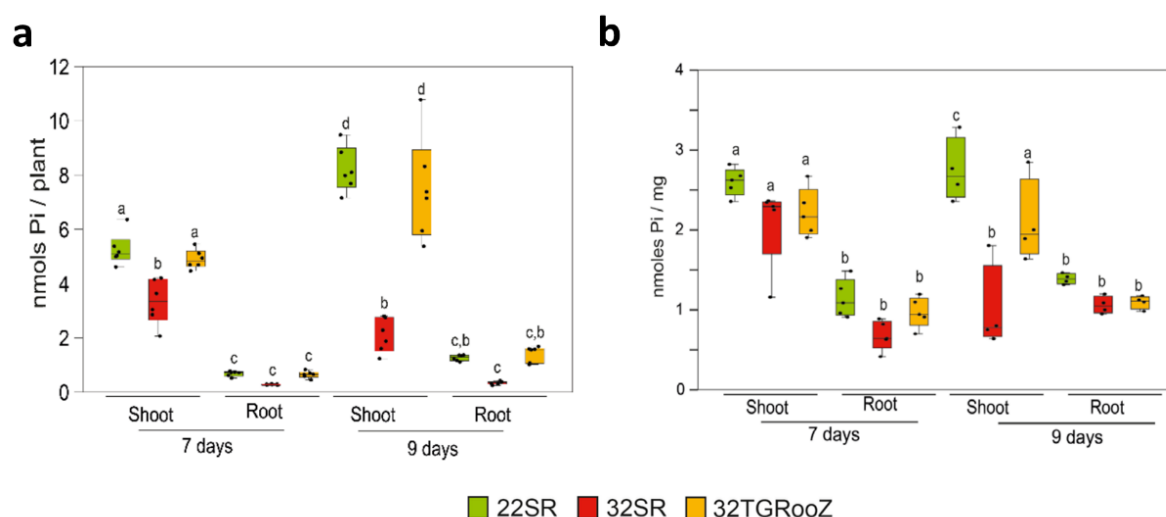


Fig. 40: High temperature affects Pi accumulation. a-b Free Pi level in roots or shoot of 22SR, 32SR or 32TGRooZ *A. thaliana* seedlings grown in $\frac{1}{2}$ MS medium for 7 or 9 days represented as nmols Pi/plant a or per mg of fresh tissue b. Significance was analyzed by ANOVA and Tukey HSD post-test (p -value < 0.05).

To further understand the relationship between high temperature and root function and extend our findings to other plant species, we decided to explore the nutrient accumulation of tomato (*Solanum lycopersicum*) plants using our system. It has been reported before that tomato root growth was limited by an excessive temperature and its nutrient accumulation is altered (Giri *et al.*, 2017). To determine the effect of root temperature, we used tomato seedlings grown in natural soil and exposed to the three different temperature regimes (22SR, 36SR, and 36TGRooZ). As our previous Pi determinations indicated that alterations were reflected in shoots, we decided to sample aerial parts of tomato. We found that, in general, mineral nutrient accumulation patterns were similar for 22SR and 36TGRooZ leaves, but different from SR36 leaves as reflected in the hierarchical clustering (Fig. 41a-b). We found that different temperatures in soil altered mineral nutrients in leaves, including P, Cu, B, Mo, As, Rb, and K (Fig. 41b). These results suggest that changes in plant mineral nutrition might be a consequence of high temperature in soil that alters root function. Therefore, the use of the TGRooZ might be adequate to understand the effects of heat on plant nutrition in the greenhouse or *in vitro* experiments.

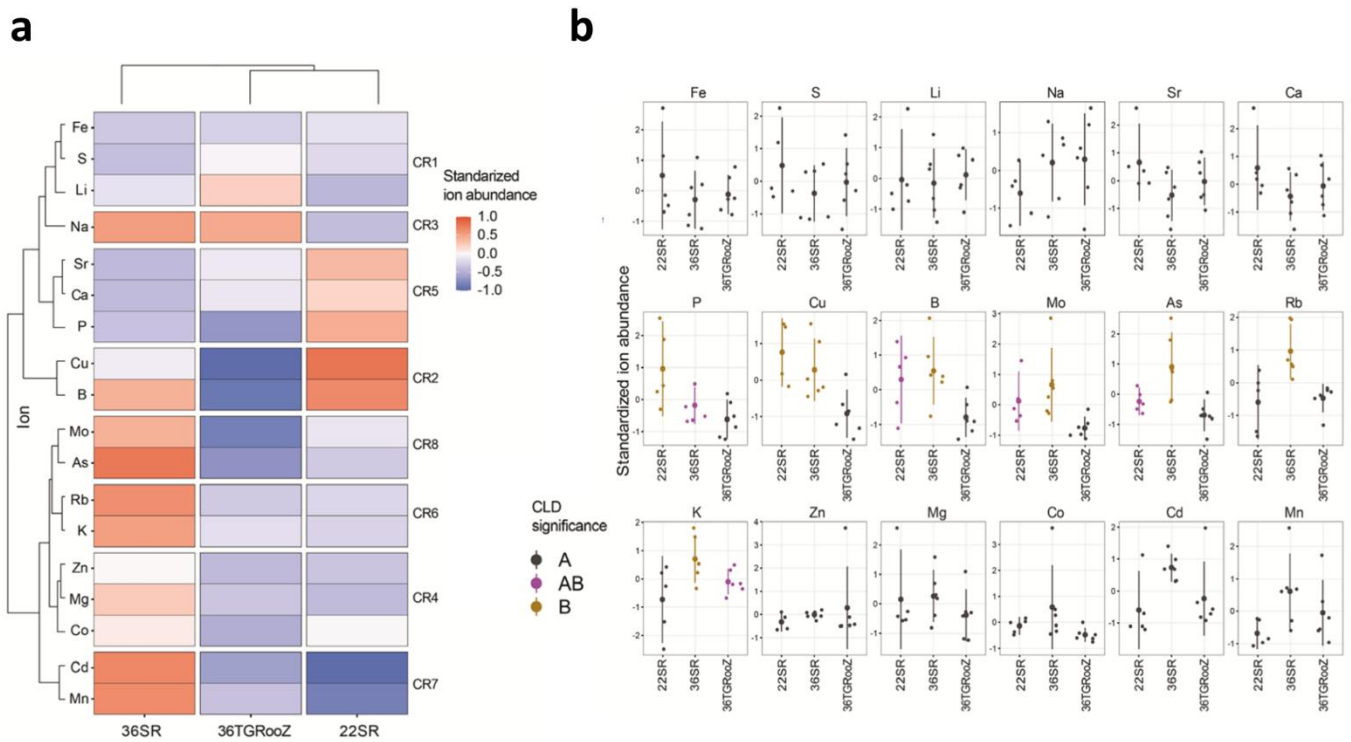


Fig. 4l: High temperature in the root affects mineral nutrient accumulation in tomato leaves. A Hierarchical clustered heatmap showing the standardized mineral nutrient concentration in leaves of tomato plants grown at 22°C (22SR), 36°C (36SR) or 36°C with a temperature gradient in the root zone (36TGRooZ) for 4 weeks. CR numbered represents ion different clusters. **B** Individual standardized mineral nutrient abundances across the temperature treatments used in **a**. Significance was determined via pairwise comparisons and the results visualized using the CLD (p -value < 0.05).

4.3.6. High temperature in the root system affects bacterial community assembly

Since plant mineral nutrition and root microbiota are closely interconnected (Zhang *et al.*, 2019^a; Salas-González *et al.*, 2021) and microbiome can have a role in stress tolerance (Liu *et al.*, 2020), we decided to explore whether high temperature in the root system alters its bacterial assembly. To determine the effect, we used tomato seedlings grown in a natural soil as described above (Fig. 4l), exposed to 22SR, 36SR and 36TGRooZ. We sampled different fractions: i) original soil (soil); ii) rhizosphere, and iii) tomato roots. We compared bacterial community profiles using 16S rRNA amplicon sequencing. Soil and rhizosphere fractions supported higher bacterial alpha diversity

and richness when compared with roots across all temperatures used (Fig. 42), and soil temperature did not affect alpha diversity or richness of bacterial assemblages in any of the analysed fractions (Fig. 42). Most of the variance in community composition was explained by the fraction ($\approx 40\%$ PERMANOVA, p -value < 0.05 ; Fig. 43a), followed by the temperature regime ($\approx 10\%$ PERMANOVA, p -value < 0.05 ; Fig. 43a). In a PcoA based on Bray-Curtis dissimilarities between samples, Pco1 (which explains more than 38% of the variance) clearly differentiated root fraction from soil and rhizosphere (Fig. 43a). Principal coordinate axis 2 (Pco2), which explains more than 10% of variance, differentiated SR36 samples from the rest, indicating that soil temperature exerts an effect on microbiome assembly both in rhizosphere and root fractions (Fig. 43a). Tomato root samples were mainly enriched, as compared to rhizosphere and soil fractions, in Proteobacteria and Bacteroidetes and depleted in Acidobacteria, Gemmatimonadetes, Firmicutes, Chloroflexi, and Verrucomicrobia (Fig. 43b-c).

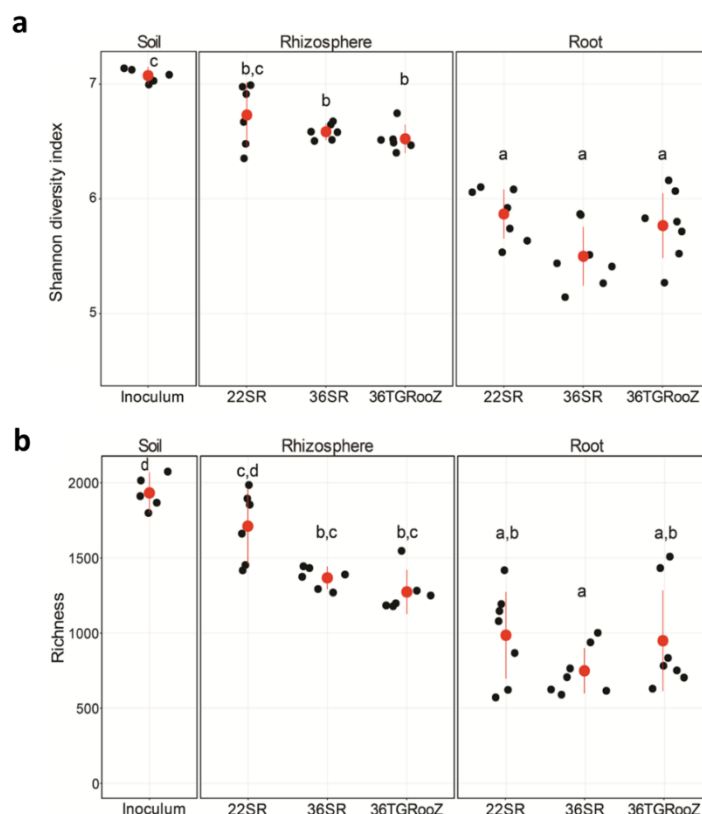


Fig. 42: Soil temperature does not affect diversity and richness of bacterial assemblages in soil or tomato rhizosphere or roots. A Bacterial alpha diversity estimated using the Shannon diversity index. **B** Richness in soil, rhizosphere, and root-associated samples of tomato plants grown at 22°C

(22SR), 36°C (36SR) or 36TGRooZ for 4 weeks. Letters represent post hoc test results, based on a full factorial ANOVA model.

We further investigated the effect of high temperature on the rhizosphere and root bacterial community composition. PcoA and PERMANOVA analysis showed significant differences in bacterial community compositions across the temperature treatments used (Fig. 44; PERMANOVA $R^2 = 0.6$, p -value = $1e-04$ and $R^2 = 0.34$, p -value = $1e-04$ for rhizosphere and root respectively). These differences were robust in both fractions, rhizosphere and root (Fig. 44a,b). Thus, in line with the ionomic data, we noticed that rhizosphere and root microbiomes composition in tomato 22SR and 36TGRooZ plants were similar and significantly different from the microbiome analysed in 36SR plants (Fig. 44). Indeed, the bacterial profile found in the roots of high-temperature-treated plants (36SR) was different from the 22SR and 36TGRooZ plants (Fig. 43b-c and 44d). Furthermore, tomato roots of 36SR showed a clear increase in actinobacteria, a phylum that is normally reduced in healthy roots (Castrillo *et al.*, 2017; Finkel *et al.*, 2019) (Fig. 43b-c and 44d). This increase was also observed, although at a lesser extent, in 36TGRooZ plants (Fig. 43b-c and 44d). This observation was consistent at different phylogenetic levels (Fig. 44c,d), indicating that soil temperature has more effect in microbiome assembly than shoot temperature and its derived signalling in roots. Taken together, these results suggest that changes in the root function might emerge as a consequence of the high temperature effect on the soil, its microbiota, and the interaction between them.

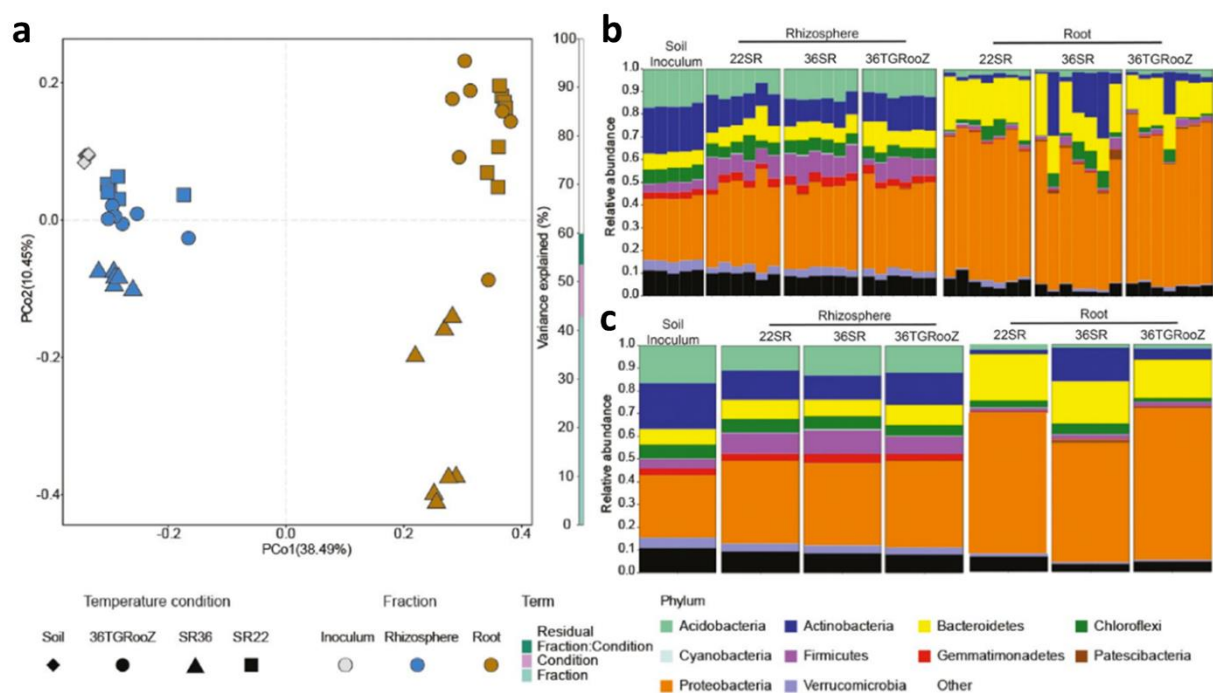


Fig. 43: Bacterial composition profiles of samples exposed to different temperature conditions.

A PcoA based on Bray-Curtis dissimilarities between bacterial communities in soil, rhizosphere, and roots of tomato plants grown at 22°C (22SR), 36°C (36SR) or 36TGRooZ for 4 weeks. The bar graph to the right of the Pco shows the percentage of variance explained in a PERMANOVA analysis (p -value < 0.05). **b** Bars show individual or **c** mean relative abundance profiles of the main bacterial phyla found in soil, rhizosphere, and roots of tomato plants from **a**.

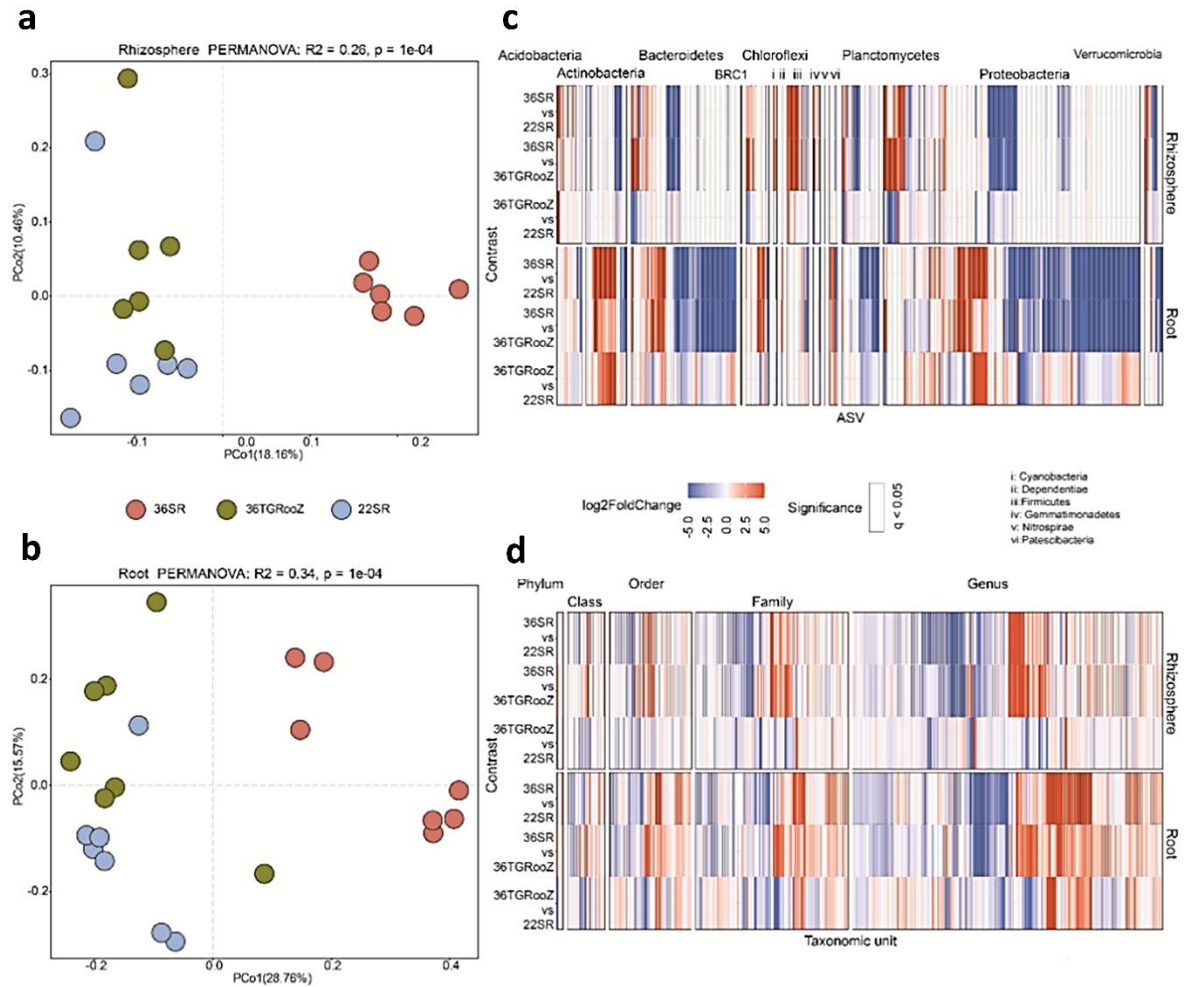


Fig. 44: High soil temperature selects different rhizosphere and root microbiomes composition. **a-b** PCoA based on Bray-Curtis dissimilarities between bacterial communities in roots **a** or **b** rhizospheres of tomato plants grown at 22°C (22SR), 36°C (36SR) or 36TGRooZ for 4 weeks. **c-d** Heatmaps showing the root and rhizosphere enrichment patterns of different taxonomic units (Phylum, Class, Order, Family, Genus) **c** or **d** ASVs across all versus all contrast between temperature conditions used in **a**. The tiles of the heatmaps are colored based on the log₂ fold change estimated from a GLM contrasting the abundance of each taxonomic unit in a given temperature respect to another in each contrast. Squares outlined in black represent taxonomic units at each taxonomic level significantly enriched (red) and depleted (blue) in each comparison (q -value < 0.05).

