The molecular physiology of belowground interactions between plants

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Plants have evolved for hundreds of millions of years to grow alongside neighbouring plants, and this is a natural state for them. Due to their sessile nature, neighbouring plants are also the most enduring associations a plant experiences throughout its lifetime. Evidence shows that plants can detect, respond to, and be influenced by their neighbours, often with fitness consequences. While there are several models of plant-plant interactions, the mechanisms driving plant strategies remain unclear. In this thesis, I have employed *Arabidopsis thaliana* to explore the molecular physiology behind neighbour responsiveness and tolerance. I have thoroughly documented above- and belowground growth and measured reproductive outcomes in various intraspecific interaction scenarios. I have shown that two of the most essential resources for roots—nutrients and space—though utilised by neighbouring plants, are relatively straightforward and not interchangeable with the more complex influence of neighbour presence. I have also explored the strategies used by different, yet closely related species within the *Brassicae* family, as well as ecotypes of *A. thaliana*, and found that instead of uniform reactions, distinct multitrait responses to neighbours can be identified. I have clarified previous misconceptions regarding the role of rhizodeposits in *A. thaliana* plant-plant communication showing that it rarely occurs in soils condition. I provide the extensive characterisation of proteins present in root exudates and emphasise its nutrient regulation, which has general implications for rhizosphere research, but also sheds light on the prevalence of proteins involved in biotic interactions in soil. My results highlight the rhizosphere role of VOC ethylene in neighbour detection and response and demonstrate its effect on plants fitness. This work also demonstrates the internal role of CEP-CEPR1 signalling pathway in neighbour response execution. Overall, this thesis provides a foundational basis on which to develop further research aimed at molecular mechanistic understanding of plant-plant interactions.

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List of Abbreviations

aa amino acid

AIC Akaike Information Criterion

ABC ATP-binding cassette transporters

ACC 1-aminocyclopropane-1-carboxylic acid

ACO1 1-aminocyclopropane-1-carboxylate oxidase 1

ACO2 1-aminocyclopropane-1-carboxylate oxidase 2

ACO4 1-aminocyclopropane-1-carboxylate oxidase 4

ACT2 ACTIN2

ANOVA Analysis of Variance

AUX/IAA AUXIN/INDOLE-3-ACETIC ACID17

ATP Adenosine triphosphate

ATS Arabidopsis thaliana salts

BAK1 BRI1-ASSOCIATED RECEPTOR KINASE

BAM1 BARELY ANY MERISTEM 1

BRI1 BRASSINOSTEROID INSENSITIVE 1

cDNA Complementary DNA

CEP C-TERMINALLY ENCODED PEPTIDE

CEPR1 C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1

CLE CLAVATA3/ESR-related peptide

CTR1 CONSTITUTIVE TRIPLE RESPONSE1

Da Dalton

DDA Data-Dependent Acquisition

ddH2O double-distilled water

d.f. Degrees of Freedom

DNA Deoxyribonucleic acid

$\mathbf{D}_{res}$ Residual Deviance

EIL1 ETHYLENE-INSENSITIVE3-LIKE 1

EIL2 ETHYLENE-INSENSITIVE3-LIKE 2

EIN2 ETHYLENE INSENSITIVE2

EIN3 ETHYLENE INSENSITIVE3

EIN4 ETHYLENE INSENSITIVE4

EIR1 ETHYLENE INSENSITIVE ROOT 1 (other name PIN2 PIN-FORMED 2)

ERFs Ethylene responsive factors

ERS1 ETHYLENE RESPONSE SENSOR 1

ERS2 ETHYLENE RESPONSE SENSOR 2

ET Ethylene

ET-free-2 Ethylene-free-2

ETR1 ETHYLENE RESPONSE1

ETR2 ETHYLENE RESPONSE2

FDR False Discovery Rate

GLM Generalized Linear Model

GLMM Generalized Linear Mixed Model

GLV Green leaf volatiles

GLV GOLVEN peptides

GO Gene Ontology

HSD Honestly Significant Difference

HSP Heat Shock Protein

HMW High molecular weight

IDF Ideal free distribution

JCL Jacalin-related lectin

KD Kinase Domain

KIN7 KINASE 7

LMW Low molecular weight

LR Lateral root

LRR-RLK Leucine-rich Repeat Receptor-like Kinase

MIK1 MDIS1-INTERACTING RECEPTOR LIKE KINASE1

MUL MUSTACHES-LIKE

NASC The Nottingham Arabidopsis Stock Centre

NEM n-ethylmorpholine

NRT1.1 NITRATE TRANSPORTER1.1

NRT2.1 NITRATE TRANSPORTER2.1

NRT3.1 NITRATE TRANSPORTER3.1

PAMP Pathogen-associated molecular patterns

PAP purple acid phosphatase

PAR Photosynthetically active radiation

PCA Principal Component Analysis

PDR1 PLEIOTROPIC DRUG RESISTANCE 1

PHD Hydroxyproline arabinosylation

PHT1;1 PHOSPHATE TRANSPORTER1;1

PHT1;4 PHOSPHATE TRANSPORTER1;4

PIN2 PIN-FORMED 2 EIR1 (other name EIR1 ETHYLENE INSENSITIVE ROOT 1)

PR Primary root

PSF Plant-soil feedback

PSK Phytosulfokine peptide

PTM Postranslational modification

RALF Rapid Alkalinization Factor peptide

RII Relative interaction index

R:FR The red: far red ratio

RGF1 ROOT MERISTEM GROWTH FACTOR 1

RGI4 RGF1 INSENSITIVE 4

RIL Recombinant Inbred Line

RNA Ribonucleic acid

RT Reverse Transcription

qPCR Quantitative Polymerase Chain Reaction

SD Standard Deviation

SEM Standard Error of the Mean

SGH Stress Gradient Theory

SOBIR1 SUPPRESSOR OF BIR1 1

SP Signal peptide

SWEET Sugars Will Eventually be Exported transporters

TMD Transmembrane Domain

wt wild type

VOC Volatile organic compound

List of Publications

Bilas, R. D., Bretman, A., & Bennett, T. (2024). Friends, Neighbours and Enemies: An Overview of the Communal and Social Biology of Plants 1. *LIFE*, 102-138.

Chapter 1
General Introduction

Since Darwin's' contributions to understanding the competitive interactions among plants in 1859, mounting evidence underscored plants' capacity for detection, response, and interaction with neighbouring plants. However, the investigation on plant competition has traditionally been compartmentalised within distinct disciplinary fields (Ackerly & Sultan, 2006; Callaway et al., 2003; Heil & Karban, 2010; Novoplansky, 2002). Ecologists typically explore how species varying capacities to outcompete others for limited resources influence population and community dynamics (Fargione & Tilman, 2006; Goldberg, 1996a), while plant physiologists concentrate on the diverse traits that underpin these adaptations (Gaudet & Keddy, 1983). Meanwhile, molecular biologists have just relatively recently initiated endeavors to unravel the molecular fundamentals of plant-to-plant signalling (Becker et al., 2023). Bridging the gap between disciplines becomes imperative to address both the longstanding and recently emerging inquiries. The uncovering of the entirety of information accessible to plants, discerning pertinent information amidst noise, understanding mechanisms underlying signal perception and the execution of plastic responses, and distinguishing between active communication and eavesdropping are just a handful of pressing questions. Therefore, addressing them has the potential to reveal further groundbreaking insights in the realm of plant-plant interactions (Armstrong et al., 2023; Becker et al., 2023; Jessup et al., 2022). This is a research area with both fundamental and practical importance. It is significant to fundamental research as it explores the underlying biological mechanisms that govern plants' perception and response to environment which advances our understanding of signal transduction, sensory biology, and plasticity in plants. Additionally, it provides insights into the evolutionary processes driving these interactions and how they shape plant behaviour and adaptation over time. Understanding plant-plant interactions offers valuable insights into ecological dynamics such as species coexistence and biodiversity, which could inform better environmental practices. In agriculture, this knowledge has practical applications, such as developing crops that are more resource-efficient or competitive with weeds.

Exploring these aspects and their broader implications highlights the significance of this field and its potential for substantial impact.

1.1 The viewpoints on plant-plant interactions

The study of plant-plant interactions has a rich ecological history, spanning centuries of scientific inquiry into the complex dynamics governing these relationships. Darwin found further evidence for his *theory of natural selection* in the lawn plot experiment and the observation that plants compete for resources and that the outcome of this competition can influence the composition and structure of plant communities (Darwin, 1859). Indeed, the *competitive exclusion principle* proposed in the 1930s, states that species competing for the same limited resources cannot coexist indefinitely (Gause, 1930). Niche differentiation is a critical evolutionary response to the competitive exclusion principle. To avoid competitive displacement, species adapt by differentiating their niches in various ways i.e. spatial or temporal. The *limiting similarity* is a related theory that describes the degree of niche overlap tolerated between coexisting species (MacArthur & Richard Levins, 1967). Though early works often focused on competition, it was soon noticed that it exists as a continuum with cooperation and facilitation. The *group selection* and *kin selection hypotheses* highlighted that plants can exhibit cooperative behaviours, particularly among closely related individuals or within genetically similar groups (Hamilton, 1964; Harper, 1961). It was not until the late 20th century, particularly in the 1980s, that the understanding of plant-plant interactions began to gain slow but significant traction within the scientific community. That is when plants started being regarded as active entities, influenced by neighbouring vegetation in ways beyond indirect effects of resource availability (de Kroon et al., 2005; Hodgson, 1986; Pierik et al., 2013; Vicherová et al., 2020).

1.1.1 The ecology of plant-plant interactions

Theoretical considerations and experimental evidence suggest a spectrum of potential interactions between adjacent plants, each yielding varying fitness outcomes. Those stem from the different strategies that are applied depending on the neighbour and context and may be positive, neutral, or negative (Figure 1.1). Plant-plant interactions are commonly categorised into competition, facilitation, and cooperation, based on their effects on the fitness of the plants involved. Competition results in a reduction of fitness for both plants due to the struggle for shared resources, while facilitation occurs when one plant enhances the fitness of another without a direct cost to itself. Cooperation, by contrast, benefits both plants, leading to mutual fitness gains. These categories provide a framework for understanding the diverse and dynamic ways in which plants influence each other's growth, survival, and reproductive success (Bilas et al., 2021; Callaway, 1995; Metlen et al., 2009; Novoplansky, 2009; West et al., 2007a).