5. DISCUSSION

5. Discussion

In nature, roots grow in a dark environment through different soil layers that are highly heterogeneous ecosystems. Plants have evolved different root system architectural traits to adapt to soil and exploit its resources (ie. water, nutrients, structure) and cope with adverse environmental conditions (Hodge, 2009; Giehl & von Wirén, 2014). Due to this necessity of continuous adaptation, roots show a high developmental plasticity, being able to arrest or promote the main root and/or LR growth throughout their life span. Likewise, roots interact with soil microbiota to establish a functional network, including symbiotic associations for enhanced nutrition or pathogen protection (Hassani *et al.*, 2019). Root developmental processes and responses are highly influenced by nutritional and stress plant status (Gruber *et al.*, 2013; Trivedi *et al.*, 2020; Karlova *et al.*, 2021). *In vitro* studies in model plants, mainly in *A. thaliana*, have provided a rapid and in-depth molecular knowledge on the regulation of the root system development (Motte *et al.*, 2019), plant nutrition (Gruber *et al.*, 2013) or plant-microbiote interaction (Liu *et al.*, 2019a). Most of these *in vitro* studies were performed using standard laboratory growth conditions implying that the entire plant, including the root system, is cultivated with constant illumination and/or temperature. However, in nature, roots grow mainly underground, where light is generally blocked by the first 0.5 cm of the soil (Tester & Morris, 1987) and the temperature is buffered and different than that of the atmosphere (Elias *et al.*, 2004). The use of novel approaches that mimic natural conditions to study root responses, namely here D-Root or TGRooZ, allows us to study with a new perspective the root system development, its responses to environmental changes and its interactions with the microbiota, that otherwise could have been masked due to the inherent limitations of reductionist approaches. In addition, due to the communication between shoot and root, the use of these devices will allow us to better understand the growth and responses of the plants as a whole organism.

5.1. Characterization of *Arabidopsis thaliana* PSR using the D-Root system

The mechanisms underlying Pi starvation signaling and responses have been studied in different species, including *A. thaliana*. Pi starvation triggers several adaptive responses to improve Pi mobilization, usage and uptake (López-Arredondo *et al.*, 2014). In addition, plants use alternative metabolic pathways to limit the consumption of Pi in cells (Rouached, 2011). It has been shown that Pi deficiency reduces root and shoot growth, and increases root:shoot growth ratio, LR density and root hairs (Lynch & Brown, 2001; López-Bucio *et al.*, 2003; Gruber *et al.*, 2013; Cruz-Ramírez *et al.*, 2013). Pi deficiency also induces a determinate root growth arrest that leads to a progressive loss of dividing cells, exhausting the root meristematic activity (Sánchez-Calderón *et al.*, 2005). At the transcriptional level, Pi starvation has a significant impact on global gene expression (Misson *et al.*, 2005; Bustos *et al.*, 2010; Woo *et al.*, 2012; Secco & Whelan, 2014; Mora-Macías *et al.*, 2017). These molecular and physiological analyses, combined with genetic studies (Rubio *et al.*, 2001; Catarcha *et al.*, 2007; Ticconi *et al.*, 2009; Nussaume *et al.*, 2011; Wang *et al.*, 2012; Gamuyao *et al.*, 2012; Ruan *et al.*, 2015), have shed light on the molecular mechanisms that regulate the PSR. However, it should be highlighted that these data have been gathered using plants with the root system illuminated. It is known that environmental conditions affect plant nutrition and growth. For instance, salt and drought (Baek *et al.*, 2016) as well as temperature (Giri *et al.*, 2017), affect the Pi starvation response. The identification of these crosstalks have allowed the discovery of new TFs that seem to modulate the PSR in plants (Baek *et al.*, 2017). Here, using the D-Root, we wanted to analyze the specific response to Pi without the influence of the illumination stress on roots.

5.1.1. Pi starvation response is influenced by light

Roots are underground organs that normally do not perceive light. Previously, it has been shown that root illumination generates a stress that affects root growth,

development and responses to abiotic stresses (Silva-Navas *et al.*, 2015, 2016; Manzano *et al.*, 2017). For example, blue light alters root development by producing fewer LRs and shortening the PRL compared to DGR (Moni *et al.*, 2015; Silva-Navas *et al.*, 2015). This effect is mediated by the plant blue light photoreceptors (Moni *et al.*, 2015; Silva-Navas *et al.*, 2015). Here, we show that root illumination has a significant impact on the PSR at morphological, physiological, and molecular levels. Our data demonstrate that root illumination generates a stress that is additive to Pi starvation, influencing some of the responses. We showed that the inhibition of primary root growth is driven by the negative and additive effect of root illumination and Pi deprivation. Recently, it has been shown that blue light is responsible for the shortening of roots under low-Pi regimes (Yeh *et al.*, 2019). This is, at least in part, the consequence of a malate-mediated photo-Fenton reaction triggered by blue light (Zheng *et al.*, 2019). In line with these results, *hps7*, a hypersensitive mutant to Pi starvation, does not show this response when plants were grown in the dark, suggesting an effect of root illumination on Pi responses (Kang *et al.*, 2014). Additionally, blue light stimulates flavonol production in roots, and this flavonol accumulation directly correlates with a reduction in cell division and premature cell differentiation in a CK-dependent manner (Silva-Navas *et al.*, 2016). Together, these results suggest that flavonols might be involved in root growth modulation under Pi starvation.

Root illumination has a significant effect on gene expression (Silva-Navas *et al.*, 2016; Qu *et al.*, 2017). Thus, it is plausible that root illumination could also further affect the transcriptional PSR responses. Comparative analyses using DGRs seedlings show that Pi starvation affects the expression of a large number of genes involved in development or responses to stress, highlighting the link between root development and abiotic stress. Interestingly, one of the downregulated categories corresponds to iron metabolism, and iron accumulation is also altered when comparing LGR and DGR (Section 3.1.1., Fig. 10). Iron has a direct effect on root growth under Pi deficiency, as its balance controls callose deposition in the root apical meristem that results in tuning its activity (Müller *et al.*, 2015). In addition, transcriptomic analyses between DGR seedlings grown in high or low Pi have uncovered new genes involved in the PSR that were not reported in previous analyses using LGR seedlings. Thus, taken together, we

can conclude that the D-Root system provides a more natural environment for investigating root traits that might help uncover novel genes/pathways involved in the PSR.

5.1.2. cZ as PSR hormone

CKs are an important class of phytohormones that control cell proliferation and differentiation and regulate several aspects of plant growth and development, including delay of senescence, shoot:root growth balance and transduction of nutritional clues (Sakakibara, 2006). CKs repress LR development through local biosynthesis in LRP. This creates a spatial signal that restricts the formation of new LRP in neighboring pericycle cells and suppresses the initiation of new LRs (Li *et al.*, 2006; Laplaze *et al.*, 2007; Chang *et al.*, 2015). CKs also control root-meristem differentiation and, therefore, its size (Dello Ioio *et al.*, 2007). Different hormonal pathways have been implicated in modulating root systems during PSRs (Franco-Zorrilla *et al.*, 2002; Jiang *et al.*, 2007; Pérez-Torres *et al.*, 2009; Rouached *et al.*, 2010). Pi-starved plants increase root hair density and length as well as LR density to enlarge the nutrient-absorbing surface. CKs have been described to signal Pi nutrient status, as their quantity decreases under starvation and mediates PSRs such as the increase of root hair density and length (Martín *et al.*, 2000; Rouached *et al.*, 2010). Tissue-specific expression experiments demonstrated that xylem-pole pericycle cells, but not LRP in early stages, are sensitive to CK (Laplaze *et al.*, 2007). A similar conclusion was reached by analyzing mutants and overexpression lines of CK biosynthetic genes (Kuroha *et al.*, 2009). Together, these data suggest that CKs are important for both LR positioning and number. In DGR plants, although LR density was slightly decreased, the total number of LRs and their lengths were significantly increased. Our results show that the concentration of tZ, the most abundant CK, is reduced in Pi-starved roots, likely by the decrease expression of *IPT3* and *IPT5* (Section 4.1.3, Fig. 11d) Using our D-Root system we also show that tZ has a more pronounced effect on reducing the LRP formation than cZ. Since Pi-starved plants increase the cZ:tZ ratio, it is tempting to speculate that the increase in cZ maintains a

balance between reducing CK signaling and maintaining root growth and LR development to increase the root surface.

CKs have been proposed to modify the sink/source nutrient mobilization and shoot:root ratio (Werner *et al.*, 2008). Under Pi starvation, CKs seem to play an important role in controlling the PSR systemically, reducing the expression level of PSR genes (Martín *et al.*, 2000; Franco-Zorrilla *et al.*, 2002; Wang *et al.*, 2006). CK treatment increases intracellular free Pi content, probably by mobilizing organic P (Wang *et al.*, 2006). In line with these results, rice plants harboring a mutation in a CK oxidase (*OsCKX2*) accumulate higher level total Pi under low Pi conditions (Yan *et al.*, 2022). Based on these data and our physiological and transcriptomic results, we believe that cZ plays an important function in the plant response to Pi starvation. The fact that cZ repression of PSR genes is lower than that of tZ (Section 4.1.4., Fig. 14) will be important to balance the responses to Pi starvation. In line with this idea, both cZ and tZ reduce root meristem size, but only cZ seems to increase cell elongation during Pi starvation, favoring root growth (Section 4.1.3., Fig. 12). Thus, the cZ biological function is relevant for maintaining optimal root growth during Pi starvation to keep searching for new Pi sources with the aim of sustaining plant fitness. Concomitantly, cZ also effectively represses the *PHO1* gene (Supplementary File 1), blocking the translocation of Pi from root to shoot, favoring the Pi allocation in roots. All these processes increase free Pi content in root cells to maintain a minimum growth under low-Pi conditions. Taken together, our results confirm the hypothesis that cZ:tZ ratio might balance the equilibrium between root:shoot growth, the Pi absorption and PSR gene expression level and root growth under Pi deprivation.

The different zeatin isomers, cZ and tZ, or cZR and tZR, are synthesized through different pathways. In *A. thaliana*, the majority of cZ is formed by isopentenylation of tRNA by AtIPT2 and AtIPT9 (Miyawaki *et al.*, 2006). Here we show that Pi starvation resulted in downregulation of two of the tZ biosynthesis genes, while the expression of the cZ biosynthesis genes *IPT2* and *IPT9* is not affected. However, the increase in LOG genes expression, involved in the later steps of zeatin synthesis, might be relevant to increased cZ concentrations. Interestingly, tRNA modifications are often correlated

with specific stress responses (El Yacoubi *et al.*, 2012). During Pi starvation, tRNA-derived small RNAs are generated, which might have important biological functions in the Pi deficiency response (Hsieh *et al.*, 2010; Megel *et al.*, 2015; Park & Kim, 2018). Thus, it is tempting to speculate that tRNA halves of 31–40 nucleotides long, which are generated by specific cleavage in the anticodon loops of mature tRNAs, might be used for prenylation and cZ formation in response to Pi starvation. Further analyses of tRNAs processing using the D-Root system will shed new light on cZ biosynthesis and PSR. It is thought that cZ accumulation is related to environmental responses such as abiotic stresses, biotic interaction, or pathogen and herbivore attacks (Havlová *et al.*, 2008; Vyroubalová *et al.*, 2009; Jiang *et al.*, 2013; Schäfer *et al.*, 2015), although this point is not completely clear. Now, our data clearly indicate that cZ has an important role in regulating the plant physiological and molecular responses during Pi starvation response. It would be interesting to study if the response to other stresses, biotic or abiotic, also implicates changes in the cZ:tZ ratio.

CKs are perceived by a small family of three receptors, *AHK2*, *AHK3*, and *AHK4* (Lomin *et al.*, 2012). A triple *ahk2 ahk3 ahk4* mutant shows small-dwarf phenotype with a severe root growth reduction and short root meristems (Nishimura *et al.*, 2004). Although these receptors can partially complement each other's function, it seems that they are not completely redundant, since each of them has a specific expression pattern (Nishimura *et al.*, 2004). *AHK4*, which is highly expressed in roots, is implicated in the PSR (Franco-Zorrilla *et al.*, 2002) and may be part of a molecular mechanism for plant adaptation to external nutritional clues. It is possible that the presence of *AHK4* modulates the sensitivity of particular cells to CK. In recent years it has been shown that cZ is able to bind to and activate the CHASE-domain-containing histidine kinases (CHKs) and activate downstream CK response genes (Romanov *et al.*, 2006; Stolz *et al.*, 2011). In *A. thaliana*, *AHK2* and *AHK3* receptors show higher affinity for cZ than *AHK4*; however, in all cases, the affinity for cZ was several-fold lower than for tZ (Romanov *et al.*, 2006; Stolz *et al.*, 2011). In line with this, our results suggest that PSR regulation by cZ is mediated by AHK receptors, although their effect downstream is clearly different than that of tZ.

PSR in roots also involves secretion of organic acids, RNAses and phosphatases (López-Arredondo *et al.*, 2014), as well as root exudates that could act as molecular cues to recruit beneficial microbes (Rolfe *et al.*, 2019). Pi deficiency inhibits plant immune responses directly by *PHR1*, which in turn affects the composition of the plant microbiome (Castrillo *et al.*, 2017). Using the D-Root system, we show that the level of defense hormones is altered in response to Pi deficiency in the root, where JA levels diminish while SA levels increase. It is possible that CKs affect immunity through specific pathways which are both dependent and independent of SA and JA signaling networks (Choi *et al.*, 2011; Naseem *et al.*, 2014). It has been reported that cZ has a mild effect inducing SA biosynthesis and repressing *Pseudomonas* growth after infection (Großkinsky *et al.*, 2013). In our transcriptomic data, tZ repressed glucosinolate biosynthesis genes compared to cZ (Section 4.1.4., Fig. 14e-f), which are necessary for establishing the beneficial interaction and control fungal growth of *C. tofieldiae* (Hiruma *et al.*, 2016). It might be possible that, in line with the effect on root development, the cZ:tZ ratio also maintains a balance to fine tune immunity to a level that allows the establishment of mutualistic associations with the interacting microbiota.

In conclusion, this work has clearly highlighted the specific role of cZ in PSR. We show that cZ regulates distinct sets of genes, which are not regulated by tZ, and cZ (but not tZ) differentially regulates root growth in response to Pi starvation. Thus, cZ plays an important role in PSR by regulating root hair elongation, allocating Pi preferentially in roots, increasing root:shoot weight ratio for P foraging, and may also be involved in the modulation of plant defenses for an optimal interaction with beneficial microorganisms. Therefore, increasing cZ:tZ ratio during Pi starvation may trigger a specific molecular response that is needed for a proper plant adaptation to low concentrations of Pi.

5.1.3. GWAS reveals genetic architecture of PSR related traits in the Iberian Peninsula population

Plants have developed different strategies to cope with Pi deficiency, from modifying the RSA to secretion of phosphatases, RNAases or organic acids (Zhang *et al.*, 2014a). Among them, root architecture development to explore soil for nutrients has been linked to PUE phenotypes (Lynch, 2019). QTLs have been used in different crops to increase tolerance to low Pi (López-Arredondo *et al.*, 2014). In contrast to crops, *A. thaliana* has been described to shorten the primary root growth in response to Pi deficiency, although it should be highlighted that in plants growing with illuminated roots. Here, we have taken advantage of the D-root to keep roots in darkness to achieve a more realistic approach *in vitro* and exploit the previously unexplored RSA response to Pi starvation in DGR. We used the natural variability of *A. thaliana* to uncover the genetic components of the RSA through primary root growth and the number of LRs without the influence of root illumination. We investigated the natural variation in shoot weight and Pi accumulation during Pi starvation response in DGR seedlings, which are complex traits directly related to Pi deficiency tolerance and integrate other traits such as Pi uptake or metabolic reprogramming. The discovery of novel genes will be useful to understand the genetic mechanisms involved in natural responses to low P, as well as future breeding targets to increase tolerance to low P in the soils.

Our results show that all traits were negatively and significantly affected by low Pi availability, specially shoot Pi concentration and weight, in line with previous results studying natural variability in *A. thaliana* (Chevalier *et al.*, 2003; Yi *et al.*, 2021). The reduction of PRL was less pronounced in our DGR system than expected from previous results (Chevalier *et al.*, 2003; Yi *et al.*, 2021), indicating that the negative effect of light in roots is a general trend across the *A. thaliana* accessions. All traits showed high phenotypic variation, and the heritability falls within the range of related traits previously described (El-Soda *et al.*, 2019; Deja-Muyllé *et al.*, 2021). However, our GWAS analyses did not detect any SNP highly significantly linked to a phenotype in low Pi conditions. There are several reasons to explain this, but one could be due to the complexity of these traits, which are governed by multiple alleles with minor effects that

are difficult to detect in GWAS of limited populations (Korte & Farlow, 2013). Another possibility could arise from the fact that not all the genes involved in PSR might be subject to evolutive trade-offs and, therefore, result in natural variation. Nonetheless, GWAS analysis of non-significant associations have uncovered the roles of *PHYB* in PSR (Sakuraba *et al.*, 2018) and *NRTR1.1* in nitrogen deficiency (Sakuraba *et al.*, 2021) or been successfully used to predict candidate genes involved in shade avoidance response (Filiault & Maloof, 2012), showing the relevance of these analyses for uncovering the genetic architecture of traits.

In our study, root traits were significantly associated among them in both Pi conditions (sufficient or starving), while shoot weight was negatively correlated with Pi concentration. A possible explanation for this is that plants with higher weight might have allocated more organic P, using available P to grow rather than to store it in inorganic forms. LRs have been associated with PUE phenotypes (Lynch & Brown, 2001) and increase Pi uptake (Heppell *et al.*, 2015), and it has been proposed as one of the major locations of Pi acquisition (Kanno *et al.*, 2016). However, our studied Iberian accessions showed a negative correlation between the number of LRs and Pi concentration of shoots under Pi deprivation. In line with this, *OsIAAll* mutants with lower LR number did not impair Pi acquisition under Pi deficiency (Liu *et al.*, 2013). The author's hypothesis to address the low contribution of LRs was that Pi deficiency was severe, and that limits the growth potential of the LR. Another possible explanation for this negative correlation may be due to the increase in metabolic cost of exploring more soil. Increasing root development may demand a higher amount of organic phosphorous, redirecting Pi to roots with that aim and negatively impacting shoot Pi accumulation and usage. Despite these hypotheses, the cause of the association remains to be discerned. However, we should keep in mind that RSA develops in 3D in real soils where other factors such as spatial nutrient accumulation could play a decisive role. In fact, Pi is located at the top part of the soil and nutrient distribution is known to modify RSA (Hanlon *et al.*, 2018). Additionally, other root traits such as root hairs have been proposed to be the main responsible of Pi uptake (López-Arredondo *et al.*, 2014). In our study, the inferred soil phosphorous content at the original site of origin of the ecotype was not linked to any trait except for Pi concentration under high Pi. However, soil

inference is a rough estimation, and real Pi availability is difficult to estimate since phosphorous content can highly vary depending on the exact location and ecological context (i.e. farmland vs grassland), and its availability also depends on different interacting factors (such as the content of cations in soil, pH or microbiome). It is possible that Pi concentration in plates and soil correlate, such in a recent controlled study in *Lotus japonicus* (Giovannetti *et al.*, 2019), although natural environments are far more complex and it is difficult to infer global correlations.