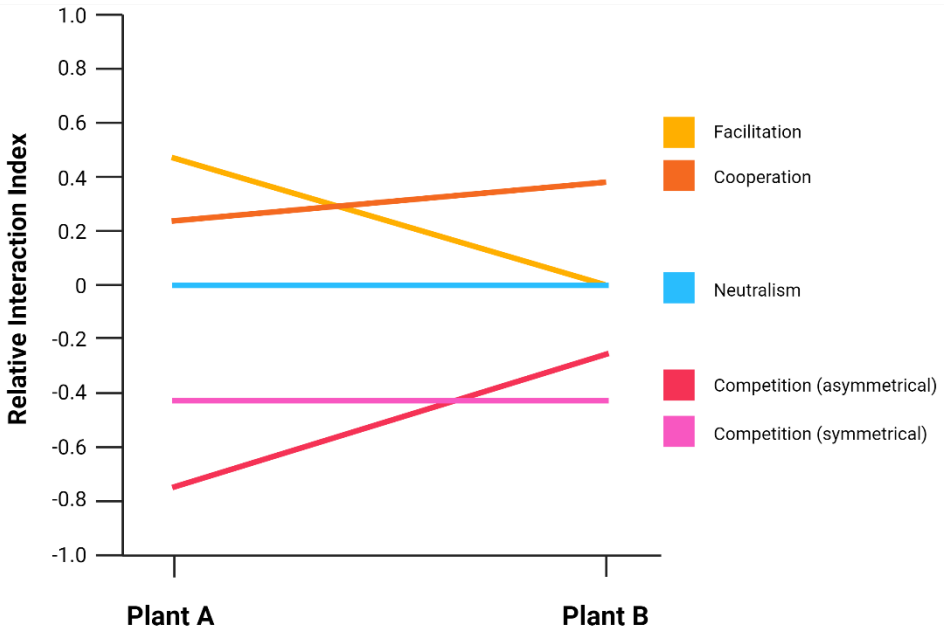


Figure 1.1. The scope of plant-plant interactions.

The Relative Interaction Index (RII) of five types of interactions between two plants A and B. For a detailed description of RII mathematical equation see Chapter 2.2. The RII values range from -1 to 1, with positive values indicating beneficial interactions,

negative values indicating negative interactions, and values around zero indicating neutral interactions. Facilitation: One plant benefits, and the other plant is not significantly affected or may also benefit. At least one plant shows a positive interaction index (between 0 and 1), indicating a benefit, while the other plant's interaction index is not negative (it can be positive, zero, or slightly negative but not significantly detrimental). Cooperation: Both plants benefit from each other's presence. Represented by high positive interaction indicators for both plants. Neutralism: Neither plant significantly affects the other. Represented by neutral (zero or near zero) interaction indices for both plants. Competition: Both plants are negatively affected by each other's presence in similar strength (symmetrical) or non-similar strength (asymmetrical). Represented by negative interaction indicators for both plants.

Competition

Competition is defined as a relationship between individuals in which both are experiencing reduction in fitness while striving for the same resource related to growth or reproduction and is driven by competitive ability — the ability of one species to exclude another (Gaudet & Keddy, 1983; West et al., 2007). Competition among organisms serves as a significant ecological and evolutionary force, with a plethora of evidence emerging from both experimental investigations and ecological observations especially with regards to interspecific competition (Aschehoug et al., 2016; Thorpe et al., 2011). Far less is known about intraspecific interactions even though theoretically competition in this context should be more pronounced and as well as crucial for species coexistence (Ehlers & Bilde, 2019; Grime, 1977). The evidential basis for this assertion remains inconclusive, as intraspecific competition generally exhibited comparable strength to interspecific competition which implies that resource partitioning may not represent a pivotal mechanism governing coexistence within plant communities (Goldberg & Barton, 1992, Subrahmaniam et al., 2018, 2021).

The competitive responses are often associated with the proliferation of harvesting organs, a self-interest strategy aimed at preemption of resources manifesting particularly in the case of limited reserves (De Kroon, 2012; Schenk, 2006; Shemesh et al., 2010). However, this simplification is not supported in light of

the insufficient evidence for the tragedy of the commons in the plant realm (Hardin, 1968). For example, numerous studies have found a reduction in root mass in response to competition (Chen et al., 2021; Fitter, 1976; Haugland & Froud-Williams, 1999; Semchenko et al., 2007; Semere & Froud-Williams, 2001). This suggests that more complex processes govern plant competitive responses (Callaway, 2002; McNickle & Brown, 2014; Zhu et al., 2019).

Facilitation

Facilitation describes interactions where one plant provides a benefit to another without necessarily receiving a direct benefit or harm in return (Callaway, 1995). The evidence supporting facilitation is relatively robust, with numerous examples of such effects being observed in interspecific interactions. The positive correlation between biodiversity and productivity is unequivocally established and has been substantiated through multiple empirical studies (Hector et al., 1999; Mcintire & Fajardo, 2014; Schöb et al., 2018; Tilman, 1996; Weisser et al., 2017; Wright et al., 2017).