Due to social-economic factors, there is an increasing interest in identifying new crops with high PUE. Natural variability has proven to be a successful approach to further translate genetic information into breeding traits for plants (Paz-Ares *et al.*, 2022). Here, we identified a list of putative genes involved in different Pi tolerance related traits. Among the candidates affecting plant weight under Pi deficiency, we identified LRL1, a TF regulating root hair growth (Karas *et al.*, 2009). Root hairs have been proposed to be key in PSR, increasing root surface to enhance Pi absorption (López-Arredondo *et al.*, 2014). For shoot Pi concentration, the list of genes putatively involved shows an enrichment in GO terms related to plant Pi metabolism and responses, such as MIPS2, PS2 and LPR1. MIPS2 enzyme catalyzes the rate-limiting step in the *myo*-inositol synthesis that results in InsP₈, the cellular sensor of Pi availability (Donahue *et al.*, 2010; Dong *et al.*, 2019). Two additional genes of the InsP metabolism pathway are present in our GWAS associations (8.5 fold enrichment, $p < 0.01$, Hypergeometric test): ITPK4 and PIP5K8 (Fig. 7). Thus, these three enzymes act at the initial step of synthesis (MIPS2) and in each of the synthesis pathways, lipid-dependent (PIP5K8) and independent (ITPK4), that converge in InsP₈ synthesis (Wang *et al.*, 2021). It might be possible that these three genes are involved in regulating InsP₈ levels, thus modulating PSR response in natural variation. *mips2* and *itpk4* mutants do not display PSR under Pi sufficiency despite having an altered InsPs profile, in contrast with *ipk1* or *vih1vih2* InsP₈ mutants (Kuo *et al.*, 2018). Nonetheless, the genetic functional redundancy in *MIPS*, *ITPK4* and *PIP5Ks* might hinder single mutant data interpretation (Sweetman *et al.*, 2007; Fleet *et al.*, 2018; Kuroda *et al.*, 2021). This is illustrated in *VIH1/VIH2* genes that catalyze the final step of InsP₈, as their single mutants do not have the Pi overaccumulation phenotype of a constitutive PSR response present in the

double *vihlvih2* (Dong *et al.*, 2019). Interestingly, InsP₈ levels are depleted in *itpk4* mutants and are not altered in PSR, suggesting that not only InsP₈ but InsP stoichiometry and its coordination with ATP/energy system and/or subcellular compartmentalization are also important for Pi-signaling (Wang *et al.*, 2021). *LPRI* was another gene putatively involved in shoot Pi concentration. This gene encodes for a multicopper oxidase involved in root meristem growth and might regulate root growth under Pi deficiency (Müller *et al.*, 2015). Therefore, this gene is involved in the PRL stop that occurs in LGR, mediated by blue light (Yeh *et al.*, 2019; Zheng *et al.*, 2019). Whether this pathway has a role stopping PRL under DGR is unclear. It could be possible that LPRI-PDR2 module blockage of PRL, enhanced by the additive effect of blue light in LGR, also occurs in DGR, although to a lower extent (Zheng *et al.*, 2019). In our GWAS analysis, LPRI is associated with Pi concentration in shoots. However, we cannot discard that this association in shoots is due to the altered capacity of PRL to explore and uptake Pi. The last candidate, *PS2*, encodes for an organic phosphatase that dephosphorylates phospholipid and seems to be involved in Pi recycling under Pi scarcity. It should be highlighted that *PS2* is upregulated in our RNASeq (Supplementary File 1 – Table b, Supplementary File 2 – Table e). Furthermore, the TF *ZFP10* is also upregulated in response to Pi starvation (Supplementary File 1 – Table b, Supplementary File 2 – Table e) and is a GWAS candidate. *ZFP10* overexpression leads to a dwarf phenotype, suggesting that, during Pi starvation, expression of this gene might negatively regulate shoot growth (Dinkins *et al.*, 2002). Taken together, these data suggest that our candidate gene list inferred from GWAS analysis might be reflecting the genetic architecture of these traits under Pi deficiency and point towards InsP metabolism underlying the natural variation in PSR regulation.

Root architecture (principal or LR growth and development) determines the capacity of the plant to explore soil resources. Regarding PRL, the GWAS analysis identified ATIG34046, an ANK protein, as a candidate gene for this trait under sufficient Pi conditions. The ANK motif is involved in protein interactions and ANK proteins have been described in a wide range of processes in plants, from acquired immunity to development (Cao *et al.*, 1997; Yu *et al.*, 2010). Whether this ANK protein is underlying natural variation of PRL remains to be elucidated. LR development is a PSR associated

with enhanced PUE, and multiple studies have tried to decipher the molecular bases of this response (Lynch & Brown, 2001; Crombez *et al.*, 2019). In our study, 102 genes were associated with the number of LR under low Pi supply (Supplementary File 2 – Table i). Of these, ~15% were associated with LR development or PSR in the literature and/or deregulated in our root expression data (Supplementary File 2 – Table i). For example, *GRF6* is upregulated in Pi deficiency, although its function is still unknown (Cao *et al.*, 2007). Likewise, *EXPA10* is an expansin upregulated in our data (Supplementary File 1 – Table b, Supplementary File 2 – Table i) and associated to number of LRs in the present GWAS study (Fig. 19, Supplementary File 2 – Table i). Another member of this family, *EXPA1*, has been linked to LR initiation in *A. thaliana* (Ramakrishna *et al.*, 2019). Although under no stress conditions, *EXPA10* acts on cell differentiation in the transition zone and it is not specifically expressed in LRP (Pacifici *et al.*, 2018; Ramakrishna *et al.*, 2019). Thus, it might be possible that *EXPA10* is specifically involved in LR initiation under low Pi conditions. Similarly, the *XHT4* gene associated in our Pi starved LR GWAS has a family related gene (*XHT9*) that is induced specifically under nitrogen deficiency, promoting LR development (Xu & Cai, 2019). We also identified two other genes with known functions in LR root development and auxin related, *ARF17* and *PIP5K2* (Mei *et al.*, 2012; Couzigou & Combier, 2016). Interestingly, *PIP5K2* is also part of the InsP synthesis route and InsP₆ is a co-factor of the auxin co-receptor TIR1 (Tan *et al.*, 2007; Crombez *et al.*, 2019; Starodubtseva *et al.*, 2022). This suggests a role for certain InsP species that could act in specific cell-types to produce spatially restricted Pi derived signaling (Crombez *et al.*, 2019; Starodubtseva *et al.*, 2022), and hints a role for auxins in PSR responses through InsPs. Another identified gene that is involved in LRP cell divisions is *COPT2* (Waidmann *et al.*, 2019). Interestingly, *COPT2* (a copper transporter) and *FERONIA* (a lectin/receptor like kinase) have a role under low Pi conditions and are related to Cu and Fe homeostasis respectively (Perea-García *et al.*, 2013; Li & Lan, 2015; Tang *et al.*, 2022; Xie *et al.*, 2022). Altogether, the evidence seems to indicate that the candidate list is enriched in genes related to root architecture that seem to be regulated under low Pi conditions and suggest that some of these genes could be involved in the RSA development during the plant response to Pi starvation.

To identify candidate genes involved in more than one trait, we looked for common genes in our GWAS list of candidates. We found that the TF *WRKY18* was associated with plant weight and PRL, traits that have a positive correlation in the ecotype population (Section 4.1.7., Fig. 18b and Fig. 21). *WRKY18* has been involved in multiple stress responses, especially against pathogens (Khan *et al.*, 2022; Biniaz *et al.*, 2022; Boonyaves *et al.*, 2022). However, the role of this TF in PSR remains to be elucidated. *WRKY18* is a direct target of PHR1 (Barragán-Rosillo *et al.*, 2021) and is upregulated during the first stages of PSR, peaking after 30 minutes after Pi depletion (Lin *et al.*, 2011). Furthermore, it physically interacts with WRK42, which regulates Pi uptake through the PHT1;1 transporter and translocation through PHO1 (Su *et al.*, 2015). WRKY42 also interacts physically with PIF4, a direct repressor of *PHT1;1* (Sakuraba *et al.*, 2018). Our GWAS analysis combined with these pieces of evidence scattered through the literature have led us to hypothesize a role for *WRKY18* in PSR that might be of relevance for natural variation. However, further experiments are needed to corroborate this possibility as well as to analyze the possible role of this genes in the modulation the plant-microbiota interaction during the Pi starvation response.

5.2. Screening for fungal positive interactors under Pi deprivation

Plant's ability to absorb phosphorus is limited mainly by the low availability of free Pi chemical specie in soil (Raghothama, 1999). In nature, plants establish associations with a wide variety of microorganisms to enhance their mineral nutrition ability (Mishra *et al.*, 2017). Fungal endophytes are emerging as an alternative strategy to increase crop phosphorous uptake efficiency, since they are capable of solubilizing, mineralizing and/or transporting phosphorous into plants (Alori *et al.*, 2017; Hiruma *et al.*, 2018). We performed a screening to find fungal endophytes that improved plant growth under Pi limiting conditions. The collection of isolates used was obtained from natural populations of *A. thaliana* from phosphorous poor soils, from where the beneficial fungal endophyte *C. tofieldiae* had also been obtained (Hiruma *et al.*, 2016). We hypothesized that other isolates with similar beneficial effects but different modes

of action in the interaction with the plant could also be found among the isolates in that collection. For the screening, we decided to exacerbate plant stress conditions by combining low Pi and root illumination, with the idea of intensifying the positive or negative effect of the inoculated fungi. Thus, we observed a continuous of interactions (negative, neutral and positive) among the different isolates (Fig. 24), standing out the isolate Ps-1 for its overall performance in improving plant growth traits under Pi starving conditions (Section 4.2.2., Fig. 24 and Fig. 25).

5.2.1. Plant PSR might be required for Ps-1 PGP effects in *Arabidopsis thaliana*

Our screening has identified Ps-1, a putative endophyte with PGP effects under Pi limiting conditions. Ps-1 induces both shoot and RSA growth under Pi deprivation (Section 4.2.2., Fig. 25). Thus, Ps-1-plant interaction outcome seems to be dependent on external Pi availability. Interestingly, this is in line with other reports of AM and other fungal endophytes that are capable of supplying Pi to the plant (Parniske, 2008; Bakshi *et al.*, 2015; Hiruma *et al.*, 2016; Almario *et al.*, 2017; Redkar *et al.*, 2022). In rice, *PHR1* mediates AM symbiosis success by controlling the expression of several genes needed to establish the symbiosis. Similarly, *A. thaliana* beneficial interaction with the fungal endophyte *C. tofieldiae* only occurs under low Pi regimen, and *PHR1/PHL* are needed to produce PGP benefits (Hiruma *et al.*, 2016). Thus, in both cases, PSR is needed to establish a beneficial interaction and Pi exchange. Although direct Pi transport from Ps-1 hyphae to the plant has not been addressed yet, the increase in shoot weight and phosphorous content in Ps-1 treated plants hints towards this possibility. Thus, it might be possible that Ps-1 interaction is mediated by PSR through *PHR1*, similarly to *C. tofieldiae* or AM associations. Nodule-forming rhizobia, another symbiotic association in plants, fix atmospheric nitrogen and give it to the plant in exchange for carbon. The regulation of the nodulation process is essential to establish rhizobia symbiosis, and this pathway is also activated by *PHR1* (Isidra-Arellano *et al.*, 2021). This evidence suggests that PSR modulation of plant-microbiota relationships may be a

general mechanism and therefore it would be interesting to analyze the possible role of PHRI in Ps-I-plant interaction.

5.2.2. *Ps-I enhances RSA*

To cope with a varying soil nutrient distribution, plants modify their RSA and interact with their surrounding microbiota to improve their root nutrient acquisition (Verbon & Liberman, 2016). In addition to transfer nutrients directly to the plant, PGP microbes can modify primary root or LR development to increase nutrient absorption capacity. In this work, we show that Ps-I increases primary root length as well as meristem size under Pi starvation (Fig. 24 and Fig. 25). Root growth promoting endophytes, bacterial or fungi, tend to increase eLR while restricting primary root development (Verbon & Liberman, 2016). Changes in primary root by PGP microbes have been less studied in comparison to LR formation. In presence of *Achromobacter*, *A. thaliana* plants increase their PRL by stimulating cell division, auxin signaling and PIN distribution in the root meristem (Jiménez-Vázquez *et al.*, 2020). In other cases, a volatile organic compound (VOC) produced by the microorganism but not the direct interaction with the microbe, enhances PRL (Bailly *et al.*, 2014; Garnica-Vergara *et al.*, 2016). In the case of Ps-I, we did not observe significant changes in the expression of the auxin signaling marker DR5:LUC in the root tip. However, we cannot fully discard the involvement of auxins, since we did not observe the DR5::LUC expression pattern at cell resolution level. Future work will address if Ps-I induces cell division in the root meristem and drives root growth through auxins or whether this is mediated by other mechanisms.

Besides primary root, Ps-I strain also altered *A. thaliana* LR development under Pi deficiency, enhancing the number of eLRs and PBS. Plant beneficial microorganisms are reported to alter LR development and LRP specification, a process that is driven by auxin (Verbon & Liberman, 2016). *Trichoderma virens* increases the number of eLR in *A. thaliana* through production of auxin compounds that stimulate LR development (Contreras-Cornejo *et al.*, 2009). Furthermore, endophytic fungal strains known for

RSA manipulation such as *Laccaria bicolor*, *Trichoderma* sp. or *S. indica* are known to produce auxins or exert its function through auxin signaling/perception (Contreras-Cornejo *et al.*, 2009; Felten *et al.*, 2009; Garnica-Vergara *et al.*, 2016; Pérez-Alonso *et al.*, 2020). In this work we show that *A. thaliana* -Ps-1 interaction does not trigger a differential DR5 auxin response nor inhibits PRL, suggesting that the fungus does not significantly increase the auxin level and/or signaling. Nonetheless, it is possible that the fungus is capable of synthesizing auxin-like compounds that might count for root growth under low Pi conditions. Besides, Ps-1 seems to promote PBS specification. The root clock determines PBS through oscillation of complex auxin signaling mechanisms that establish the PBS patterning in roots (Bustillo-Avendaño *et al.*, 2022). Endogenous cues, such as auxin as well as other hormones or retinoids (a subclass of β -apocarotenoids) can regulate root clock oscillations (Bustillo-Avendaño *et al.*, 2022). Recently, it has been described that cedrene, a sesquiterpene VOC from *Trichoderma guizhouense*, promotes PRL and eLR (Li *et al.*, 2022). Although this volatile does not seem to alter root clock oscillation periodicity, it increases its intensity; showing that it is possible that external signals from root endophytes alter root clock oscillation or intensity (Li *et al.*, 2022). Our data suggest that the increase in PBS might be derived from an increase in periodicity, meaning more peaks of oscillations specifying PBS per time. Nonetheless, the exact mechanism and/or compound altering the root clock remains to be discerned and will require new experiments.

Fungal endophytes can establish different types of association with plant roots. This colonization can be inter or intracellular and local, limited to certain tissues, or systemically extended to different tissues (Kariman *et al.*, 2018). Our re-isolation assay did not show shoot colonization of root inoculated Ps-1 in *A. thaliana* seedlings growing *in vitro*. However, Ps-1 was originally isolated from a surface sterilized leaf of an *A. thaliana* plant growing in natural conditions (García *et al.*, 2013). It is possible that Ps-1 is a root endophyte that is occasionally able to reach the shoot, as already described for *C. tofieldiae* (Hiruma *et al.*, 2016). Other fungi, endophytic or not, produce auxin, VOC or other compounds to exert their benefits in plants, so direct contact might not be necessary to produce PGP effects (Felten *et al.*, 2009; Kariman *et al.*, 2018; Li *et al.*,

2022). Further work is necessary to better describe the infection strategy colonization way of Ps-I, and whether the colonization is in fact needed for the beneficial effect.

5.2.3. PGP effects of Ps-I is shared on *Hordeum vulgare*

Fungal symbionts can be specific to one host, but there are several examples of fungal endophytes that are able to colonize, and provide benefits, to different host species (Parniske, 2008; Zuccaro *et al.*, 2014; Bonito *et al.*, 2017; Díaz-González *et al.*, 2020). Our results indicate that Ps-I also increases shoot and root growth in barley (*H. vulgare*). Moreover, this PGP effect is similar to the one observed in *A. thaliana*. However, *H. vulgare* plants did not require Pi starvation to establish the beneficial relationship with Ps-I. Similarly, the fungal endophyte *C. tofieldiae*, which establishes a beneficial interaction with *A. thaliana* under Pi depleted conditions, is able to colonize and produce PGP on *Zea mays* (maize) and *Solanum lycopersicum* (tomato) under high Pi nutrition (Díaz-González *et al.*, 2020). The *A. thaliana* - *C. tofieldiae* positive interaction depends on intact PSR and indol-glucosinolate derived defense metabolites pathways (Hiruma *et al.*, 2016). Although this mechanism seems to be necessary for *A. thaliana*, other mechanisms, and the benefits obtained thereof, may vary in the different hosts (Redkar *et al.*, 2022). *S. indica* produces different hyphal invasion depending on which host is entering, and in each host triggers a different hormonal response (Liu *et al.*, 2019b). Thus, in a similar way, Ps-I may make use of different mechanisms and hormonal pathways to interact with *H. vulgare*, which may also vary depending on the nutritional needs of the plant. Thus, there are some open questions that should be addressed in the future: Does Ps-I use different colonization processes depending on the host? Are the molecular and physiological responses of the host similar during Ps-I association? Is the specific species-Ps-I association dependent on Pi level? Do they use the same molecular mechanisms if they are Pi-dependent?

Our screening of PGP fungi under Pi starvation conditions identified Ps-I, a putative fungal endophyte that enhances shoot biomass and root development. These traits are highly relevant for improving crop yield in low Pi soils or with low fertilization

regimens. Furthermore, our *A. thaliana* - fungi screening pipeline was highly successful as we identified at least one PGP fungus out of 23 screened fungi. This screening method, combined with the use of a semi-hydroponic *H. vulgare* growth platform, provides a novel methodology to quickly identify PGP microorganisms in plants to further study their mechanisms and/or move toward greenhouse and field trials to explore its economic viability.

5.3. Root temperature effect on plant responses to high temperature, nutrition and microbiome

Using a novel approach that mimics in many aspects the natural soil properties, we have demonstrated that a temperature gradient in the root ecosystem is essential to study and to understand the whole plant response to heat stress. In general, the majority of the *in vitro* and greenhouse studies have not considered the temperature in the root zone when analyzing the effect of heat stress (Chen & Li, 2016; Valdés-López *et al.*, 2016; Calleja-Cabrera *et al.*, 2020; Estravis-Barcala *et al.*, 2021; Pei *et al.*, 2021). In response to warming temperature, which is normally optimal and non-detrimental for plant growth, one of the thermomorphogenic responses is the promotion of root elongation. Opposite, when the entire plants are cultivated at slightly higher temperatures over the optimal, root growth is arrested and LR formation is blocked, reducing root functionality and negatively affecting shoot biomass. Here we present robust data showing that high temperature in roots has a strong impact on plant development, nutrition, physiological and molecular responses to heat as well as microbiome assembly. The TGRooZ provides a novel and useful approach to study heat stress in the shoot and roots. This approach can help to identify new solutions and facilitate plant breeding programs to obtain more heat tolerance crops or to test new microbes to enhance plant tolerance to heat in a closer-to-field conditions. Thus, the knowledge generated by using the TGRooZ can be useful to cope with the uncertain effects that global warming and heat waves will produce in crops.

5.3.1. Soil temperature in the root growing zone is important for plant growth and development

In natural ecosystems, the temperature of the soil is affected by several factors such as the environmental temperature, water content, soil compaction, or organic matter among others (Elias *et al.*, 2004). In turn, soil temperature affects many soil physical-chemical properties and living processes that have an impact on plant growth and adaptation to the environment (Smith & Mullins, 2001; Onwuka, 2018). A recent work found that temperature in the top soil increases, proportionally, more rapidly than air temperature (Zhang *et al.*, 2016). This work predicts that soil respiration will rise by up to 28%, reinforcing the idea that climate warming affects the soil ecosystem and perturbs plant growth. To analyze the effect of heat on soil temperature, we have measured the temperature during July 2019 in a natural soil in Spain, with an atmospheric temperature average of 34°C, but with peaks of heat waves over 40°C. Temperature was measured at different depths from the top surface down to 14 cm deep, in order to estimate the range of the temperature gradient that is naturally formed in the soil. We observed that a decreasing temperature gradient was formed in the root growth zone (Section 4.3.1., Table 5). These data were used to set the parameters in the TGRooZ device to generate a temperature gradient in the plant growth substrate (agar-containing plates or soil) that mimics somehow the natural soil conditions during a heat wave in an enclosed ecosystem (plant growth chambers or greenhouses) where we can control environmental temperature. In addition, we measured the day-night temperature fluctuation during 4 days during June 2022 in a natural soil in Spain, finding that, at 15 cm depth, the soil has a stronger temperature buffer capacity than at 5 cm, while in the top surface the fluctuation was significantly higher (Section 4.3.1., Table 6 and Table 7). This is in agreement with soil thermal diffusivity analyses that show that superficial soil (5 cm) suffers higher temperature variations, while at 15 cm or deeper soils the changes are buffered along the different seasons (Brunetti *et al.*, 2021). Gradient and soil buffer capacity will depend on physical soil characteristics, such as humidity or compaction among others (Luo *et al.*, 1992).