Facilitative responses include shade tolerance, support for climbing, provision of water or nitrogen fixation (Brooker et al., 2008; West et al., 2007). Facilitative neighbours may alleviate stressful conditions or create novel niches for other plants. For example, facilitation is considered a key mechanism underlying the overyielding in intercropping systems comprised of cereals and legumes (Duchene et al., 2017; Raseduzzaman & Jensen, 2017). In phosphorus-impoverished soils, plants may benefit from phosphorus-mobilizing heterospecific neighbours (Khashi et al., 2021; Pantigoso et al., 2020; Thorpe et al., 2006; Zhang et al., 2020). Nurse plants are plants that enhance the growth and survival of other plants by creating more favourable microhabitats. Shrubs, in particular, have been frequently shown to facilitate other species' seedling establishment, especially in the harsh environments of arid regions (Chacón-Labella et al., 2016; Gómez-Aparicio et al., 2004; Loayza et al., 2017).

Much less is known about intraspecific facilitation, likely because positive interactions reported so far typically depend on differences in form and strategy between individuals, which are generally not present within the same species (Creissen et al., 2013). In intraspecific interactions this can be usually observed with plants of different age and mostly relates to the facilitation of reproduction and seedlings recruitment by development of microhabitats of ameliorated nutrients and water, generated by older plants (Gómez-Aparicio et al., 2004; Swanson et al., 2020). In populations of *Arabidopsis thaliana*, seeds that germinate late in the autumn benefit from the presence of neighbouring winter annuals that have already sprouted. These existing neighbours are presumed to shield them from the lethal effects of cold temperatures (Leverett, 2017). Facilitative effects may be subtle and context-dependent, making them difficult to observe and differentiate from other ecological processes.

Cooperation

Cooperation denotes mutualistic relationships where both plants actively contribute to each other's fitness, often through reciprocal actions (Callaway, 1995). The cooperative growth strategies may manifest as a decrease in the development of competitive structures. For example, in certain species, intricate root and shoot growth patterns, such as segregation and avoidance, manifest during interaction (Biernaskie, 2011; Ljubotina & Cahill, 2019; van der Zee et al., 2021; Zhang et al., 2020). However, conclusive evidence supporting such effects remains sparse and primarily restricted either to studies on clonal species or kin selection in plants (File et al., 2012a; Holzapfel & Alpert, 2003; Yang, 2018).

The concept of inclusive fitness has been adapted from the animal kingdom to plants and suggests that the relative benefits of cooperative interactions increase with greater relatedness (Gardner & West, 2010, 2014; Hamilton, 1964; Smith, 1964). Indeed, the cooperative interactions between closely related plants were reported in numerous species (File et al., 2012; Kong et al., 2018; Murphy et al., 2017; Simonsen

et al., 2014; Yang et al., 2018) with numerous studies being focused on wild herbaceous plants (Biedrzycki et al., 2010; Dudley & File, 2007; Lepik et al., 2012; Masclaux et al., 2010; Murphy & Dudley, 2007). In direct intraspecific interactions, kin recognition has been suggested to lead to reduced investment in competitive traits (Bhatt et al., 2011; Cahill et al., 2010; Fang et al., 2013; File et al., 2012), adjusted resource capture (Crepy & Casal, 2015; Lepik et al., 2012) including differentiation in nutrient niche and uptake (Li et al., 2018) and positive impact on plant fitness (Donohue et al., 2005; Torices et al., 2018; Wender et al., 2005). Kin recognition was shown to increase grain yield and was measured directly in rice (Yang et al., 2018), and seed biomass was higher in sibling groups than mixtures in ivy leaf (Biernaskie, 2011).

The early land plant lineages are mainly characterised by short-range dispersal through asexual reproduction which promoted continuous assemblies of closely related individuals and colonization of new habitats through *consequent vegetative multiplication* (Frey & Kürschner, 2011; Renzaglia et al., 2000; Taylor et al., 2005). The population viscosity is hypothesised to be the main driver of evolution of range of altruisms, including kin selection (Mitteldorf & Wilson, 2000). A mechanistic conceptualization suggests that for kin selection to evolve, kins and non-kins had to be mutually challenged at similar frequencies (Ehlers & Bilde, 2019; Semchenko et al., 2014; Yamawo & Mukai, 2020). Similarly, for facilitation to evolve mixtures with coexistence history are required (Schöb et al., 2018). Theoretically, kin selection would require a rather sophisticated mechanisms, that would not only enable neighbour detection, but more importantly—recognition (Rousset & Roze, 2007). Further studies on kin selection would benefit from efforts directed at identification of the signal that represents individual's identity, which is considered the main research gap in this field. Previous efforts point to the role of root exudates in this process (Biedrzycki, 2010; Gruntman et al., 2017; Semchenko et al., 2014). However, the expected high variability and low abundance of this signal renders its discovery challenging.

Interconnectedness

So far, phenomenon such as a natural selection, niche differentiation and spatiotemporal community dynamics have significantly advanced mainly with the immense input from studies on competition (Chesson, 2000; Thorpe et al., 2011). However, as more efforts are directed into studies on facilitation and cooperation it becomes clear that both are indispensable drivers of plants' coexistence patterns.