Using the TGRooZ, we provided strong evidence that soil-root temperature is essential to activate a coordinated response to heat in the whole plant, avoiding severe cellular damage and root organ failure by an excess of temperature (Section 4.3.1., Fig. 32-35) (Hasanuzzaman *et al.*, 2013; Qi & Zhang, 2019; Liu *et al.*, 2022). However, the severity of the damage depends on the plant species and genotype, and the context of the stress, since these responses are different if the plant encounters a gradual temperature variation or if it suddenly faces a high temperature wave (Larkindale & Vierling, 2008; Gomaa *et al.*, 2011; Zhang *et al.*, 2015). During their lifetime, roots face many hostile environmental conditions that affect, not only root growth and development, but also the whole plant. In response to high temperature in the root zone *in vitro*, plants decrease root growth and root meristem size (Yang *et al.*, 2017; Liu *et al.*, 2022). It has been reported that heat stress shortens the cell elongation zone, reducing the cell flux from the meristem to the elongation area and stopping root growth (Baskin *et al.*, 1992), likely by reducing cell division in the root meristem. We found similar phenotypes in plants exposed to shoot-root homogeneous temperature of 32°C (Section 4.3.1., Fig. 32). However, if seedlings are grown in the TGRooZ, with conditions more similar to field temperatures, root growth is recovered to the level found at optimal temperatures (Section 4.3.1., Fig. 32). Hypocotyl elongation, a trait associated with warming (Gray *et al.*, 1998; Bellstaedt *et al.*, 2019; Gaillochet *et al.*, 2020; Lee *et al.*, 2021), is the unique parameter in the aerial part that is not fully recovered in TGRooZ plants (Section 4.3.2., Fig. 33). This suggests that this response is mainly dependent on the atmospheric temperature surrounding the shoot, although root temperature can partially influence it, whereas other responses to heat such as hyponastic angle are more dependent on soil-root temperature. Taken together, these results highlight the importance of having different soil-atmospheric temperatures to analyze the molecular, physiological and morphological responses to heat as it occurs in nature.

5.3.2. Root temperature drives transcriptomic responses at organ level

We observed that the transcriptional response to heat is conditioned by root temperature, and that the global gene expression is shoot- or root-dependent (Section

4.3.3., Fig. 3.5). We also show that, by cultivating the root system in a temperature gradient, the gene expression of shoots exposed to an identical high temperature is modified, pointing out the root-to-shoot communication and *vice versa*. These data indicate that the response to temperature is organ dependent but should be coordinated and integrated to give a global response.. It should be highlighted that many of the DEGs are related to auxin signaling and are downregulated in plants subjected to homogeneous 32°C (Section 4.3.3., Fig. 35e). Since auxin plays an essential role in shoot growth and biomass production (Tivendale & Millar, 2022), this down-regulation might explain why these plants develop a reduced shoot biomass. However, this aspect should be validated in the future.

Our transcriptomic comparisons allowed us to identify genes involved in heat responses that are also maintained in TGRooZ seedlings (Supplemental File 4 – Table g). Among them, we found an enrichment in heat shock proteins and chaperones, which are important players of the heat stress response (Charng *et al.*, 2007; Liu *et al.*, 2011; Hasanuzzaman *et al.*, 2013). Interestingly, root growth does not fully recover in the *hsfa2* mutant growing in a root temperature gradient (Section 4.3.4., Fig. 37). *HSFA2* regulates the expression of heat shock proteins and is required for the acquisition and extension of plant thermotolerance (Charng *et al.*, 2007) (Charng *et al.*, 2007). Analyses of mutants of individual members of the *HEAT SHOCK PROTEIN 70 (HSP70)* family did not reveal morphological phenotypes (Leng *et al.*, 2017). In our conditions, heat stress reduces root growth and the number of LR_s formed in 32TGRooZ *hsp70* mutant seedlings (Section 4.3.4., Fig. 37), indicating that this gene, similarly to *HSFA2*, might connect shoot heat stress responses with root development. It should be highlighted that the identification of these genes has been possible due to the fact that the root responses are not blocked by the excessive high temperature, supporting the idea that plants under heat stress should be cultivated with the root system in a temperature gradient that mimics field conditions. In that sense, TGRooZ might be able to uncover new genes in the plant response to heat affecting roots or shoots independently and differentially. Thus, based on our data, we strongly believe that the use of TGRooZ can improve our knowledge on how heat stress affects plant development and allow the further translation from the lab data to the field, improving field-trials.

5.3.3. Root temperature affects plant nutrition

Plant nutrition has been reported to be altered during heat stress (Hassan *et al.*, 2021). Recently, a study in quinoa showed that the mineral content of the seeds was altered in plants with heated roots (Tovar *et al.*, 2022). Interestingly, heating roots or shoot or both produced a different ionic profile compared with plants cultivated at 22°C homogeneously, reflecting that grain filling process could be altered by root temperature (Tovar *et al.*, 2022). This observation is in line with our results, that showed that the KIG collection of genes involved in plant ionome composition was significantly regulated by temperature in *A. thaliana* shoots and roots (Supplementary File 5 – Table a). Furthermore, our results show that tomato leaf ionic profile was altered by root temperature (Section 4.3.5., Fig. 41). Altogether, these results emphasize that keeping root temperature as close as natural conditions is essential to understand ionic processes affecting the whole plant, a trait that is essential for crop production and human nutrition.

Different transcriptomic analyses have pointed the interconnection between high temperature and PSR (Pacak *et al.*, 2016; Giri *et al.*, 2017). Herein, we show that high temperatures affect several genes related to Pi starvation and reduce the level of Pi in roots and shoots (Section 4.3.5., Fig. 39 and Fig. 40). Interestingly, two of the main Pi starvation regulators (*PHR1* and *PHO2*) were downregulated in accordance with our data (Supplementary File 5 – Table a), showing an increase in Pi nutrition pathways and accumulation (Whitt *et al.*, 2020). Remarkably, a significant number of these KIG genes were also regulated by temperature in root and shoot. *PHR1* and *PHO2*, two key genes that control the Pi starvation response were downregulated in response to high temperature. Mutation of *PHO2* increases the Pi level in the shoot by increasing the activity of the *PHO1* transporter (Bari *et al.*, 2006; Pacak *et al.*, 2016). Mutation in *PHR1* gene, a key TF that regulates the PSR, reduces the level of Pi (Rouached *et al.*, 2010). In our case, 32SR seedlings show a reduction in the level of expression of *PHO2* and *PHR1* but an increase in the level of *PHO1:3* (Supplementary File 5 – Table a). However, contrary to what is expected, these 32SR seedlings accumulated lower Pi level in their shoot than TGRooz seedlings. It is possible that high temperature severely affects root

functionality, leading to low Pi uptake and therefore plants try to mobilize Pi from roots to shoots by downregulating *PHO2*. Nevertheless, these results indicate the close correlation between heat stress and Pi nutrition.

5.3.4. Microbiome assembly and plant growth is influenced by soil temperature

Nutrient availability and plant productivity is determined in a large extent by the soil properties and the soil microbial community inhabiting the roots. The soil microbiota can be affected by diverse soil factors such as nutrient content, pH or soil texture (Chaparro *et al.*, 2012). The effects of climate change factors such as elevated CO₂, drought, and higher temperature on beneficial plant-microbe interactions are increasingly being explored. For example, soil temperature affects microbiota recruitment by the plant roots (Compant *et al.*, 2010; Rousk *et al.*, 2012) and increased soil temperature might positively help roots to select microbial species bearing heat tolerance mechanisms with high growth rates over those that are temperature-sensitive and slow-growing microbes (van der Voort *et al.*, 2016). The heat driven community disturbance might lead to changes in plant growth, pathogen-protection, or their abiotic stress responses (Hariprasad *et al.*, 2021; Vogel *et al.*, 2021). Plant nutritional status has been shown to have an effect on the microbiota assembly recruitment (Zhang *et al.*, 2019a; Finkel *et al.*, 2019), implying that nutritional responses cues to recruit specific microorganisms could be also altered due to root temperature. However, temperature effect on soil-microbial fitness could also play an important role in determining what is selected by plant nutritional-derived recruitment.

A hypothetical model based on the responses of microbial communities to temperature sensitivity suggests that the growth of bacterial and fungal soil communities tend to increase under moderate high temperatures but, however, microbial diversity is significantly reduced when the soil is exposed to a continuous high temperature (Nottingham *et al.*, 2019). This reduction in diversity was also observed when soil was incubated at 35 °C for a long period of time (Lin *et al.*, 2017) or during a wildfire, a process that increases the soil temperature to abnormal high levels leading to a decrease in microbial activity and major changes in the assembly of microbial

communities (Ferrenberg *et al.*, 2013; Jolly *et al.*, 2015), or during solarization (soil heating) to sterilize soil, a procedure largely used in agriculture to eliminate or reduce soil detrimental microorganisms (Kanaan *et al.*, 2018). Our data suggest that root temperature also affects mineral plant nutrition (Section 4.3.5., Fig. 41). In our experiments, neither soil or air temperature affected microbiome richness or diversity (Section 4.3.5., Fig. 42). However, our data clearly show that plants exposed to shoot/root homogenous high temperature accommodate different rhizosphere- and root-associated microbiome than plants grown at optimal temperature or with TGRooz, which show similar microbiome composition. Thus, it seems that root temperature is driving root microbiome composition, independently of atmospheric temperature.

It is well established that, under stress, plants can change the root microbiome composition enhancing the reproduction of beneficial microbes that represent an advantage for plant fitness (Zhang *et al.*, 2019a). It has been reported that Actinobacteria abundance increase in the rhizosphere of tomato plants showing tolerance to bacterial wilt disease infection as compared with tomato plants susceptible to this disease, while Proteobacteria phylum abundance was lower (Huot *et al.*, 2017). Therefore, changes in actinobacteria and proteobacteria found in 36SR tomato plants might be associated with the plant response to stresses. Thus, changes in the ratio actino/proteo bacteria indicate that high temperature drives the root to recruit a “plant-protection” microbiota. This observation strengthens the idea that the root might function as a sensing hub for abiotic and biotic stresses and that high soil temperature, but not the gradient of temperature in the root, disturbs the equilibrium and affects root functionality. However, it should be highlighted that genes involved in glucosinolate biosynthesis and metabolism are upregulated in *A. thaliana* TGRooz roots compared to 22RS, indicating an activation of root protection against pathogens (Section 4.3.5., Fig. 35). Glucosinolates are necessary for the establishment of the beneficial interactions between *A. thaliana* and fungal endophytes such as *S. indica* and *C. tofieldiae* (Hiruma, 2019) and protect plants from fungal driven dysbiosis (Wolinska *et al.*, 2021). It would be interesting to study the effect of root and air temperature on *A. thaliana* root microbiome, besides tomato, paying special attention to fungi.

Taken together, we demonstrate that over-warming the soil compromises root function, which in turn might reduce the plant capacity to cope with environmental changes. Our approach will be helpful to study root growth and adaptation in soil-growing plants in response to heat, a trait that has been understudied. We envision that the system provided here will help the design of new experiments aiming to mitigate the coming heat waves due to climate change resulting in improved harvests and food security.

6. CONCLUSIONS

6. Conclusions

1. Root illumination alters phosphate starvation response at the molecular, physiological, and morphological levels, significantly affecting root system architecture.
2. cisZeatin:transZeatin ratio increases under Pi deficiency, and this balance regulates root growth and root hair elongation.
3. cisZeatin plays an important role in mediating Pi starvation response by regulating root growth, root hair elongation, and Pi allocation.
4. CisZeatin regulates gene transcription in a different way than transZeatin.
5. The *Arabidopsis thaliana* collection of ecotypes from the Iberian Peninsula presents phenotypic variation under high and low Pi for shoot weight, shoot Pi concentration, primary root length and number of lateral roots, suggesting underlying genetic variation associated to these traits.
6. GWAS analysis of PSR-related traits uncovered their genomic architecture basis, revealing genes that might be involved in the phenotypic variation of these traits for the Iberian Peninsula population.
7. GWAS analysis of shoot Pi concentration suggests that inositol phosphate synthesis route might be a hot-spot for natural variation regulation under Pi deficiency.
8. GWAS data of seedlings cultivated under low Pi concentration, together with a gene network analysis, suggest a novel role for the transcription factor *WKRY18* under Pi starvation.
9. Our screening for beneficial fungi among the natural fungal endophytes collection of *Arabidopsis thaliana* under Pi deprivation, identified Ps-1 as a plant growth promoting fungus.
10. Ps-1 increases *Arabidopsis thaliana* shoot biomass, total phosphate accumulation, principal root growth and lateral root formation under Pi deficiency.
11. Ps-1 positive effects are conserved in *Hordeum vulgare* in *in vitro* growth conditions.

12. Soil temperature regulates root growth as well as shoot physiological and transcriptomic responses to high atmospheric temperature in *Arabidopsis thaliana*.
13. Transcriptomic analysis identified genes conserved in plant responses to high temperature that are important for root growth in response to high temperatures.
14. Plant ionic composition is altered by soil temperature in response to heat, indicating that a temperature gradient in the soil is important for plant nutrition.
15. Soil temperature, rather than atmospheric temperature, determines plant root and rhizosphere microbiome composition.

7. REFERENCES

7. References

- 1001 Genomes Consortium. 2016.** 1,135 Genomes Reveal the Global Pattern of Polymorphism in *Arabidopsis thaliana*. *Cell* **166**: 481–491.
- Almario J, Jeena G, Wunder J, Langen G, Zuccaro A, Coupland G, Bucher M. 2017.** Root-associated fungal microbiota of nonmycorrhizal *Arabis alpina* and its contribution to plant phosphorus nutrition. *Proceedings of the National Academy of Sciences of the United States of America* **114**: E9403–E9412.
- Alonso-Blanco C, Aarts MGM, Bentsink L, Keurentjes JJB, Reymond M, Vreugdenhil D, Koornneef M. 2009.** What has natural variation taught us about plant development, physiology, and adaptation? *The Plant Cell* **21**: 1877–1896.
- Alori ET, Glick BR, Babalola OO. 2017.** Microbial Phosphorus Solubilization and Its Potential for Use in Sustainable Agriculture. *Frontiers in Microbiology* **8**: 971.
- Al-Whaibi MH. 2011.** Plant heat-shock proteins: A mini review. *Journal of King Saud University - Science* **23**: 139–150.
- Aly AH, Debbab A, Proksch P. 2011.** Fungal endophytes: unique plant inhabitants with great promises. *Applied Microbiology and Biotechnology* **90**: 1829–1845.
- Ames BN. 1966.** Assay of inorganic phosphate, total phosphate and phosphatases. In: *Methods in Enzymology. Complex Carbohydrates*. Academic Press, 115–118.
- Anders S, Pyl PT, Huber W. 2015.** HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics (Oxford, England)* **31**: 166–169.
- Andrews S. 2010.** *FastQC: a quality control tool for high throughput sequence data*. Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.
- Arsovski AA, Galstyan A, Guseman JM, Nemhauser JL. 2012.** Photomorphogenesis. *The Arabidopsis Book* **10**: e0147.

Arteaga N, Méndez-Vigo B, Fuster-Pons A, Savic M, Murillo-Sánchez A, Picó FX, Alonso-Blanco C. 2022. Differential environmental and genomic architectures shape the natural diversity for trichome patterning and morphology in different Arabidopsis organs. *Plant, Cell & Environment*.

Baek D, Chun HJ, Kang S, Shin G, Park SJ, Hong H, Kim C, Kim DH, Lee SY, Kim MC, *et al.* 2016. A Role for Arabidopsis miR399f in Salt, Drought, and ABA Signaling. *Molecules and Cells* **39**: 111–118.

Baek D, Chun HJ, Yun D-J, Kim MC. 2017. Cross-talk between Phosphate Starvation and Other Environmental Stress Signaling Pathways in Plants. *Molecules and Cells* **40**: 697–705.

Bailly A, Groenhagen U, Schulz S, Geisler M, Eberl L, Weisskopf L. 2014. The inter-kingdom volatile signal indole promotes root development by interfering with auxin signalling. *The Plant Journal: For Cell and Molecular Biology* **80**: 758–771.

Bakshi M, Vahabi K, Bhattacharya S, Sherameti I, Varma A, Yeh K-W, Baldwin I, Johri AK, Oelmüller R. 2015. WRKY6 restricts Piriformospora indica-stimulated and phosphate-induced root development in Arabidopsis. *BMC plant biology* **15**: 305.

Balestrini R. 2021. Grand Challenges in Fungi-Plant Interactions. *Frontiers in Fungal Biology* **2**: 750003.

Banda J, Bellande K, von Wangenheim D, Goh T, Guyomarc'h S, Laplaze L, Bennett MJ. 2019. Lateral Root Formation in Arabidopsis: A Well-Ordered LRexit. *Trends in Plant Science* **24**: 826–839.

Bari R, Datt Pant B, Stitt M, Scheible W-R. 2006. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* **141**: 988–999.

Barragán-Rosillo AC, Peralta-Alvarez CA, Ojeda-Rivera JO, Arzate-Mejía RG, Recillas-Targa F, Herrera-Estrella L. 2021. Genome accessibility dynamics in response to phosphate limitation is controlled by the PHR1 family of transcription

factors in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **118**: e2107558118.

Baskin T, Betzner A, Hoggart R, Cork A, Williamson R. 1992. Root Morphology Mutants in Arabidopsis thaliana. *Functional Plant Biology* **19**: 427.

Bates TR, Lynch JP. 2001. Root hairs confer a competitive advantage under low phosphorus availability. *Plant and Soil* **236**: 243–250.

Bellstaedt J, Trenner J, Lippmann R, Poeschl Y, Zhang X, Friml J, Quint M, Delker C. 2019. A Mobile Auxin Signal Connects Temperature Sensing in Cotyledons with Growth Responses in Hypocotyls. *Plant Physiology* **180**: 757–766.

Berckmans B, Vassileva V, Schmid SPC, Maes S, Parizot B, Naramoto S, Magyar Z, Alvim Kamei CL, Koncz C, Bögre L, et al. 2011. Auxin-dependent cell cycle reactivation through transcriptional regulation of Arabidopsis E2Fa by lateral organ boundary proteins. *The Plant Cell* **23**: 3671–3683.

Bhattacharya A. 2019. *Effect of high temperature on crop productivity and metabolism of macro molecules*. London, United Kingdom ; San Diego, CA: Elsevier/Academic Press.

Bhosale R, Giri J, Pandey BK, Giehl RFH, Hartmann A, Traini R, Truskina J, Leftley N, Hanlon M, Swarup K, et al. 2018. A mechanistic framework for auxin dependent Arabidopsis root hair elongation to low external phosphate. *Nature Communications* **9**: 1409.

Biniaz Y, Tahmasebi A, Tahmasebi A, Albrechtsen BR, Pocza P, Afsharifar A. 2022. Transcriptome Meta-Analysis Identifies Candidate Hub Genes and Pathways of Pathogen Stress Responses in Arabidopsis thaliana. *Biology* **11**: 1155.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)* **30**: 2114–2120.

Bonito G, Hameed K, Toome-Heller M, Healy R, Reid C, Liao H-L, Aime MC, Schadt C, Vilgalys R. 2017. Atractiella rhizophila, sp. nov., an endorhizal fungus isolated from the Populus root microbiome. *Mycologia* **109**: 18–26.

Bonomi HR, Posadas DM, Paris G, Carrica M del C, Frederickson M, Pietrasanta LI, Bogomolni RA, Zorreguieta A, Goldbaum FA. 2012. Light regulates attachment, exopolysaccharide production, and nodulation in *Rhizobium leguminosarum* through a LOV-histidine kinase photoreceptor. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 12135–12140.

Boonyaves K, Wu T-Y, Dong Y, Urano D. 2022. Interplay between ARABIDOPSIS G β and WRKY transcription factors differentiates environmental stress responses. *Plant Physiology* **190**: 813–827.

Borrelli P, Robinson DA, Panagos P, Lugato E, Yang JE, Alewell C, Wuepper D, Montanarella L, Ballabio C. 2020. Land use and climate change impacts on global soil erosion by water (2015-2070). *Proceedings of the National Academy of Sciences of the United States of America* **117**: 21994–22001.

Brunetti C, Lamb J, Wielandt S, Uhlemann S, Shirley I, McClure P, Dafflon B. 2021. Estimation of depth-resolved profiles of soil thermal diffusivity from temperature time series and uncertainty quantification. *Physical: Geophysics*.

Burko Y, Gaillochet C, Seluzicki A, Chory J, Busch W. 2020. Local HY5 Activity Mediates Hypocotyl Growth and Shoot-to-Root Communication. *Plant Communications* **1**: 100078.

Bustillo-Avendaño E, Serrano-Ron L, Moreno-Risueno MA. 2022. The Root Clock as a Signal Integrator System: Ensuring Balance for Survival. *Frontiers in Plant Science* **13**: 886700.

Bustos R, Castrillo G, Linhares F, Puga MI, Rubio V, Pérez-Pérez J, Solano R, Leyva A, Paz-Ares J. 2010. A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in *Arabidopsis*. *PLoS genetics* **6**: e1001102.

Cabrera J, Conesa CM, Del Pozo JC. 2022. May the dark be with roots: a perspective on how root illumination may bias in vitro research on plant-environment interactions. *The New Phytologist* **233**: 1988–1997.

- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016.** DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**: 581–583.
- Calleja-Cabrera J, Boter M, Oñate-Sánchez L, Pernas M. 2020.** Root Growth Adaptation to Climate Change in Crops. *Frontiers in Plant Science* **11**: 544.
- Cao H, Glazebrook J, Clarke JD, Volko S, Dong X. 1997.** The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**: 57–63.
- Cao A, Jain A, Baldwin JC, Raghothama KG. 2007.** Phosphate differentially regulates 14-3-3 family members and GRF9 plays a role in Pi-starvation induced responses. *Planta* **226**: 1219–1230.
- Castrillo G, Teixeira PJPL, Paredes SH, Law TF, de Lorenzo L, Feltcher ME, Finkel OM, Breakfield NW, Mieczkowski P, Jones CD, *et al.* 2017.** Root microbiota drive direct integration of phosphate stress and immunity. *Nature* **543**: 513–518.
- Catarecha P, Segura MD, Franco-Zorrilla JM, García-Ponce B, Lanza M, Solano R, Paz-Ares J, Leyva A. 2007.** A mutant of the Arabidopsis phosphate transporter PHT1;1 displays enhanced arsenic accumulation. *The Plant Cell* **19**: 1123–1133.
- Chandrika NNP, Sundaravelpandian K, Yu S-M, Schmidt W. 2013.** ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis. *The New Phytologist* **198**: 709–720.
- Chang L, Ramireddy E, Schmülling T. 2015.** Cytokinin as a positional cue regulating lateral root spacing in Arabidopsis. *Journal of Experimental Botany* **66**: 4759–4768.
- Chaparro JM, Sheflin AM, Manter DK, Vivanco JM. 2012.** Manipulating the soil microbiome to increase soil health and plant fertility. *Biology and Fertility of Soils* **48**: 489–499.

- Charng Y-Y, Liu H-C, Liu N-Y, Chi W-T, Wang C-N, Chang S-H, Wang T-T. 2007.** A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in Arabidopsis. *Plant Physiology* **143**: 251–262.
- Chen S, Li H. 2016.** Heat Stress Regulates the Expression of Genes at Transcriptional and Post-Transcriptional Levels, Revealed by RNA-seq in Brachypodium distachyon. *Frontiers in Plant Science* **7**: 2067.
- Chen X, Yao Q, Gao X, Jiang C, Harberd NP, Fu X. 2016.** Shoot-to-Root Mobile Transcription Factor HY5 Coordinates Plant Carbon and Nitrogen Acquisition. *Current biology: CB* **26**: 640–646.
- Chevalier F, Pata M, Nacry P, Doumas P, Rossignol M. 2003.** Effects of phosphate availability on the root system architecture: large-scale analysis of the natural variation between Arabidopsis accessions: Phosphate and root growth in Arabidopsis accessions. *Plant, Cell & Environment* **26**: 1839–1850.
- Chien P-S, Chao Y-T, Chou C-H, Hsu Y-Y, Chiang S-F, Tung C-W, Chiou T-J. 2022.** Phosphate transporter PHT1;1 is a key determinant of phosphorus acquisition in Arabidopsis natural accessions. *Plant Physiology* **190**: 682–697.
- Choi J, Choi D, Lee S, Ryu C-M, Hwang I. 2011.** Cytokinins and plant immunity: old foes or new friends? *Trends in Plant Science* **16**: 388–394.
- Compant S, van der Heijden MGA, Sessitsch A. 2010.** Climate change effects on beneficial plant-microorganism interactions. *FEMS microbiology ecology* **73**: 197–214.
- Conesa CM, Saez A, Navarro-Neila S, de Lorenzo L, Hunt AG, Sepúlveda EB, Baigorri R, Garcia-Mina JM, Zamarreño AM, Sacristán S, et al. 2020.** Alternative Polyadenylation and Salicylic Acid Modulate Root Responses to Low Nitrogen Availability. *Plants (Basel, Switzerland)* **9**: E251.
- Contreras-Cornejo HA, Macías-Rodríguez L, Cortés-Penagos C, López-Bucio J. 2009.** Trichoderma virens, a plant beneficial fungus, enhances biomass production and

promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiology* **149**: 1579–1592.

Cordell D, White S. 2013. Sustainable Phosphorus Measures: Strategies and Technologies for Achieving Phosphorus Security. *Agronomy* **3**: 86–116.

Couzigou J-M, Combier J-P. 2016. Plant microRNAs: key regulators of root architecture and biotic interactions. *The New Phytologist* **212**: 22–35.

Cridland C, Gillaspay G. 2020. Inositol Pyrophosphate Pathways and Mechanisms: What Can We Learn from Plants? *Molecules (Basel, Switzerland)* **25**: E2789.

Crombez H, Motte H, Beeckman T. 2019. Tackling Plant Phosphate Starvation by the Roots. *Developmental Cell* **48**: 599–615.

Cruz-Ramírez A, Díaz-Triviño S, Wachsman G, Du Y, Arteága-Vázquez M, Zhang H, Benjamins R, Blilou I, Neef AB, Chandler V, et al. 2013. A SCARECROW-RETINOBLASTOMA protein network controls protective quiescence in the Arabidopsis root stem cell organizer. *PLoS biology* **11**: e1001724.

D E Lima CFF, Kleine-Vehn J, De Smet I, Feraru E. 2021. Getting to the Root of Belowground High Temperature Responses in Plants. *Journal of Experimental Botany*: erab202.

Das D, Paries M, Hobecker K, Gigl M, Dawid C, Lam H-M, Zhang J, Chen M, Gutjahr C. 2022. PHOSPHATE STARVATION RESPONSE transcription factors enable arbuscular mycorrhiza symbiosis. *Nature Communications* **13**: 477.

Deja-Muyllé A, Opdenacker D, Parizot B, Motte H, Lobet G, Storme V, Clauw P, Njo M, Beeckman T. 2021. Genetic Variability of Arabidopsis thaliana Mature Root System Architecture and Genome-Wide Association Study. *Frontiers in Plant Science* **12**: 814110.

Delgado D, Sánchez-Bermejo E, de Marcos A, Martín-Jimenez C, Fenoll C, Alonso-Blanco C, Mena M. 2019. A Genetic Dissection of Natural Variation for Stomatal Abundance Traits in Arabidopsis. *Frontiers in Plant Science* **10**: 1392.

Delker C, Sonntag L, James GV, Janitza P, Ibañez C, Ziermann H, Peterson T, Denk K, Mull S, Ziegler J, et al. 2014. The DET1-COP1-HY5 pathway constitutes a multipurpose signaling module regulating plant photomorphogenesis and thermomorphogenesis. *Cell Reports* **9**: 1983–1989.

Dello Ioio R, Linhares FS, Scacchi E, Casamitjana-Martinez E, Heidstra R, Costantino P, Sabatini S. 2007. Cytokinins determine Arabidopsis root-meristem size by controlling cell differentiation. *Current biology: CB* **17**: 678–682.

Díaz-González S, Marín P, Sánchez R, Arribas C, Kruse J, González-Melendi P, Brunner F, Sacristán S. 2020. Mutualistic Fungal Endophyte *Colletotrichum tofieldiae* Ct0861 Colonizes and Increases Growth and Yield of Maize and Tomato Plants. *Agronomy* **10**: 1493.

Ding Y, Shi Y, Yang S. 2020. Molecular Regulation of Plant Responses to Environmental Temperatures. *Molecular Plant* **13**: 544–564.

Dinkins R, Pflipsen C, Thompson A, Collins GB. 2002. Ectopic expression of an Arabidopsis single zinc finger gene in tobacco results in dwarf plants. *Plant & Cell Physiology* **43**: 743–750.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics (Oxford, England)* **29**: 15–21.

Donahue JL, Alford SR, Torabinejad J, Kerwin RE, Nourbakhsh A, Ray WK, Hernick M, Huang X, Lyons BM, Hein PP, et al. 2010. The Arabidopsis thaliana Myo-inositol 1-phosphate synthase1 gene is required for Myo-inositol synthesis and suppression of cell death. *The Plant Cell* **22**: 888–903.

Dong J, Ma G, Sui L, Wei M, Satheesh V, Zhang R, Ge S, Li J, Zhang T-E, Wittwer C, et al. 2019. Inositol Pyrophosphate InsP8 Acts as an Intracellular Phosphate Signal in Arabidopsis. *Molecular Plant* **12**: 1463–1473.

Duan X, Wang X, Jin K, Wang W, Liu H, Liu L, Zhang Y, Hammond JP, White PJ, Ding G, et al. 2021. Genetic Dissection of Root Angle of *Brassica napus* in Response to Low Phosphorus. *Frontiers in Plant Science* **12**: 697872.

Ebi KL, Vanos J, Baldwin JW, Bell JE, Hondula DM, Errett NA, Hayes K, Reid CE, Saha S, Spector J, et al. 2021. Extreme Weather and Climate Change: Population Health and Health System Implications. *Annual Review of Public Health* **42**: 293–315.

El Yacoubi B, Bailly M, de Crécy-Lagard V. 2012. Biosynthesis and function of posttranscriptional modifications of transfer RNAs. *Annual Review of Genetics* **46**: 69–95.

Elias EA, Cichota R, Torriani HH, de Jong van Lier Q. 2004. ANALYTICAL SOIL-TEMPERATURE MODEL: CORRECTION FOR TEMPORAL VARIATION OF DAILY AMPLITUDE. *Soil Science Society of America Journal* **68**: 784–788.

Elser JJ. 2012. Phosphorus: a limiting nutrient for humanity? *Current Opinion in Biotechnology* **23**: 833–838.

El-Soda M, Neris Moreira C, Goredema-Matongera N, Jamar D, Koornneef M, Aarts MGM. 2019. QTL and candidate genes associated with leaf anion concentrations in response to phosphate supply in *Arabidopsis thaliana*. *BMC plant biology* **19**: 410.

Estravis-Barcala M, Heer K, Marchelli P, Ziegenhagen B, Arana MV, Bellora N. 2021. Deciphering the transcriptomic regulation of heat stress responses in *Nothofagus pumilio*. *PloS One* **16**: e0246615.

Fahad S, Bajwa AA, Nazir U, Anjum SA, Farooq A, Zohaib A, Sadia S, Nasim W, Adkins S, Saud S, et al. 2017. Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Frontiers in Plant Science* **8**: 1147.

Fei Q, Zhang J, Zhang Z, Wang Y, Liang L, Wu L, Gao H, Sun Y, Niu B, Li X. 2019. Effects of auxin and ethylene on root growth adaptation to different ambient temperatures in *Arabidopsis*. *Plant Science: An International Journal of Experimental Plant Biology* **281**: 159–172.

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legué V. 2009. The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and *Arabidopsis* through auxin transport and signaling. *Plant Physiology* **151**: 1991–2005.

Ferrenberg S, O'Neill SP, Knelman JE, Todd B, Duggan S, Bradley D, Robinson T, Schmidt SK, Townsend AR, Williams MW, et al. 2013. Changes in assembly processes in soil bacterial communities following a wildfire disturbance. *The ISME journal* **7**: 1102–1111.

Filiault DL, Maloof JN. 2012. A genome-wide association study identifies variants underlying the *Arabidopsis thaliana* shade avoidance response. *PLoS genetics* **8**: e1002589.

Finkel OM, Salas-González I, Castrillo G, Conway JM, Law TF, Teixeira PJPL, Wilson ED, Fitzpatrick CR, Jones CD, Dangl JL. 2020. A single bacterial genus maintains root growth in a complex microbiome. *Nature* **587**: 103–108.

Finkel OM, Salas-González I, Castrillo G, Spaepen S, Law TF, Teixeira PJPL, Jones CD, Dangl JL. 2019. The effects of soil phosphorus content on plant microbiota are driven by the plant phosphate starvation response. *PLoS biology* **17**: e3000534.

Fleet CM, Yen JY, Hill EA, Gillaspie GE. 2018. Co-suppression of AtMIPS demonstrates cooperation of MIPS1, MIPS2 and MIPS3 in maintaining myo-inositol synthesis. *Plant Molecular Biology* **97**: 253–263.

Franco-Zorrilla JM, Martín AC, Leyva A, Paz-Ares J. 2005. Interaction between phosphate-starvation, sugar, and cytokinin signaling in *Arabidopsis* and the roles of cytokinin receptors CRE1/AHK4 and AHK3. *Plant Physiology* **138**: 847–857.

Franco-Zorrilla JM, Martín AC, Solano R, Rubio V, Leyva A, Paz-Ares J. 2002. Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in *Arabidopsis*. *The Plant Journal: For Cell and Molecular Biology* **32**: 353–360.

Frerigmann H, Piotrowski M, Lemke R, Bednarek P, Schulze-Lefert P. 2021. A Network of Phosphate Starvation and Immune-Related Signaling and Metabolic Pathways Controls the Interaction between *Arabidopsis thaliana* and the Beneficial Fungus *Colletotrichum tofieldiae*. *Molecular plant-microbe interactions: MPMI* **34**: 560–570.

Friedrich T, Oberkofler V, Trindade I, Altmann S, Brzezinka K, Lämke J, Gorka M, Kappel C, Sokolowska E, Skirycz A, et al. 2021. Heteromeric HSFA2/HSFA3 complexes drive transcriptional memory after heat stress in *Arabidopsis*. *Nature Communications* **12**: 3426.

de la Fuente Cantó C, Simonin M, King E, Moulin L, Bennett MJ, Castrillo G, Laplaze L. 2020. An extended root phenotype: the rhizosphere, its formation and impacts on plant fitness. *The Plant Journal: For Cell and Molecular Biology* **103**: 951–964.

Gaillochet C, Burko Y, Platre MP, Zhang L, Simura J, Willige BC, Kumar SV, Ljung K, Chory J, Busch W. 2020. HY5 and phytochrome activity modulate shoot-to-root coordination during thermomorphogenesis in *Arabidopsis*. *Development (Cambridge, England)* **147**: dev192625.

Gamuyao R, Chin JH, Pariasca-Tanaka J, Pesaresi P, Catausan S, Dalid C, Slamet-Loedin I, Tecson-Mendoza EM, Wissuwa M, Heuer S. 2012. The protein kinase Pstoll from traditional rice confers tolerance of phosphorus deficiency. *Nature* **488**: 535–539.

García E, Alonso Á, Platas G, Sacristán S. 2013. The endophytic mycobiota of *Arabidopsis thaliana*. *Fungal Diversity* **60**: 71–89.

Garnica-Vergara A, Barrera-Ortiz S, Muñoz-Parra E, Raya-González J, Méndez-Bravo A, Macías-Rodríguez L, Ruiz-Herrera LF, López-Bucio J. 2016. The volatile 6-pentyl-2H-pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and ETHYLENE INSENSITIVE 2 functioning. *The New Phytologist* **209**: 1496–1512.

- van Gelderen K, Kang C, Paalman R, Keuskamp D, Hayes S, Pierik R. 2018a.** Far-Red Light Detection in the Shoot Regulates Lateral Root Development through the HY5 Transcription Factor. *The Plant Cell* **30**: 101–116.
- van Gelderen K, Kang C, Pierik R. 2018b.** Light Signaling, Root Development, and Plasticity. *Plant Physiology* **176**: 1049–1060.
- Giehl RFH, von Wirén N. 2014.** Root nutrient foraging. *Plant Physiology* **166**: 509–517.
- Giovannetti M, Göschl C, Dietzen C, Andersen SU, Kopriva S, Busch W. 2019.** Identification of novel genes involved in phosphate accumulation in *Lotus japonicus* through Genome Wide Association mapping of root system architecture and anion content. *PLoS genetics* **15**: e1008126.
- Giri A, Heckathorn S, Mishra S, Krause C. 2017.** Heat Stress Decreases Levels of Nutrient-Uptake and -Assimilation Proteins in Tomato Roots. *Plants (Basel, Switzerland)* **6**: E6.
- Gomaa NH, Montesinos-Navarro A, Alonso-Blanco C, Picó FX. 2011.** Temporal variation in genetic diversity and effective population size of Mediterranean and subalpine *Arabidopsis thaliana* populations. *Molecular Ecology* **20**: 3540–3554.
- Gordon A, Hannon G. 2017.** Fastx-toolkit. FASTQ/A short-reads pre-processing tools. 2010. Unpublished available online at: http://hannonlab.cshl.edu/fastx_toolkit.
- Gray SB, Brady SM. 2016.** Plant developmental responses to climate change. *Developmental Biology* **419**: 64–77.
- Gray WM, Ostin A, Sandberg G, Romano CP, Estelle M. 1998.** High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **95**: 7197–7202.
- Großkinsky D, Edelsbrunner K, Pfeifhofer H, Van der Graaff E, Roitsch T. 2013.** Cis- and trans-zeatin differentially modulate plant immunity. *Plant Signaling & Behavior* **8**: e24798.

Gruber BD, Giehl RFH, Friedel S, von Wirén N. 2013. Plasticity of the Arabidopsis root system under nutrient deficiencies. *Plant Physiology* **163**: 161–179.

Gu R, Chen F, Long L, Cai H, Liu Z, Yang J, Wang L, Li H, Li J, Liu W, et al. 2016a. Enhancing phosphorus uptake efficiency through QTL-based selection for root system architecture in maize. *Journal of Genetics and Genomics = Yi Chuan Xue Bao* **43**: 663–672.

Gu Z, Eils R, Schlesner M. 2016b. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics (Oxford, England)* **32**: 2847–2849.

Haider S, Iqbal J, Naseer S, Shaukat M, Abbasi BA, Yaseen T, Zahra SA, Mahmood T. 2022. Unfolding molecular switches in plant heat stress resistance: A comprehensive review. *Plant Cell Reports* **41**: 775–798.

Hanlon MT, Ray S, Saengwilai P, Luthe D, Lynch JP, Brown KM. 2018. Buffered delivery of phosphate to Arabidopsis alters responses to low phosphate. *Journal of Experimental Botany* **69**: 1207–1219.

Hariprasad P, Gowtham HG, Gourav C. 2021. Beneficial plant-associated bacteria modulate host hormonal system enhancing plant resistance toward abiotic stress. In: *Biocontrol Agents and Secondary Metabolites*. Elsevier, 113–151.

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M. 2013. Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *International Journal of Molecular Sciences* **14**: 9643–9684.

Hassan MU, Chattha MU, Khan I, Chattha MB, Barbanti L, Aamer M, Iqbal MM, Nawaz M, Mahmood A, Ali A, et al. 2021. Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* **155**: 211–234.

Hassani MA, Özkurt E, Seybold H, Dagan T, Stukenbrock EH. 2019. Interactions and Coadaptation in Plant Metaorganisms. *Annual Review of Phytopathology* **57**: 483–503.

Hatfield JL, Prueger JH. 2015. Temperature extremes: Effect on plant growth and development. *Weather and Climate Extremes* **10**: 4–10.

Havlová M, Dobrev PI, Motyka V, Storchová H, Libus J, Dobrá J, Malbeck J, Gaudinová A, Vanková R. 2008. The role of cytokinins in responses to water deficit in tobacco plants over-expressing trans-zeatin O-glucosyltransferase gene under 35S or SAG12 promoters. *Plant, Cell & Environment* **31**: 341–353.

Heppell J, Talboys P, Payvandi S, Zygalakis KC, Fliege J, Withers PJA, Jones DL, Roose T. 2015. How changing root system architecture can help tackle a reduction in soil phosphate (P) levels for better plant P acquisition. *Plant, Cell & Environment* **38**: 118–128.

Heyl A, Riefler M, Romanov GA, Schmülling T. 2012. Properties, functions and evolution of cytokinin receptors. *European Journal of Cell Biology* **91**: 246–256.

Hiruma K. 2019. Roles of Plant-Derived Secondary Metabolites during Interactions with Pathogenic and Beneficial Microbes under Conditions of Environmental Stress. *Microorganisms* **7**: E362.

Hiruma K, Gerlach N, Sacristán S, Nakano RT, Hacquard S, Kracher B, Neumann U, Ramírez D, Bucher M, O'Connell RJ, *et al.* 2016. Root Endophyte Colletotrichum tofieldiae Confers Plant Fitness Benefits that Are Phosphate Status Dependent. *Cell* **165**: 464–474.

Hiruma K, Kobae Y, Toju H. 2018. Beneficial associations between Brassicaceae plants and fungal endophytes under nutrient-limiting conditions: evolutionary origins and host-symbiont molecular mechanisms. *Current Opinion in Plant Biology* **44**: 145–154.