This is partly due to the inseparable balancing role of facilitation-competition shifts observed in environmental stress conditions (Aschehoug et al., 2016; Callaway, 1995; Grime, 1977; Tirado & Pugnaire, 2005). The *stress gradient hypothesis* (SGH) posits greater competition under benign conditions and facilitation under stressful conditions (Bertness & Callaway 1994; Maestre et al., 2009; Malkinson & Tielbörger, 2010; Qi et al., 2018). This concept was supported by various studies in harsh environments characterised by exposure, temperature fluctuations, and drought (Eränen & Kozlov, 2008; Holmgren & Scheffer, 2010; Pugnaire & Luque, 2001a). The extent of the actual impact of stress depends strongly on species' intrinsic stress tolerance ability and as the tolerance lowers, the reliance and gain from facilitation increases, suggesting a trade-off between competitive ability and stress tolerance (Soliveres et al., 2015; Weinig, 2000; Zhang & Tielbörger, 2019). Even within the lifespan of individual plants, shifts in competitive dynamics may occur, such as improved seedling establishment facilitated by neighbours but reduced adult reproductive success due to competition (Lara-Romero et al., 2016). On the opposite side is a phenomenon of self-thinning (Deng et al., 2012; Yu et al., 2012). In densely populated areas, the amount of resources per plant diminishes, eventually leading to a point where the resource demand exceeds what is available, and elimination of poorer competitors.

Currently, much effort is directed to increase the resolution of facilitation-competition shifts predictability. This can be achieved by better understanding of the roles of the influencing factors, such as the ontogeny (Dayrell et al., 2018; Eränen &

Kozlov, 2008; Lara-Romero et al., 2016; Miriti, 2006; Schiffers & Tielbörger, 2006), type of preceding vegetation and length of soil occupation (Song et al., 2019), resource levels (Koffel et al., 2018; Yu et al., 2019) and neighbour character (García-Cervigón et al., 2017; Murphy & Burns, 2019). Recently it was also shown that co-adaptation lessened interspecific competition, while intraspecific competition remained unchanged (Schmutz & Schöb, 2024). This suggests that the evolutionary pressures and dynamics within a species function independently from those occurring between species. It is pivotal to estimate the conditions in which facilitative interactions are most likely to be achieved and maintained (Maestre et al., 2009b; Malkinson & Tielbörger, 2010b; Qi et al., 2018).

1.1.2 The physiology of plant-plant interactions

The physiological underpinnings of neighbour responses represent a vibrant and complex field of study. In fact, the majority of insights into neighbour detection mechanisms have been derived from physiological studies.

At first, it was assumed that plants can only indirectly interact with neighbours *via* nutrient depletion and plants competition was viewed from the size perspective where it was categorised into size-symmetric and asymmetric (de Kroon et al., 2003). It was later suggested that some species may have direct responses to neighbouring plants particularly in interspecific interactions (Fransen et al., 2001; Hess & De Kroon, 2007; McNickle et al., 2016; Mommer et al., 2010; Padilla et al., 2013; Schenk, 2006). This was further corroborated by elucidation of multiple mechanisms enabling plants to actively detect neighbouring plants. Primarily, plants rely on cues for this purpose—signals that neighbouring plants inevitably release and cannot avoid being detected (Bilas et al., 2021; Falik et al., 2011; Shelef et al., 2019; Wang et al., 2020).

Aboveground, plants exhibit well-defined responses to neighbour aimed at light capture, such as stem elongation or reorientation of leaves (Ballaré & Pierik, 2017; Gommers et al., 2013; Pierik et al., 2013; Roig-Villanova & Martínez-García, 2016). Neighbour detection may lead to different branching patterns (Wheeldon et al. 2022)

or flowering time alterations (Torices et al.). Another strategy may include changes in shoot:root biomass allocation (Tonsor, 1989; Weiner et al. 2010).

Research on aboveground responsiveness is highly advanced, with numerous physiological responses being elucidated down to the genetic and molecular levels (Bilas et al. 2020; Pierik et al., 2017). However, in natural scenarios interactions occur simultaneously above- and belowground with probable feedback loops that determine the final physiological response and outcome. Therefore the progress in belowground studies and their integration with what is known aboveground is critical for comprehensive understanding of mechanisms that govern plant interactions.

1.2 The perspectives on root-root interactions

1.2.1 The ecology of root-root interactions

Hiltner (1904) defined the rhizosphere as the zone of soil influenced by root depletion and secretion. In the resource depletion zone, the concentration of resources decreases around the roots as they absorb them, creating areas of scarcity. These depletion zones influence root growth and branching patterns, with roots proliferating in less depleted areas to optimise resource uptake (Lynch et al., 2021; Schenk, 2006). On the other hand, this zone also contains exudates that locally modify both the biotic and abiotic environment (York et al., 2016; de Parseval et al., 2017). Soil properties, including slow signal dilution, oxidation, and photodecomposition, contribute to the persistence of root exudates. This enables chemical adaptation to play a crucial role in belowground interactions (Karlovsky, 2008; Watt et al., 2006).