Hodge A. 2009. Root decisions. *Plant, Cell & Environment* **32**: 628–640.

Howe EA, Sinha R, Schlauch D, Quackenbush J. 2011. RNA-Seq analysis in MeV. *Bioinformatics (Oxford, England)* **27**: 3209–3210.

Hsieh L-C, Lin S-I, Kuo H-F, Chiou T-J. 2010. Abundance of tRNA-derived small RNAs in phosphate-starved Arabidopsis roots. *Plant Signaling & Behavior* **5**: 537–539.

Huang B, Rachmilevitch S, Xu J. 2012. Root carbon and protein metabolism associated with heat tolerance. *Journal of Experimental Botany* **63**: 3455–3465.

Huot B, Castroverde CDM, Velásquez AC, Hubbard E, Pulman JA, Yao J, Childs KL, Tsuda K, Montgomery BL, He SY. 2017. Dual impact of elevated temperature on plant defence and bacterial virulence in *Arabidopsis*. *Nature Communications* **8**: 1808.

Idnurm A, Heitman J. 2005. Light controls growth and development via a conserved pathway in the fungal kingdom. *PLoS biology* **3**: e95.

Ihsan MZ, Daur I, Alghabari F, Alzamanan S, Rizwan S, Ahmad M, Waqas M, Shafqat W. 2019. Heat stress and plant development: role of sulphur metabolites and management strategies. *Acta Agriculturae Scandinavica, Section B — Soil & Plant Science* **69**: 332–342.

Isidra-Arellano MC, Delaux P-M, Valdés-López O. 2021. The Phosphate Starvation Response System: Its Role in the Regulation of Plant–Microbe Interactions. *Plant and Cell Physiology* **62**: 392–400.

Jabeen N. 2020. Agricultural, Economic and Societal Importance of Brassicaceae Plants. In: Hasanuzzaman M, ed. *The Plant Family Brassicaceae*. Singapore: Springer Singapore, 45–128.

Jagadish SVK, Way DA, Sharkey TD. 2021. Plant heat stress: Concepts directing future research. *Plant, Cell & Environment* **44**: 1992–2005.

Jiang C, Gao X, Liao L, Harberd NP, Fu X. 2007. Phosphate starvation root architecture and anthocyanin accumulation responses are modulated by the gibberellin-DELLA signaling pathway in *Arabidopsis*. *Plant Physiology* **145**: 1460–1470.

Jiang C-J, Shimono M, Sugano S, Kojima M, Liu X, Inoue H, Sakakibara H, Takatsuji H. 2013. Cytokinins act synergistically with salicylic acid to activate defense gene expression in rice. *Molecular plant-microbe interactions: MPMI* **26**: 287–296.

Jiménez-Vázquez KR, García-Cárdenas E, Barrera-Ortiz S, Ortiz-Castro R, Ruiz-Herrera LF, Ramos-Acosta BP, Coria-Arellano JL, Sáenz-Mata J, López-Bucio J.

2020. The plant beneficial rhizobacterium *Achromobacter* sp. 5B1 influences root development through auxin signaling and redistribution. *The Plant Journal: For Cell and Molecular Biology* **103**: 1639–1654.

Jolly WM, Cochrane MA, Freeborn PH, Holden ZA, Brown TJ, Williamson GJ, Bowman DMJS. 2015. Climate-induced variations in global wildfire danger from 1979 to 2013. *Nature Communications* **6**: 7537.

Kanaan H, Frenk S, Raviv M, Medina S, Minz D. 2018. Long and short term effects on solarization on soil microbiome and agricultural production. *Applied Soil Ecology* **124**: 54–61.

Kang J, Yu H, Tian C, Zhou W, Li C, Jiao Y, Liu D. 2014. Suppression of Photosynthetic Gene Expression in Roots Is Required for Sustained Root Growth under Phosphate Deficiency. *Plant Physiology* **165**: 1156–1170.

Kanno S, Arrighi J-F, Chiarenza S, Bayle V, Berthomé R, Péret B, Javot H, Delannoy E, Marin E, Nakanishi TM, *et al.* 2016. A novel role for the root cap in phosphate uptake and homeostasis. *eLife* **5**: e14577.

Karas B, Amyot L, Johansen C, Sato S, Tabata S, Kawaguchi M, Szczylowski K. 2009. Conservation of lotus and Arabidopsis basic helix-loop-helix proteins reveals new players in root hair development. *Plant Physiology* **151**: 1175–1185.

Kariman K, Barker SJ, Tibbett M. 2018. Structural plasticity in root-fungal symbioses: diverse interactions lead to improved plant fitness. *PeerJ* **6**: e6030.

Karlova R, Boer D, Hayes S, Testerink C. 2021. Root plasticity under abiotic stress. *Plant Physiology* **187**: 1057–1070.

Khan MSS, Islam F, Chen H, Chang M, Wang D, Liu F, Fu ZQ, Chen J. 2022. Transcriptional Coactivators: Driving Force of Plant Immunity. *Frontiers in Plant Science* **13**: 823937.

Kim JH, Castroverde CDM, Huang S, Li C, Hilleary R, Seroka A, Sohrabi R, Medina-Yerena D, Huot B, Wang J, et al. 2022. Increasing the resilience of plant immunity to a warming climate. *Nature* **607**: 339–344.

Kiss JZ, Mullen JL, Correll MJ, Hangarter RP. 2003. Phytochromes A and B mediate red-light-induced positive phototropism in roots. *Plant Physiology* **131**: 1411–1417.

Koch MA. 2019. The plant model system *Arabidopsis* set in an evolutionary, systematic, and spatio-temporal context. *Journal of Experimental Botany* **70**: 55–67.

Kochian LV. 2016. Root architecture. *Journal of Integrative Plant Biology* **58**: 190–192.

Kohli PS, Kumar Verma P, Verma R, Parida SK, Thakur JK, Giri J. 2020. Genome-wide association study for phosphate deficiency responsive root hair elongation in chickpea. *Functional and Integrative Genomics*.

Köllmer I, Novák O, Strnad M, Schmülling T, Werner T. 2014. Overexpression of the cytosolic cytokinin oxidase/dehydrogenase (CKX7) from *Arabidopsis* causes specific changes in root growth and xylem differentiation. *The Plant Journal: For Cell and Molecular Biology* **78**: 359–371.

Korte A, Farlow A. 2013. The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods* **9**: 29.

Krishnamoorthy P, Sanchez-Rodriguez C, Heilmann I, Persson S. 2014. Regulatory roles of phosphoinositides in membrane trafficking and their potential impact on cell-wall synthesis and re-modelling. *Annals of Botany* **114**: 1049–1057.

Kuiper D, Steingröver E. 1991. Responses of Growth, Shoot to Root Ratio and Cytokinin Concentrations in Root Tissue of Two Barley Varieties, Differing in Their Salt Resistance. In: *Developments in Agricultural and Managed Forest Ecology*. Elsevier, 463–471.

Kuo H-F, Hsu Y-Y, Lin W-C, Chen K-Y, Munnik T, Brearley CA, Chiou T-J. 2018. *Arabidopsis* inositol phosphate kinases IPK1 and ITPK1 constitute a metabolic pathway

in maintaining phosphate homeostasis. *The Plant Journal: For Cell and Molecular Biology*.

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyojuka J. 2007. Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* **445**: 652–655.

Kuroda R, Kato M, Tsuge T, Aoyama T. 2021. Arabidopsis phosphatidylinositol 4-phosphate 5-kinase genes PIP5K7, PIP5K8, and PIP5K9 are redundantly involved in root growth adaptation to osmotic stress. *The Plant Journal: For Cell and Molecular Biology* **106**: 913–927.

Kuroha T, Tokunaga H, Kojima M, Ueda N, Ishida T, Nagawa S, Fukuda H, Sugimoto K, Sakakibara H. 2009. Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in Arabidopsis. *The Plant Cell* **21**: 3152–3169.

Lahti M, Aphalo PJ, Finér L, Ryyppö A, Lehto T, Mannerkoski H. 2005. Effects of soil temperature on shoot and root growth and nutrient uptake of 5-year-old Norway spruce seedlings. *Tree Physiology* **25**: 115–122.

Lanza M, Haro R, Conchillo LB, Benito B. 2019. The endophyte *Serendipita indica* reduces the sodium content of Arabidopsis plants exposed to salt stress: fungal ENA ATPases are expressed and regulated at high pH and during plant co-cultivation in salinity. *Environmental Microbiology*.

Laplaze L, Benkova E, Casimiro I, Maes L, Vanneste S, Swarup R, Weijers D, Calvo V, Parizot B, Herrera-Rodriguez MB, et al. 2007. Cytokinins act directly on lateral root founder cells to inhibit root initiation. *The Plant Cell* **19**: 3889–3900.

Larkindale J, Hall JD, Knight MR, Vierling E. 2005. Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. *Plant Physiology* **138**: 882–897.

Larkindale J, Vierling E. 2008. Core genome responses involved in acclimation to high temperature. *Plant Physiology* **146**: 748–761.

Lavy M, Estelle M. 2016. Mechanisms of auxin signaling. *Development (Cambridge, England)* **143**: 3226–3229.

Lee H-J, Park Y-J, Ha J-H, Baldwin IT, Park C-M. 2017. Multiple Routes of Light Signaling during Root Photomorphogenesis. *Trends in Plant Science* **22**: 803–812.

Lee S, Wang W, Huq E. 2021. Spatial regulation of thermomorphogenesis by HY5 and PIF4 in Arabidopsis. *Nature Communications* **12**: 3656.

Legris M, Klose C, Burgie ES, Rojas CCR, Neme M, Hiltbrunner A, Wigge PA, Schäfer E, Vierstra RD, Casal JJ. 2016. Phytochrome B integrates light and temperature signals in Arabidopsis. *Science (New York, N.Y.)* **354**: 897–900.

Leng L, Liang Q, Jiang J, Zhang C, Hao Y, Wang X, Su W. 2017. A subclass of HSP70s regulate development and abiotic stress responses in Arabidopsis thaliana. *Journal of Plant Research* **130**: 349–363.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC bioinformatics* **12**: 323.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics (Oxford, England)* **25**: 1754–1760.

Li W, Lan P. 2015. Genome-wide analysis of overlapping genes regulated by iron deficiency and phosphate starvation reveals new interactions in Arabidopsis roots. *BMC research notes* **8**: 555.

Li X, Mo X, Shou H, Wu P. 2006. Cytokinin-mediated cell cycling arrest of pericycle founder cells in lateral root initiation of Arabidopsis. *Plant & Cell Physiology* **47**: 1112–1123.

Li Y, Shao J, Fu Y, Chen Y, Wang H, Xu Z, Feng H, Xun W, Liu Y, Zhang N, et al. 2022. The volatile cedrene from *Trichoderma guizhouense* modulates Arabidopsis root

development through auxin transport and signalling. *Plant, Cell & Environment* **45**: 969–984.

Lin Y-T, Jia Z, Wang D, Chiu C-Y. 2017. Effects of temperature on the composition and diversity of bacterial communities in bamboo soils at different elevations. *Biogeosciences* **14**: 4879–4889.

Lin W-D, Liao Y-Y, Yang TJW, Pan C-Y, Buckhout TJ, Schmidt W. 2011. Coexpression-based clustering of Arabidopsis root genes predicts functional modules in early phosphate deficiency signaling. *Plant Physiology* **155**: 1383–1402.

Liu D. 2021. Root developmental responses to phosphorus nutrition. *Journal of Integrative Plant Biology* **63**: 1065–1090.

Liu H, Brettell LE, Qiu Z, Singh BK. 2020. Microbiome-Mediated Stress Resistance in Plants. *Trends in Plant Science* **25**: 733–743.

Liu Y, Donner E, Lombi E, Li R, Wu Z, Zhao F-J, Wu P. 2013. Assessing the contributions of lateral roots to element uptake in rice using an auxin-related lateral root mutant. *Plant and Soil* **372**: 125–136.

Liu H-C, Liao H-T, Charng Y-Y. 2011. The role of class A1 heat shock factors (HSFAs) in response to heat and other stresses in Arabidopsis. *Plant, Cell & Environment* **34**: 738–751.

Liu J, Liu Y, Wang S, Cui Y, Yan D. 2022. Heat Stress Reduces Root Meristem Size via Induction of Plasmodesmal Callose Accumulation Inhibiting Phloem Unloading in Arabidopsis. *International Journal of Molecular Sciences* **23**: 2063.

Liu Y-X, Qin Y, Bai Y. 2019a. Reductionist synthetic community approaches in root microbiome research. *Current Opinion in Microbiology* **49**: 97–102.

Liu H, Senthilkumar R, Ma G, Zou Q, Zhu K, Shen X, Tian D, Hua MS, Oelmüller R, Yeh KW. 2019b. Piriformospora indica-induced phytohormone changes and root colonization strategies are highly host-specific. *Plant Signaling & Behavior* **14**: 1632688.

- Lomin SN, Krivosheev DM, Steklov MY, Osolodkin DI, Romanov GA. 2012.** Receptor properties and features of cytokinin signaling. *Acta Naturae* **4**: 31–45.
- López-Arredondo DL, Leyva-González MA, González-Morales SI, López-Bucio J, Herrera-Estrella L. 2014.** Phosphate nutrition: improving low-phosphate tolerance in crops. *Annual Review of Plant Biology* **65**: 95–123.
- López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L. 2003.** The role of nutrient availability in regulating root architecture. *Current Opinion in Plant Biology* **6**: 280–287.
- Lorenzo-Orts L, Couto D, Hothorn M. 2020.** Identity and functions of inorganic and inositol polyphosphates in plants. *The New Phytologist* **225**: 637–652.
- Losi A, Gärtner W. 2021.** A light life together: photosensing in the plant microbiota. *Photochemical & Photobiological Sciences: Official Journal of the European Photochemistry Association and the European Society for Photobiology* **20**: 451–473.
- Love MI, Huber W, Anders S. 2014.** Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 550.
- Luo Y, Loomis RS, Hsiao TC. 1992.** Simulation of soil temperature in crops. *Agricultural and Forest Meteorology* **61**: 23–38.
- Luo B, Ma P, Nie Z, Zhang X, He X, Ding X, Feng X, Lu Q, Ren Z, Lin H, et al. 2019.** Metabolite profiling and genome-wide association studies reveal response mechanisms of phosphorus deficiency in maize seedling. *The Plant Journal: For Cell and Molecular Biology* **97**: 947–969.
- Lynch J. 1995.** Root Architecture and Plant Productivity. *Plant Physiology* **109**: 7–13.
- Lynch JP. 2019.** Root phenotypes for improved nutrient capture: an underexploited opportunity for global agriculture. *The New Phytologist* **223**: 548–564.
- Lynch JP. 2022.** Harnessing root architecture to address global challenges. *The Plant Journal: For Cell and Molecular Biology* **109**: 415–431.

Lynch JP, Brown KM. 2001. Topsoil foraging – an architectural adaptation of plants to low phosphorus availability. *Plant and Soil* **237**: 225–237.

Lynch J, Marschner P, Rengel Z. 2012. Effect of Internal and External Factors on Root Growth and Development. In: Marschner's Mineral Nutrition of Higher Plants. Elsevier, 331–346.

Manzano C, Pallero-Baena M, Silva-Navas J, Navarro Neila S, Casimiro I, Casero P, Garcia-Mina JM, Baigorri R, Rubio L, Fernandez JA, et al. 2017. A light-sensitive mutation in Arabidopsis LEW3 reveals the important role of N-glycosylation in root growth and development. *Journal of Experimental Botany* **68**: 5103–5116.

Manzano C, Ramirez-Parra E, Casimiro I, Otero S, Desvoyes B, De Rybel B, Beeckman T, Casero P, Gutierrez C, C Del Pozo J. 2012. Auxin and epigenetic regulation of SKP2B, an F-box that represses lateral root formation. *Plant Physiology* **160**: 749–762.

Martín AC, del Pozo JC, Iglesias J, Rubio V, Solano R, de La Peña A, Leyva A, Paz-Ares J. 2000. Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. *The Plant Journal: For Cell and Molecular Biology* **24**: 559–567.

Megel C, Morelle G, Lalande S, Duchêne A-M, Small I, Maréchal-Drouard L. 2015. Surveillance and cleavage of eukaryotic tRNAs. *International Journal of Molecular Sciences* **16**: 1873–1893.

Mei Y, Jia W-J, Chu Y-J, Xue H-W. 2012. Arabidopsis phosphatidylinositol monophosphate 5-kinase 2 is involved in root gravitropism through regulation of polar auxin transport by affecting the cycling of PIN proteins. *Cell Research* **22**: 581–597.

Méndez-Vigo B, Picó FX, Ramiro M, Martínez-Zapater JM, Alonso-Blanco C. 2011. Altitudinal and climatic adaptation is mediated by flowering traits and FRI, FLC, and PHYC genes in Arabidopsis. *Plant Physiology* **157**: 1942–1955.

Michaletz ST, Weiser MD, Zhou J, Kaspari M, Helliker BR, Enquist BJ. 2015. Plant Thermoregulation: Energetics, Trait-Environment Interactions, and Carbon Economics. *Trends in Ecology & Evolution* **30**: 714–724.

Miller S, Chua K, Coggins J, Mohtadi H. 2021. Heat Waves, Climate Change, and Economic Output. *Journal of the European Economic Association* **19**: 2658–2694.

Mishra J, Singh R, Arora NK. 2017. Plant Growth-Promoting Microbes: Diverse Roles in Agriculture and Environmental Sustainability. In: Kumar V, Kumar M, Sharma S, Prasad R, eds. Probiotics and Plant Health. Singapore: Springer Singapore, 71–111.

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, *et al.* 2005. A genome-wide transcriptional analysis using Arabidopsis thaliana Affymetrix gene chips determined plant responses to phosphate deprivation. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11934–11939.

Miyawaki K, Tarkowski P, Matsumoto-Kitano M, Kato T, Sato S, Tarkowska D, Tabata S, Sandberg G, Kakimoto T. 2006. Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 16598–16603.

Moni A, Lee A-Y, Briggs WR, Han I-S. 2015. The blue light receptor Phototropin 1 suppresses lateral root growth by controlling cell elongation. *Plant Biology (Stuttgart, Germany)* **17**: 34–40.

Montes N, Cobos A, Gil-Valle M, Caro E, Pagán I. 2021. Arabidopsis thaliana Genes Associated with Cucumber mosaic virus Virulence and Their Link to Virus Seed Transmission. *Microorganisms* **9**: 692.

Mora-Macías J, Ojeda-Rivera JO, Gutiérrez-Alanís D, Yong-Villalobos L, Oropeza-Aburto A, Raya-González J, Jiménez-Domínguez G, Chávez-Calvillo G, Rellán-Álvarez R, Herrera-Estrella L. 2017. Malate-dependent Fe accumulation is a

critical checkpoint in the root developmental response to low phosphate. *Proceedings of the National Academy of Sciences of the United States of America* **114**: E3563–E3572.

Moreno-Risueno MA, Van Norman JM, Moreno A, Zhang J, Ahnert SE, Benfey PN. 2010. Oscillating gene expression determines competence for periodic Arabidopsis root branching. *Science (New York, N.Y.)* **329**: 1306–1311.

Motte H, Vanneste S, Beeckman T. 2019. Molecular and Environmental Regulation of Root Development. *Annual Review of Plant Biology* **70**: 465–488.

Müller J, Toev T, Heisters M, Teller J, Moore KL, Hause G, Dinesh DC, Bürstenbinder K, Abel S. 2015. Iron-dependent callose deposition adjusts root meristem maintenance to phosphate availability. *Developmental Cell* **33**: 216–230.

Nagel KA, Kastenholz B, Jahnke S, van Dusschoten D, Aach T, Mühlich M, Truhn D, Scharf H, Terjung S, Walter A, et al. 2009. Temperature responses of roots: impact on growth, root system architecture and implications for phenotyping. *Functional plant biology: FPB* **36**: 947–959.

Narang RA, Bruene A, Altmann T. 2000. Analysis of phosphate acquisition efficiency in different Arabidopsis accessions. *Plant Physiology* **124**: 1786–1799.

Naseem M, Wölfling M, Dandekar T. 2014. Cytokinins for immunity beyond growth, galls and green islands. *Trends in Plant Science* **19**: 481–484.

Nishimura C, Ohashi Y, Sato S, Kato T, Tabata S, Ueguchi C. 2004. Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. *The Plant Cell* **16**: 1365–1377.

Nottingham AT, Bååth E, Reischke S, Salinas N, Meir P. 2019. Adaptation of soil microbial growth to temperature: Using a tropical elevation gradient to predict future changes. *Global Change Biology* **25**: 827–838.

Nussaume L, Kanno S, Javot H, Marin E, Pochon N, Ayadi A, Nakanishi TM, Thibaud M-C. 2011. Phosphate Import in Plants: Focus on the PHT1 Transporters. *Frontiers in Plant Science* **2**: 83.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2013. Package 'vegan'. *Community ecology package, version 2*: 1–295.

Okushima Y, Fukaki H, Onoda M, Theologis A, Tasaka M. 2007. ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in Arabidopsis. *The Plant Cell* **19**: 118–130.

Onwuka B. 2018. Effects of Soil Temperature on Some Soil Properties and Plant Growth. *Advances in Plants & Agriculture Research* **8**.

Ortiz-Álvarez R, Ortega-Arranz H, Ontiveros VJ, de Celis M, Ravarani C, Acedo A, Belda I. 2021. Network Properties of Local Fungal Communities Reveal the Anthropogenic Disturbance Consequences of Farming Practices in Vineyard Soils. *mSystems* **6**: e00344-21.

Pacak A, Barciszewska-Pacak M, Swida-Barteczka A, Kruszka K, Segal P, Milanowska K, Jakobsen I, Jarmolowski A, Szweykowska-Kulinska Z. 2016. Heat Stress Affects Pi-related Genes Expression and Inorganic Phosphate Deposition/Accumulation in Barley. *Frontiers in Plant Science* **7**: 926.

Pacifici E, Di Mambro R, Dello Ioio R, Costantino P, Sabatini S. 2018. Acidic cell elongation drives cell differentiation in the Arabidopsis root. *The EMBO journal* **37**: e99134.

Pareek N. 2017. Climate Change Impact on Soils: Adaptation and Mitigation. *MOJ Ecology & Environmental Sciences* **2**.

Park EJ, Kim T-H. 2018. Fine-Tuning of Gene Expression by tRNA-Derived Fragments during Abiotic Stress Signal Transduction. *International Journal of Molecular Sciences* **19**: E518.

Parniske M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews. Microbiology* **6**: 763–775.

Paz-Ares J, Puga MI, Rojas-Triana M, Martinez-Hevia I, Diaz S, Poza-Carrión C, Miñambres M, Leyva A. 2022. Plant adaptation to low phosphorus availability: Core signaling, crosstalks, and applied implications. *Molecular Plant* **15**: 104–124.

Pei X, Zhang Y, Zhu L, Zhao D, Lu Y, Zheng J. 2021. Physiological and transcriptomic analyses characterized high temperature stress response mechanisms in *Sorbus pohuashanensis*. *Scientific Reports* **11**: 10117.

Perea-García A, Garcia-Molina A, Andrés-Colás N, Vera-Sirera F, Pérez-Amador MA, Puig S, Peñarrubia L. 2013. Arabidopsis copper transport protein COPT2 participates in the cross talk between iron deficiency responses and low-phosphate signaling. *Plant Physiology* **162**: 180–194.

Péret B, Clément M, Nussaume L, Desnos T. 2011. Root developmental adaptation to phosphate starvation: better safe than sorry. *Trends in Plant Science* **16**: 442–450.

Pérez-Alonso M-M, Guerrero-Galán C, Scholz SS, Kiba T, Sakakibara H, Ludwig-Müller J, Krapp A, Oelmüller R, Vicente-Carbajosa J, Pollmann S. 2020. Harnessing symbiotic plant-fungus interactions to unleash hidden forces from extreme plant ecosystems. *Journal of Experimental Botany* **71**: 3865–3877.

Perez-Garcia P, Serrano-Ron L, Moreno-Risueno MA. 2022. The nature of the root clock at single cell resolution: Principles of communication and similarities with plant and animal pulsatile and circadian mechanisms. *Current Opinion in Cell Biology* **77**: 102102.

Pérez-Torres C-A, López-Bucio J, Cruz-Ramírez A, Ibarra-Laclette E, Dharmasiri S, Estelle M, Herrera-Estrella L. 2009. Phosphate Availability Alters Lateral Root Development in *Arabidopsis* by Modulating Auxin Sensitivity via a Mechanism Involving the TIR1 Auxin Receptor. *The Plant Cell* **20**: 3258–3272.

Perilli S, Di Mambro R, Sabatini S. 2012. Growth and development of the root apical meristem. *Current Opinion in Plant Biology* **15**: 17–23.

Petricka JJ, Winter CM, Benfey PN. 2012. Control of Arabidopsis root development. *Annual Review of Plant Biology* **63**: 563–590.

Picó FX, Méndez-Vigo B, Martínez-Zapater JM, Alonso-Blanco C. 2008. Natural genetic variation of Arabidopsis thaliana is geographically structured in the Iberian peninsula. *Genetics* **180**: 1009–1021.

Pikovskaya RI. 1948. Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Mikrobiologiya* **17**: 362–370.

Poveda J, Abril-Urias P, Escobar C. 2020. Biological Control of Plant-Parasitic Nematodes by Filamentous Fungi Inducers of Resistance: Trichoderma, Mycorrhizal and Endophytic Fungi. *Frontiers in Microbiology* **11**: 992.

Poveda J, Díaz-González S, Díaz-Urbano M, Velasco P, Sacristán S. 2022. Fungal endophytes of Brassicaceae: Molecular interactions and crop benefits. *Frontiers in Plant Science* **13**: 932288.

Pozo MJ, Zabalgoceazcoa I, Vazquez de Aldana BR, Martinez-Medina A. 2021. Untapping the potential of plant mycobiomes for applications in agriculture. *Current Opinion in Plant Biology* **60**: 102034.

Qi F, Zhang F. 2019. Cell Cycle Regulation in the Plant Response to Stress. *Frontiers in Plant Science* **10**: 1765.

Qu Y, Liu S, Bao W, Xue X, Ma Z, Yokawa K, Baluška F, Wan Y. 2017. Expression of Root Genes in Arabidopsis Seedlings Grown by Standard and Improved Growing Methods. *International Journal of Molecular Sciences* **18**: E951.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**: D590-596.

Quint M, Delker C, Franklin KA, Wigge PA, Halliday KJ, van Zanten M. 2016. Molecular and genetic control of plant thermomorphogenesis. *Nature Plants* **2**: 15190.

R Core Team. 2020. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Raghothama KG. 1999. PHOSPHATE ACQUISITION. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**: 665–693.

Ramakrishna P, Ruiz Duarte P, Rance GA, Schubert M, Vordermaier V, Vu LD, Murphy E, Vilches Barro A, Swarup K, Moirangthem K, et al. 2019. EXPANSIN A1-mediated radial swelling of pericycle cells positions anticlinal cell divisions during lateral root initiation. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 8597–8602.

Ramireddy E, Hosseini SA, Eggert K, Gillandt S, Gnad H, von Wirén N, Schmülling T. 2018. Root Engineering in Barley: Increasing Cytokinin Degradation Produces a Larger Root System, Mineral Enrichment in the Shoot and Improved Drought Tolerance. *Plant Physiology* **177**: 1078–1095.

Redkar A, Sabale M, Zuccaro A, Di Pietro A. 2022. Determinants of endophytic and pathogenic lifestyle in root colonizing fungi. *Current Opinion in Plant Biology* **67**: 102226.

Reymond M, Svistoonoff S, Loudet O, Nussaume L, Desnos T. 2006. Identification of QTL controlling root growth response to phosphate starvation in *Arabidopsis thaliana*. *Plant, Cell & Environment* **29**: 115–125.

Ried MK, Wild R, Zhu J, Pipercevic J, Sturm K, Broger L, Harmel RK, Abriata LA, Hothorn LA, Fiedler D, et al. 2021. Inositol pyrophosphates promote the interaction of SPX domains with the coiled-coil motif of PHR transcription factors to regulate plant phosphate homeostasis. *Nature Communications* **12**: 384.

Riemer E, Qiu D, Laha D, Harmel RK, Gaugler P, Gaugler V, Frei M, Hajirezaei M-R, Laha NP, Krusenbaum L, et al. 2021. ITPK1 is an InsP6/ADP phosphotransferase that controls phosphate signaling in *Arabidopsis*. *Molecular Plant* **14**: 1864–1880.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**: 139–140.

Rodríguez Martín JA, López Arias M, Grau Corbí JM. 2009. *Heavy metals, organic matter and other parameters of farmlands and pasturelands of Spain*. Madrid, Spain: Ministerio de Medio Ambiente y Medio Rural y Marino.

Rogers ED, Benfey PN. 2015. Regulation of plant root system architecture: implications for crop advancement. *Current Opinion in Biotechnology* **32**: 93–98.

Rolfe SA, Griffiths J, Ton J. 2019. Crying out for help with root exudates: adaptive mechanisms by which stressed plants assemble health-promoting soil microbiomes. *Current Opinion in Microbiology* **49**: 73–82.

Romanov GA, Lomin SN, Schmülling T. 2006. Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. *Journal of Experimental Botany* **57**: 4051–4058.

Rouached H. 2011. Multilevel coordination of phosphate and sulfate homeostasis in plants. *Plant Signaling & Behavior* **6**: 952–955.

Rouached H, Arpat AB, Poirier Y. 2010. Regulation of phosphate starvation responses in plants: signaling players and cross-talks. *Molecular Plant* **3**: 288–299.

Rousk J, Frey SD, Bååth E. 2012. Temperature adaptation of bacterial communities in experimentally warmed forest soils. *Global Change Biology* **18**: 3252–3258.

RStudio Team. 2020. *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, PBC.

Ruan W, Guo M, Cai L, Hu H, Li C, Liu Y, Wu Z, Mao C, Yi K, Wu P, et al. 2015. Genetic manipulation of a high-affinity PHR1 target cis-element to improve phosphorous uptake in *Oryza sativa* L. *Plant Molecular Biology* **87**: 429–440.

Rubio V, Linhares F, Solano R, Martín AC, Iglesias J, Leyva A, Paz-Ares J. 2001. A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes & Development* **15**: 2122–2133.

Sabri NSA, Zakaria Z, Mohamad SE, Jaafar AB, Hara H. 2018. Importance of Soil Temperature for the Growth of Temperate Crops under a Tropical Climate and Functional Role of Soil Microbial Diversity. *Microbes and Environments* **33**: 144–150.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ. 1998. FUNGAL ENDOPHYTES: A Continuum of Interactions with Host Plants. *Annual Review of Ecology and Systematics* **29**: 319–343.

Sakakibara H. 2006. Cytokinins: activity, biosynthesis, and translocation. *Annual Review of Plant Biology* **57**: 431–449.

Sakuraba Y, Chaganzhana null, Mabuchi A, Iba K, Yanagisawa S. 2021. Enhanced NRTL1/NPF6.3 expression in shoots improves growth under nitrogen deficiency stress in Arabidopsis. *Communications Biology* **4**: 256.

Sakuraba Y, Kanno S, Mabuchi A, Monda K, Iba K, Yanagisawa S. 2018. A phytochrome-B-mediated regulatory mechanism of phosphorus acquisition. *Nature Plants* **4**: 1089–1101.

Salas-González I, Reyt G, Flis P, Custódio V, Gopaulchan D, Bakhoun N, Dew TP, Suresh K, Franke RB, Dangl JL, et al. 2021. Coordination between microbiota and root endodermis supports plant mineral nutrient homeostasis. *Science (New York, N.Y.)* **371**: eabd0695.

Samach A, Wigge PA. 2005. Ambient temperature perception in plants. *Current Opinion in Plant Biology* **8**: 483–486.

Sánchez-Calderón L, López-Bucio J, Chacón-López A, Cruz-Ramírez A, Nieto-Jacobo F, Dubrovsky JG, Herrera-Estrella L. 2005. Phosphate starvation induces a determinate developmental program in the roots of Arabidopsis thaliana. *Plant & Cell Physiology* **46**: 174–184.

Schäfer M, Brütting C, Meza-Canales ID, Großkinsky DK, Vankova R, Baldwin IT, Meldau S. 2015. The role of cis-zeatin-type cytokinins in plant growth regulation and mediating responses to environmental interactions. *Journal of Experimental Botany* **66**: 4873–4884.

Scharf K-D, Berberich T, Ebersberger I, Nover L. 2012. The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochimica Et Biophysica Acta* **1819**: 104–119.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**: 676–682.

Schramm F, Ganguli A, Kiehlmann E, Englich G, Walch D, von Koskull-Döring P. 2006. The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in Arabidopsis. *Plant Molecular Biology* **60**: 759–772.

Secco D, Whelan J. 2014. Toward deciphering the genome-wide transcriptional responses of rice to phosphate starvation and recovery. *Plant Signaling & Behavior* **9**: e28319.

Seren Ü. 2018. GWA-Portal: Genome-Wide Association Studies Made Easy. *Methods in Molecular Biology (Clifton, N.J.)* **1761**: 303–319.

Serrano-Ron L, Perez-Garcia P, Sanchez-Corrionero A, Gude I, Cabrera J, Ip P-L, Birnbaum KD, Moreno-Risueno MA. 2021. Reconstruction of lateral root formation through single-cell RNA sequencing reveals order of tissue initiation. *Molecular Plant* **14**: 1362–1378.

Shen C, Yue R, Yang Y, Zhang L, Sun T, Tie S, Wang H. 2014. OsARF16 is involved in cytokinin-mediated inhibition of phosphate transport and phosphate signaling in rice (*Oryza sativa* L.). *PloS One* **9**: e112906.

Silva-Navas J, Conesa CM, Saez A, Navarro-Neila S, Garcia-Mina JM, Zamarreño AM, Baigorri R, Swarup R, Del Pozo JC. 2019. Role of cis-zeatin in root responses to phosphate starvation. *The New Phytologist* **224**: 242–257.

Silva-Navas J, Moreno-Risueno MA, Manzano C, Pallero-Baena M, Navarro-Neila S, Téllez-Robledo B, Garcia-Mina JM, Baigorri R, Gallego FJ, del Pozo JC. 2015. D-Root: a system for cultivating plants with the roots in darkness or under different light conditions. *The Plant Journal: For Cell and Molecular Biology* **84**: 244–255.

Silva-Navas J, Moreno-Risueno MA, Manzano C, Téllez-Robledo B, Navarro-Neila S, Carrasco V, Pollmann S, Gallego FJ, Del Pozo JC. 2016. Flavonols Mediate Root Phototropism and Growth through Regulation of Proliferation-to-Differentiation Transition. *The Plant Cell* **28**: 1372–1387.

Singh SK, Wu X, Shao C, Zhang H. 2022. Microbial enhancement of plant nutrient acquisition. *Stress Biology* **2**: 3.

Smith KA, Mullins CE (Eds.). 2001. *Soil and environmental analysis: physical methods*. New York: M. Dekker.

Sriden N, Charoensawan V. 2022. Large-scale comparative transcriptomic analysis of temperature-responsive genes in *Arabidopsis thaliana*. *Plant Molecular Biology*.

Starodubtseva A, Kalachova T, Retzer K, Jelínková A, Dobrev P, Lacey J, Pospíchalová R, Angelini J, Guivarc'h A, Pateyron S, *et al.* 2022. An *Arabidopsis* mutant deficient in phosphatidylinositol-4-phosphate kinases $\beta 1$ and $\beta 2$ displays altered auxin-related responses in roots. *Scientific Reports* **12**: 6947.

Stoeckle D, Thellmann M, Vermeer JE. 2018. Breakout-lateral root emergence in *Arabidopsis thaliana*. *Current Opinion in Plant Biology* **41**: 67–72.

Stolz A, Riefler M, Lomin SN, Achazi K, Romanov GA, Schmülling T. 2011. The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. *The Plant Journal: For Cell and Molecular Biology* **67**: 157–168.

- Su T, Xu Q, Zhang F-C, Chen Y, Li L-Q, Wu W-H, Chen Y-F. 2015.** WRKY42 modulates phosphate homeostasis through regulating phosphate translocation and acquisition in Arabidopsis. *Plant Physiology* **167**: 1579–1591.
- Sweetman D, Stavridou I, Johnson S, Green P, Caddick SEK, Brearley CA. 2007.** Arabidopsis thaliana inositol 1,3,4-trisphosphate 5/6-kinase 4 (AtITPK4) is an outlier to a family of ATP-grasp fold proteins from Arabidopsis. *FEBS letters* **581**: 4165–4171.
- Tabas-Madrid D, Méndez-Vigo B, Arteaga N, Marcer A, Pascual-Montano A, Weigel D, Xavier Picó F, Alonso-Blanco C. 2018.** Genome-wide signatures of flowering adaptation to climate temperature: Regional analyses in a highly diverse native range of Arabidopsis thaliana. *Plant, Cell & Environment* **41**: 1806–1820.
- Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A. 2012.** GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics. *Nucleic Acids Research* **40**: W478–483.
- Takahashi N, Ogita N, Takahashi T, Taniguchi S, Tanaka M, Seki M, Umeda M. 2019.** A regulatory module controlling stress-induced cell cycle arrest in Arabidopsis. *eLife* **8**: e43944.
- Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N. 2007.** Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640–645.
- Tang J, Wu D, Li X, Wang L, Xu L, Zhang Y, Xu F, Liu H, Xie Q, Dai S, et al. 2022.** Plant immunity suppression via PHRI-RALF-FERONIA shapes the root microbiome to alleviate phosphate starvation. *The EMBO journal* **41**: e109102.
- Tester M, Morris C. 1987.** The penetration of light through soil. *Plant, Cell and Environment* **10**: 281–286.
- Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M. 2004.** MAPMAN: a user-driven tool to display genomics data sets

onto diagrams of metabolic pathways and other biological processes. *The Plant Journal: For Cell and Molecular Biology* **37**: 914–939.

Ticconi CA, Lucero RD, Sakhonwasee S, Adamson AW, Creff A, Nussaume L, Desnos T, Abel S. 2009. ER-resident proteins PDR2 and LPR1 mediate the developmental response of root meristems to phosphate availability. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 14174–14179.

Tivendale ND, Millar AH. 2022. How is auxin linked with cellular energy pathways to promote growth? *The New Phytologist* **233**: 2397–2404.

Tiwari M, Kumar R, Min D, Jagadish SVK. 2022. Genetic and molecular mechanisms underlying root architecture and function under heat stress-A hidden story. *Plant, Cell & Environment* **45**: 771–788.

Tovar JC, Berry JC, Quillatupa C, Castillo SE, Acosta-Gamboa L, Fahlgren N, Gehan MA. 2022. Heat stress changes mineral nutrient concentrations in *Chenopodium quinoa* seed. *Plant Direct* **6**: e384.

Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK. 2020. Plant-microbiome interactions: from community assembly to plant health. *Nature Reviews. Microbiology* **18**: 607–621.

Tsukagoshi H, Busch W, Benfey PN. 2010. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. *Cell* **143**: 606–616.

Ubeda-Tomás S, Beemster GTS, Bennett MJ. 2012. Hormonal regulation of root growth: integrating local activities into global behaviour. *Trends in Plant Science* **17**: 326–331.

Valdés-López O, Batek J, Gomez-Hernandez N, Nguyen CT, Isidra-Arellano MC, Zhang N, Joshi T, Xu D, Hixson KK, Weitz KK, *et al.* 2016. Soybean Roots Grown under Heat Stress Show Global Changes in Their Transcriptional and Proteomic Profiles. *Frontiers in Plant Science* **7**: 517.

Verbon EH, Liberman LM. 2016. Beneficial Microbes Affect Endogenous Mechanisms Controlling Root Development. *Trends in Plant Science* **21**: 218–229.

Vogel CM, Potthoff DB, Schäfer M, Barandun N, Vorholt JA. 2021. Protective role of the Arabidopsis leaf microbiota against a bacterial pathogen. *Nature Microbiology* **6**: 1537–1548.

van der Voort M, Kempenaar M, van Driel M, Raaijmakers JM, Mendes R. 2016. Impact of soil heat on reassembly of bacterial communities in the rhizosphere microbiome and plant disease suppression. *Ecology Letters* **19**: 375–382.

Vyroubalová S, Václavíková K, Turecková V, Novák O, Smehilová M, Hluska T, Ohnoutková L, Frébort I, Galuszka P. 2009. Characterization of new maize genes putatively involved in cytokinin metabolism and their expression during osmotic stress in relation to cytokinin levels. *Plant Physiology* **151**: 433–447.

Waidmann S, Ruiz Rosquete M, Schöller M, Sarkel E, Lindner H, LaRue T, Petřík I, Dünser K, Martopawiro S, Sasidharan R, et al. 2019. Cytokinin functions as an asymmetric and anti-gravitropic signal in lateral roots. *Nature Communications* **10**: 3540.