The optimal foraging theory was originally developed for animal behaviour and has been adapted to describe how plants allocate resources to their roots to maximise nutrient uptake efficiency (de Kroon et al., 2003; McNickle et al., 2009; McNickle & Brown, 2014; Oborny, 1991). In this context, it predicts that plants will allocate growth to roots in a way that maximises the acquisition of the most limiting resource (i.e. water, particular nutrients). In plant ecology, this theory is used to

explain root growth patterns, particularly how roots proliferate in nutrient-rich patches or grow deeper in search of water (Hodge, 2009; Schenk et al., 1999). Plants have undergone evolutionary adaptations resulting in territorial root systems and foraging strategies characterised by heightened root tip sensitivity to environmental cues (Canarini et al., 2019; Falik et al., 2005; Schenk, 2006a; Simões et al., 2011). Root tropisms denote the directed growth responses of plant roots elicited by external stimuli. A growing body of evidence suggests the modulation of root systems in the presence of neighbouring plants (Cabal et al., 2020; Caffaro et al., 2011; McNickle et al., 2016). Nevertheless, the precise nature of these tropisms remains inconclusive, with inquiries revolving around whether they primarily entail nutrient tropisms or chemotropisms guided by exudates containing plant-plant signalling molecules (Baluška & Mancuso, 2021; Callaway & Li, 2020; Ljubotina & Cahill, 2019; Schenk, 2006; Semchenko et al., 2014).

The dynamic resource allocation focuses on how plants dynamically allocate resources between aboveground and belowground parts in response to changing environmental conditions (Bennett et al., 2012). The allocation to roots versus shoots is adjusted based on the availability of light, water, and nutrients, aiming to optimise overall plant growth and reproduction (Poorter et al., 2012). It is widely used to predict how plants will alter their growth patterns in response to environmental stressors, such as drought or nutrient deficiency. Plant-plant interactions have effects on allocation as well. For example, increased allocation to shoots in response to closely related neighbours in *P. lanceolata* and *I. hederacea* (Tonsor, 1989) and in various crops (Jacob et al., 2017; Ninkovic et al., 2003; Weiner et al., 2010).

Allelopathy refers to the chemical interactions between plants, where root exudates or other plant-derived chemicals inhibit or promote the growth of neighbouring plants (Blum & Blum, 2011; Zeng & Sen 2014). This suggests that plants can influence their surroundings through the release of specific compounds that affect the growth and development of other plants. It seems that root influence zone can be extended in time to future generations with the findings from studies on plant-

soil feedback (PSF). PSF describes how the presence of a plant species influences the performance of itself or related species in the same soil environment over time (Crawford et al., 2019; Hu et al., 2018; Kardol et al., 2013). The soil conditions can become more or less favourable for certain plant species due to the preceding vegetation (Hu et al., 2018; Scheres & Van Der Putten, 2017; van der Putten, 2017). This intricate interplay is often driven by root exudates, which contribute to the chemical legacy of the soil. Root exudates contain a diverse array of compounds that can persist in the soil, influencing plant growth and interactions over extended periods (Delory et al., 2021; Hu et al., 2018).

It is hypothesised that plants integrate both abiotic and biotic cues to anticipate future conditions (Novoplansky, 2009; Wheeldon et al., 2021). The quantity and composition of root exudates may serve as indicators of root density and identity, influencing nutrient allocation and growth optimization in both intra- and interspecific interactions (Cahill et al., 2010; Chen et al., 2020; Ljubotina & Cahill, 2019; Nord et al., 2011; Poorter et al., 2012; Wheeldon et al., 2020). Roots may also physically avoid contact with neighbouring roots based on the concentration of exudates, indicating a mechanism for competitive advantage (Cabal et al., 2021; Fang et al., 2013; Semchenko et al., 2008).

1.2.2 The physiology of root-root interactions

Understanding the complexity of root behaviour presents challenges in isolating factors related solely to the neighbour identity. Soil volume and nutrient availability have often been cited as causative agents of responses attributed to neighbour or kin recognition (Chen et al., 2012; Gruntman et al., 2017; Hess & De Kroon, 2007a; Semchenko et al., 2008). However, numerous studies have demonstrated that root exudates, when controlled for external nutrient application, independently convey plant identity signals, aiding in the assessment of genetic relatedness (Biedrzycki, 2010; Fang et al., 2013; Maina et al., 2002; Semchenko et al., 2007, 2014; Yamawo & Mukai, 2020; Yang et al., 2018). The involvement of ABC transporters in the exudation

process has been proposed (Biedrzycki et al., 2011, Yang et al., 2013), but the specific signals mediating plant interactions at this level of specificity remain unknown.

Candidate compounds such as ethylene, (-)-loliolide and strigolactones or more recently reported L-phenylalanine seem to play generic roles in density sensing, competitive root growth stimulation, or allelopathy (Kong et al., 2018; Li et al., 2020; Pandey et al., 2021; Pierik et al., 2007, 2013; Visser & Pierik, 2007; Wheeldon et al., 2022; Yoneyama et al., 2022; Yu et al., 2019). Intraspecific belowground interactions may lead to root distribution alterations and root avoidance strategy (Hess & De Kroon, 2007a). Exudates reflect both abiotically and biotically dependent physiological states (Lucas García et al., 2001) which plants perceive and respond to in order to enhance their fitness (Metlen et al., 2009). Conspecifics utilise exudates for various signalling, including synchronizing flowering (Falik et al., 2014). The exudate-mediated responses can extend to aboveground tritrophic interactions and stress responses (Elhakeem et al., 2018; Falik et al., 2011; Haisel et al., 2006). Despite advancements in methods for analyzing root exudates, much remains to be discovered about the specific compounds involved and their role in plant interactions (Karlovsky, 2008; van Dam & Bouwmeester, 2016; Wang et al., 2021). So far, non-exhaustive metabolomics approaches revealed *A. thaliana* root exudates to contain at least 130 primary and more than 100 secondary metabolites (Badri et al., 2012; Chaparro et al., 2013; Strehmel et al., 2014). Understanding the role of constitutive and inducible signals, along with their ability to influence fitness, will offer valuable insights into their adaptive significance in an evolutionary context (Metlen et al., 2009). Social stimuli in plants, including root exudates, are integral to the evolution of interactions, particularly among kin, emphasizing the importance of reliable signal propagation (Karban et al., 2013; Mokkonen & Lindstedt, 2016). The morphological plasticity and secondary metabolite release serve as transparent indicators of plant behaviour.