Wang L, Dong J, Gao Z, Liu D. 2012. The Arabidopsis gene hypersensitive to phosphate starvation 3 encodes ethylene overproduction 1. *Plant & Cell Physiology* **53**: 1093–1105.

Wang Z, Kuo H-F, Chiou T-J. 2021. Intracellular phosphate sensing and regulation of phosphate transport systems in plants. *Plant Physiology* **187**: 2043–2055.

Wang X, Yi K, Tao Y, Wang F, Wu Z, Jiang D, Chen X, Zhu L, Wu P. 2006. Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. *Plant, Cell & Environment* **29**: 1924–1935.

Ward JT, Lahner B, Yakubova E, Salt DE, Raghothama KG. 2008. The effect of iron on the primary root elongation of Arabidopsis during phosphate deficiency. *Plant Physiology* **147**: 1181–1191.

Wei T, Simko V. 2021. *R package ‘corrplot’: Visualization of a Correlation Matrix.*

- Werner T, Holst K, Pörs Y, Guivarc'h A, Mustroph A, Chriqui D, Grimm B, Schmülling T. 2008.** Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. *Journal of Experimental Botany* **59**: 2659–2672.
- Whitt L, Ricachenevsky FK, Ziegler GZ, Clemens S, Walker E, Maathuis FJM, Kear P, Baxter I. 2020.** A curated list of genes that affect the plant ionome. *Plant Direct* **4**: e00272.
- Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, et al. 2019.** Welcome to the tidyverse. *Journal of Open Source Software* **4**: 1686.
- Wickham H, Chang W, Wickham MH. 2016.** Package ‘ggplot2’. *Create elegant data visualisations using the grammar of graphics. Version 2*: 1–189.
- Wolinska KW, Vannier N, Thiergart T, Pickel B, Gremmen S, Piasecka A, Piślewska-Bednarek M, Nakano RT, Belkhadir Y, Bednarek P, et al. 2021.** Tryptophan metabolism and bacterial commensals prevent fungal dysbiosis in Arabidopsis roots. *Proceedings of the National Academy of Sciences of the United States of America* **118**: e2111521118.
- Woo J, MacPherson CR, Liu J, Wang H, Kiba T, Hannah MA, Wang X-J, Bajic VB, Chua N-H. 2012.** The response and recovery of the Arabidopsis thaliana transcriptome to phosphate starvation. *BMC plant biology* **12**: 62.
- Woodward AW, Bartel B. 2018.** Biology in Bloom: A Primer on the Arabidopsis thaliana Model System. *Genetics* **208**: 1337–1349.
- Xie Y, Sun P, Li Z, Zhang F, You C, Zhang Z. 2022.** FERONIA Receptor Kinase Integrates with Hormone Signaling to Regulate Plant Growth, Development, and Responses to Environmental Stimuli. *International Journal of Molecular Sciences* **23**: 3730.

- Xu J, Belanger F, Huang B. 2008.** Differential gene expression in shoots and roots under heat stress for a geothermal and non-thermal *Agrostis* grass species contrasting in heat tolerance. *Environmental and Experimental Botany* **63**: 240–247.
- Xu P, Cai W. 2019.** Nitrate-responsive OBP4-XTH9 regulatory module controls lateral root development in *Arabidopsis thaliana*. *PLoS genetics* **15**: e1008465.
- Xue P, Bai Y, Rottwinkel G, Averbukh E, Ma Y, Roeder T, Scheerer P, Krauß N, Lamparter T. 2021.** Phytochrome Mediated Responses in *Agrobacterium fabrum*: Growth, Motility and Plant Infection. *Current Microbiology* **78**: 2708–2719.
- Yamada K, Fukao Y, Hayashi M, Fukazawa M, Suzuki I, Nishimura M. 2007.** Cytosolic HSP90 regulates the heat shock response that is responsible for heat acclimation in *Arabidopsis thaliana*. *The Journal of Biological Chemistry* **282**: 37794–37804.
- Yan H, Wang Y, Chen B, Wang W, Sun H, Sun H, Li J, Zhao Q. 2022.** OsCKX2 regulates phosphate deficiency tolerance by modulating cytokinin in rice. *Plant Science: An International Journal of Experimental Plant Biology* **319**: 111257.
- Yang X, Dong G, Palaniappan K, Mi G, Baskin TI. 2017.** Temperature-compensated cell production rate and elongation zone length in the root of *Arabidopsis thaliana*. *Plant, Cell & Environment* **40**: 264–276.
- Yang Y, Liu H. 2020.** Coordinated Shoot and Root Responses to Light Signaling in *Arabidopsis*. *Plant Communications* **1**: 100026.
- Yang Y, Tilman D. 2020.** Soil and root carbon storage is key to climate benefits of bioenergy crops. *Biofuel Research Journal* **7**: 1143–1148.
- Yang Y, Zhang L, Chen P, Liang T, Li X, Liu H. 2020.** UV-B photoreceptor UVR8 interacts with MYB73/MYB77 to regulate auxin responses and lateral root development. *The EMBO journal* **39**: e101928.

Yeh C-M, Kobayashi K, Fujii S, Fukaki H, Mitsuda N, Ohme-Takagi M. 2019. Blue Light Regulates Phosphate Deficiency-Dependent Primary Root Growth Inhibition in Arabidopsis. *Frontiers in Plant Science* **10**: 1803.

Yi C, Wang X, Chen Q, Callahan DL, Fournier-Level A, Whelan J, Jost R. 2021. Diverse phosphate and auxin transport loci distinguish phosphate tolerant from sensitive Arabidopsis accessions. *Plant Physiology* **187**: 2656–2673.

Yin L, Zhang H, Tang Z, Xu J, Yin D, Zhang Z, Yuan X, Zhu M, Zhao S, Li X, et al. 2021. rMVP: A Memory-efficient, Visualization-enhanced, and Parallel-accelerated Tool for Genome-wide Association Study. *Genomics, Proteomics & Bioinformatics* **19**: 619–628.

Yokawa K, Kagenishi T, Kawano T, Mancuso S, Baluška F. 2011. Illumination of Arabidopsis roots induces immediate burst of ROS production. *Plant Signaling & Behavior* **6**: 1460–1464.

Yu F, Shi J, Zhou J, Gu J, Chen Q, Li J, Cheng W, Mao D, Tian L, Buchanan BB, et al. 2010. ANK6, a mitochondrial ankyrin repeat protein, is required for male-female gamete recognition in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 22332–22337.

Yu G, Wang L-G, Han Y, He Q-Y. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *Omics: A Journal of Integrative Biology* **16**: 284–287.

Zanten M van, Ai H, Quint M. 2021. Plant thermotropism: an underexplored thermal engagement and avoidance strategy. *Journal of Experimental Botany*: erab209.

van Zanten M, Voesenek LACJ, Peeters AJM, Millenaar FF. 2009. Hormone- and light-mediated regulation of heat-induced differential petiole growth in Arabidopsis. *Plant Physiology* **151**: 1446–1458.

Zhang N, Belsterling B, Raszewski J, Tonsor SJ. 2015. Natural populations of *Arabidopsis thaliana* differ in seedling responses to high-temperature stress. *AoB PLANTS* **7**: plv101.

Zhang Z, Liao H, Lucas WJ. 2014a. Molecular mechanisms underlying phosphate sensing, signaling, and adaptation in plants. *Journal of Integrative Plant Biology* **56**: 192–220.

Zhang J, Liu Y-X, Zhang N, Hu B, Jin T, Xu H, Qin Y, Yan P, Zhang X, Guo X, et al. 2019a. NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nature Biotechnology* **37**: 676–684.

Zhang D, Song H, Cheng H, Hao D, Wang H, Kan G, Jin H, Yu D. 2014b. The acid phosphatase-encoding gene *GmACP1* contributes to soybean tolerance to low-phosphorus stress. *PLoS genetics* **10**: e1004061.

Zhang Y, Wang C, Xu H, Shi X, Zhen W, Hu Z, Huang J, Zheng Y, Huang P, Zhang K-X, et al. 2019b. HY5 Contributes to Light-Regulated Root System Architecture Under a Root-Covered Culture System. *Frontiers in Plant Science* **10**: 1490.

Zhang H, Wang E, Zhou D, Luo Z, Zhang Z. 2016. Rising soil temperature in China and its potential ecological impact. *Scientific Reports* **6**: 35530.

Zhao J, Lu Z, Wang L, Jin B. 2020. Plant Responses to Heat Stress: Physiology, Transcription, Noncoding RNAs, and Epigenetics. *International Journal of Molecular Sciences* **22**: E117.

Zheng Z, Wang Z, Wang X, Liu D. 2019. Blue Light-Triggered Chemical Reactions Underlie Phosphate Deficiency-Induced Inhibition of Root Elongation of *Arabidopsis* Seedlings Grown in Petri Dishes. *Molecular Plant* **12**: 1515–1523.

Zhou J, Diao X, Wang T, Chen G, Lin Q, Yang X, Xu J. 2018. Phylogenetic diversity and antioxidant activities of culturable fungal endophytes associated with the mangrove species *Rhizophora stylosa* and *R. mucronata* in the South China Sea. *PloS One* **13**: e0197359.

Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, Benner C, Chanda SK. 2019. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature Communications* **10**: 1523.

Zhu T, van Zanten M, De Smet I. 2022. Wandering between hot and cold: temperature dose-dependent responses. *Trends in Plant Science* **27**: 1124–1133.

Zuccaro A, Lahrmann U, Langen G. 2014. Broad compatibility in fungal root symbioses. *Current Opinion in Plant Biology* **20**: 135–145.

8. SUPPLEMENTARY MATERIAL

8. Supplementary material

Supplementary Table 1. List of Iberian Peninsula *A. thaliana* accessions used in our GWAS study, indicating geolocation and inferred soil phosphorous concentration of the site.

Accession ID	Abbreviated Name	Latitude	Longitude	Soil P (ppm)
6909	Col-0	38.30	-92.3	-
6933	LL-0	41.59	2.49	82.5
9506	IP-Alo-0	40.11	-7.47	-
9507	IP-Coa-0	38.45	-7.5	-
9508	IP-Mos-1	40.04	-7.11	-
9509	IP-Reg-0	39.29	-7.4	-
9511	IP-Vav-0	38.53	-8.02	-
9512	IP-Vid-1	38.22	-7.84	-
9513	IP-Adc-5	38.77	-4.07	25
9514	IP-Adm-0	39.15	-4.54	17.5
9515	IP-Ala-0	39.72	-6.89	25
9517	IP-All-0	42.19	-7.8	100
9519	IP-Ang-0	41.94	2.64	82.5
9520	IP-Ara-4	41.70	-3.68	42.5
9521	IP-Bar-1	41.43	2.13	42.5
9522	IP-Bea-0	36.52	-5.27	22.5
9523	IP-Ben-0	38.37	-2.66	17.5
9524	IP-Ber-0	42.52	-0.56	62.5
9525	IP-Bis-0	42.49	0.54	60
9526	IP-Cab-3	41.54	2.39	60
9527	IP-Cad-0	40.37	-5.74	25
9528	IP-Cal-0	40.94	-1.37	77.5
9529	IP-Cap-1	36.97	-3.36	22.5
9530	IP-Car-1	38.25	-4.32	22.5
9531	IP-Cdc-3	41.21	-4.54	82.5
9532	IP-Cdo-0	42.23	-4.64	47.5
9533	IP-Cem-0	41.15	-4.32	87.5
9534	IP-Cmo-3	40.05	-4.65	37.5

9535	IP-Coc-1	42.31	3.19	85
9536	IP-Cor-0	40.83	-2	65
9537	IP-Cum-1	38.07	-6.66	27.5
9539	IP-Deh-1	40.29	-6.67	25
9540	IP-Elb-0	41.81	2.34	62.5
9541	IP-Fue-2	38.26	-5.42	25
9542	IP-Fun-0	40.79	-4.05	62.5
9543	IP-Gra-0	36.77	-5.39	12.5
9544	IP-Gua-1	39.40	-5.33	20
9545	IP-Her-12	39.40	-5.78	20
9546	IP-Hom-4	40.82	-1.68	60
9547	IP-Hor-0	41.67	2.62	82.5
9548	IP-Hoy-0	40.40	-5	30
9549	IP-Hum-2	42.23	-3.69	55
9550	IP-Iso-4	43.05	-5.37	32.5
9551	IP-Jim-1	42.28	-5.92	55
9552	IP-Lab-7	40.87	-4.5	50
9553	IP-Ldd-0	41.58	-4.71	75
9554	IP-Lso-0	38.86	-3.16	27.5
9556	IP-Men-2	39.66	-4.34	35
9557	IP-Moa-0	42.46	0.7	60
9558	IP-Moc-11	41.57	-5.64	55
9559	IP-Mon-5	38.06	-4.38	32.5
9560	IP-Mot-0	38.19	-6.24	17.5
9561	IP-Mun-0	40.71	-5.04	37.5
9562	IP-Mur-0	41.67	2	32.5
9564	IP-Nog-17	40.45	-1.6	62.5
9565	IP-Orb-10	42.97	-1.23	42.5
9567	IP-Pal-0	42.34	1.3	70
9568	IP-Pan-0	42.76	-0.23	52.5
9569	IP-Pds-1	42.87	-6.45	77.5
9571	IP-Pro-0	43.28	-6.01	47.5
9573	IP-Rds-0	41.86	2.99	85
9574	IP-Rel-0	38.60	-2.7	12.5
9576	IP-Rev-0	40.86	-4.11	55
9577	IP-Ria-0	42.34	2.17	82.5

9578	IP-Sac-0	42.13	-6.7	82.5
9579	IP-San-l0	38.33	-3.51	15
9581	IP-Sdv-3	42.84	-5.12	35
9582	IP-Ses-0	41.48	-1.63	37.5
9583	IP-Sne-0	37.09	-3.38	20
9584	IP-Stp-0	41.19	-3.58	35
9585	IP-Svi-0	43.40	-7.39	100
9586	IP-Tam-0	41.03	-3.27	32.5
9587	IP-Tdc-0	41.50	-1.88	35
9588	IP-Tol-7	42.11	0.6	47.5
9589	IP-Tor-1	41.60	-2.83	32.5
9590	IP-Trs-0	43.37	-5.49	20
9591	IP-Vad-0	42.86	-3.59	45
9593	IP-Vaz-0	42.26	-2.99	77.5
9594	IP-Vdm-0	42.04	1.01	75
9595	IP-Vdt-0	40.89	-5.5	57.5
9596	IP-Ver-5	41.95	-7.45	95
9597	IP-Vig-1	42.31	-2.53	57.5
9598	IP-Vim-0	41.88	-6.51	70
9599	IP-Vin-0	42.80	-5.77	20
9600	IP-Vis-0	39.85	-6.04	25
9601	IP-Voz-0	41.85	-1.88	32.5
9602	IP-Vpa-1	40.50	-3.96	25
9817	IP-Ace-0	39.84	-6.6	25
9819	IP-Amu-0	42.35	-3.03	82.5
9820	IP-Are-0	41.00	-4.71	42.5
9821	IP-Aru-0	41.81	2.49	77.5
9822	IP-Aul-0	40.52	-4.02	45
9823	IP-Bae-0	43.34	-5.84	42.5
9824	IP-Bes-5	42.91	-4.91	57.5
9825	IP-Boa-0	40.40	-3.88	17.5
9826	IP-Bor-0	42.49	-6.71	87.5
9827	IP-Bos-0	42.78	0.69	67.5
9828	IP-Bra-0	42.50	-6.15	55
9830	IP-Bus-0	36.97	-3.28	12.5
9831	IP-Cas-0	38.54	-3.39	10

9832	IP-Cat-0	40.54	-3.69	20
9833	IP-Cha-0	40.38	-4.21	27.5
9834	IP-Cho-0	40.51	-3.9	25
9835	IP-Cir-0	40.61	-6.57	30
9836	IP-Cod-0	41.25	-1.32	67.5
9837	IP-Con-0	37.94	-5.6	42.5
9838	IP-Cot-0	41.83	-5.38	17.5
9839	IP-Coy-0	40.44	-4.27	25
9840	IP-Dar-0	41.13	-1.43	82.5
9841	IP-Ees-0	40.59	-4.15	35
9843	IP-Elp-0	40.53	-3.92	22.5
9844	IP-Esn-2	42.27	0.19	32.5
9845	IP-Evs-0	40.48	-3.96	22.5
9846	IP-Ezc-2	42.31	-3.02	75
9847	IP-Fel-2	43.31	-5.7	25
9848	IP-Glo-1	40.11	-5.77	42.5
9849	IP-Gud-3	40.65	-4.11	60
9850	IP-Hec-0	42.86	-0.7	42.5
9851	IP-Hue-3	42.96	-6.1	22.5
9852	IP-Ini-0	40.46	-3.75	20
9853	IP-Lac-0	43.33	-5.91	42.5
9854	IP-Laf-1	43.36	-5.88	42.5
9855	IP-Lam-0	40.57	-3.89	22.5
9857	IP-Leg-0	40.33	-3.8	22.5
9858	IP-Loz-0	40.98	-3.8	62.5
9859	IP-Lro-0	40.50	-3.88	22.5
9860	IP-Lum-0	42.24	-2.62	77.5
9861	IP-Mac-0	40.72	-3.21	70
9862	IP-Mad-0	40.45	-3.67	25
9864	IP-Mat-0	41.76	2.69	77.5
9866	IP-Mdd-0	41.89	-2.79	42.5
9867	IP-Mie-1	40.94	-3.22	22.5
9868	IP-Moe-0	41.78	2.37	77.5
9869	IP-Moj-0	36.76	-5.28	12.5
9870	IP-Moz-0	41.91	0.17	50
9871	IP-Nac-0	40.75	-3.99	52.5

9873	IP-Ndc-0	37.94	-5.45	30
9874	IP-Oja-0	42.34	-3	80
9875	IP-Ovi-1	43.38	-5.87	42.5
9876	IP-Pad-0	41.34	0.99	47.5
9877	IP-Pdl-0	43.02	-5.6	25
9878	IP-Pee-0	40.78	-3.62	60
9879	IP-Per-0	37.60	-1.12	55
9880	IP-Pib-1	42.72	-3.44	62.5
9881	IP-Pie-0	40.46	-5.32	25
9882	IP-Pil-0	40.46	-4.26	22.5
9883	IP-Piq-0	42.10	-2.56	65
9885	IP-Prd-0	41.14	-3.68	40
9886	IP-Pru-0	42.38	1.73	75
9887	IP-Pun-0	40.40	-4.77	25
9888	IP-Pva-1	40.93	-3.31	37.5
9890	IP-Rib-1	43.16	-5.07	45
9891	IP-Sal-0	41.93	2.92	82.5
9892	IP-Sam-0	42.68	-6.96	100
9894	IP-Sen-0	42.59	0.76	67.5
9895	IP-Sfb-6	41.78	2.57	82.5
9897	IP-Smt-1	40.95	-5.63	45
9898	IP-Som-0	41.14	-3.58	22.5
9899	IP-Tau-0	42.54	0.84	62.5
9900	IP-Tri-0	37.38	-6.01	47.5
9901	IP-Urd-1	42.27	-2.98	80
9902	IP-Usa-0	40.71	-3.24	65
9903	IP-Val-0	42.31	-3.1	82.5
9904	IP-Vas-0	40.95	-3.31	22.5
9905	IP-Ven-0	40.76	-4.01	47.5
9941	Fei-0	40.92	-8.54	-
9942	Agu-1	41.32	-1.34	62.5
9943	Cdm-0	39.73	-5.74	17.5
9944	Don-0	36.83	-6.36	65
9946	Mer-6	38.92	-6.34	60
9947	Ped-0	40.74	-3.9	45
9948	Pra-6	41.05	-3.54	22.5

9949	Qui-0	42.69	-6.93	100
9950	Vie-0	42.63	0.76	67.5

Supplementary Table 2. Correlation matrix of the measured traits under high and low phosphate conditions. Coefficients were calculated using Spearon's correlation. Bold values represent significant correlations (p -value > 0.05).

Variables	High Pi				Low Pi			
	Weight	Pi	PRL	nLRs	Weigth	Pi	PRL	nLRs
High Pi	Weight							
	Pi	0.06						
	PRL	0.44	0.19					
	nLRs	0.72	0.08	0.50				
Low Pi	Weight	0.58	-0.02	0.41	0.33			
	Pi	-0.33	0.07	-0.05	-0.27	-0.24		
	PRL	0.32	0.13	0.58	0.27	0.55	0.01	
	nLRs	0.56	0.02	0.45	0.79	0.46	-0.22	0.48
Soil P	-0.05	0.17	0.05	0.05	0.00	0.10	0.10	0.11