1.3 The molecular resolution of rhizosphere interactions

Research on plant-plant interactions remains relatively fragmented. In their meta-analyses, Subrahmaniam and colleagues (2018, 2021) identified 66 studies published over the past 35 years, spanning various species and investigating both intra- and interspecific interactions among herbaceous plants. Over the years, various molecular techniques such as the whole transcriptome gene expression analysis, QTL mapping, and candidate gene assessment have been employed to study plant-plant interactions in *A. thaliana*, but these efforts have met with limited success (Table 1.1).

In the specific context of *A. thaliana* transcriptome studies, so far, only two investigations have focused on transcriptome expression, and interestingly, yielded significantly different results. Masclaux and colleagues (2010) found no differences in gene expression between plants grown with the same ecotype *versus* a different one while examining 14 well characterised ecotypes (Masclaux et al., 2010; Nordborg et al., 2005). In contrast, Biedrzycki and colleagues (2011) identified 29 gene categories that were differentially regulated. In this study the objective was the intraspecific response to root exudates, using Cha-25 as the target ecotype and Col-0 as the neighbouring ecotype. The differences in expression included the downregulation of ABC transporters, a result in contradiction to their earlier works (Biedrzycki et al. 2011) and upregulation of pathogen response genes such as PDF1.3, PDF1.2b, and CA1 (Biedrzycki et al., 2011). Unfortunately, the Cha ecotypes, originally collected by Donohue (Harvard University) lack a detailed morphological description nor are they publicly available (Biedrzycki, et al., 2011). In a QTL analysis conducted by Mutic & Wolf (2007), a competition experiment was performed using an *A. thaliana* recombinant inbred line (RIL) population as the target plants, with the Ler accession as the neighbouring plant. The study identified QTLs located near genes involved in ethylene synthesis, suggesting ethylene as a potential signal in plant-plant interactions (Mutic & Wolf, 2007). Studies showing direct effects of

specific genes on belowground competition are limited and genetic influences on competitive ability are likely to collectively involve numerous genes. Cahill and colleagues (2005) noticed that the ethylene overproducing mutant *eto1-1* was a particularly poor competitor, with wild type plant growing better with it than when grown alone. This could be attributed to multiple explanations, for example difference in competing plants' sizes, as this genotype was amongst the smallest tested, or the actual ethylene involvement in shade avoidance response or root morphological response (Cahill et al., 2005).

1.4 Synthesis

Here, I outline some challenges that hinder the progress on molecular and genetic resolution. My purpose is not to question particular designs, but rather to reflect on designs that may be well suited for the particular question being asked.

1. Studies on plant-plant interactions are conducted across a broad range of species, ecotypes, accessions, and cultivars.

The wide variation in species, ecotypes, accessions, and cultivars (hereafter referred to as plant groups) used in studies makes it challenging to draw general conclusions, as the reported results often vary or contradict each other. This is expected since different plant groups inherently possess differently correlated traits i.e. due to their differences in life strategies, which is why they are distinct. These groups often show high variability in their responses to similar conditions or interactions, require different experimental setups, measurement techniques, or analysis methods. This inconsistency introduces biases and complicates cross-comparisons of results. The lack of continuity, with few studies focusing on the same plant group, leaves the field broad but shallow. A potential solution to this issue was implemented in this dissertation by focusing on a single plant family, *Brassicaceae*. To narrow the scope, I concentrated on plants with a similar life strategy—following an r-type reproductive strategy and classified as ruderal/stress-tolerant species (according to Grime et al.,

2007). Specifically, I focused on *A. thaliana*, a species with a body of research demonstrating its responsiveness to neighbours (Table 1.1). Additionally, *A. thaliana* offers long-term advantages due to the access to genetic and molecular resources (Bonser & Ladd, 2011; Cahill et al., 2005; Masclaux et al., 2010, 2012).

2. There is a disproportionate focus on intraspecific *versus* interspecific studies.

There are many more studies focusing on interspecific interactions than on intraspecific ones. Taking into the account differences between plant groups highlighted in paragraph 1, such an approach leads to further increase of complexity. Each plant group may interact differently and uniquely with other group, leading to a combinatorial explosion of possible interactions to study and conclude from. What is more, unless taken into account, differences between plant groups subjected to interspecific interactions are likely to promote asymmetric interactions by adding yet another confounding variable. Here, I have focused almost solely on intraspecific interactions that, especially in the case of intragenotypic interactions, tend to be symmetric. Whenever intergenotypic interaction had potential to impose asymmetric competition whether due to the size or morphology of the competitor it was acknowledged.

3. Many studies lack consideration for fitness.

A surprisingly large number of studies give insufficient attention to the fitness outcomes of plant interactions, often overlooking their significance and failing to measure them (Table 1.1). Here, I define fitness in its most widely accepted sense as plant's ability to survive, reproduce, and transmit its genes to the next generation, serving as a measure of reproductive success. Typically, conclusions about the overall nature of plant interactions are drawn based on neighbour responses rather than by assessing tolerance through fitness measurements. Furthermore, many studies refer to evolutionary and ecological theories related to plant interactions without fully considering their broader implications. From both evolutionary and ecological perspectives, it is unlikely to accurately assess the nature of a response

without evaluating its impact on fitness. The terms "neighbour response" and "competitive response" are often used interchangeably. Although the overproduction of organs is frequently cited as a competitive response, as shown in Table 1.1, increases and decreases in root trait expression are equally common neighbour responses. The true significance of these trait changes would be better understood by considering their effects (or lack thereof) on the fitness of the plants employing these strategies. I have focused on annual plants where fitness can be measured directly and easily therefore interaction outcome can be referred to interaction response directly. Whenever possible seed biomass as the most relatable trait was measured, in other cases the siliques number or seed number was assessed.

4. Controversies around experimental designs.

A wide range of experimental designs have been used in studies on plant interactions so far and each received some level of criticism in an ongoing debate. Indeed, the design of an experimental setup that will avoid confounding variables such as volume and nutrients is not a trivial task. In order to unravel the mechanism of plant-plant interactions it is required to record pure neighbour responses and interaction outcomes. This in turn leads to combinatorial designs composed of multiple treatments and conditions. In the most recent publication on this issue two setups (that have in fact been used vastly) were defined as the most reliable (McNickle, 2019). The first one is composed of a single plant and paired plants as well as a single plant grown in half of the standard volume (McNickle & Brown, 2014). The second takes advantage of pots divided by permeable and non-permeable barriers (Zhu et al. 2019). Still, there is no agreement on the proper experimental design apart from the general conclusion, that an ideal design is not available at the moment, and the next best solution is to adjust experiment to the addressed question and at least minimise the impact from the confounding effects (Chen et al. 2020). With this conclusion in mind, in this dissertation multiple experimental designs were used according to the study objectives. Designs are highlighted and commented on in methods sections.

Table 1.1. The description of intraspecific interaction studies on annual plants.

Species	Accession/ Cultivar/ Genotype	Type of competition	Density	Mechanism	Trait/process measured	Fitness	Reported response	Signal	Ref.
Pisum sativum	na	intraspecific intragenotypic	1 plant	exudates	LR direction LR number LR length	na	reduced Total LR length towards the obstacle	organic comps.	Falik et al., 2005
Arabidopsis thaliana	Col-0 *spe-1* *axr4* *aux1-7* *phy-b* *gl1-1* *eto1-1* *xv-1* *pho-2*	intraspecific intergenotypic	1-2 plants	na	Siliques number	Siliques number	higher siliques number in wt paired with *eto1-1* than in single wt	na	Cahill et al., 2005
Avena sativa	Dala	intraspecific intragenotypic	2 plants	exudates	Shoots number Leaves number Total biomass Shoot biomass Root biomass	na	no changes between plants in different belowground partitions (without AC)	organic comps.	Semchenko et al., 2007
Impatiens pallida	Hamilton, Canada	intraspecific intragenotypic intergenotypic	1-4 plants	belowground	Height Branches Leaf biomass Root biomass	na	no response single *vs*. paired decrease in root allocation in intergenotypic int.	na	Murphy et al., 2009
Arabidopsis thaliana	NFA-8, NFA-10, Zdr-1, Zdr-6, Lp2-2, Lp2-6, Kz-1, Kz-9, Ren-1, Ren-11 Bay-0, Mrk-0, Ts-1LL-0	intraspecific intragenotypic intergenotypic	1-4 plants	belowground	Total biomass Siliques number Transcriptome expression	Siliques number	total biomass and siliques number not different in similar intergenotypic pairs of similar accessions total biomass and siliques change in intergenotypic pairs of distant accessions no gene expression change	na	Masclaux et al., 2010
Arabidopsis thaliana	CHA25 Col-0 Ws-0	intraspecific intragenotypic intergenotypic	1 plant	exudates	PR length LR number Hypocotyl length	na	higher LR number in intergenotypic int. shorter PR in intergenotypic int.	phenolic comps.	Biedrzycki et al., 2011

Arabidopsis thaliana	CHA25 Col-0	intraspecific intragenotypic intergenotypic	1 plant	exudates	PR length LR number PGP1 expression ATH1 expression ATH10 expression	na	higher LR number in intergenotypic int. shorter PR in intergenotypic int. ABC transporters upregulated no matching phenotypes with ABC mutants	phenolic comps.	Biedrzycki et al., 2011
Arabidopsis thaliana	CHA25 Col-0	intraspecific intragenotypic intergenotypic	1 plant	exudates	Transcriptome expression	na	genes from 29 categories differently regulated	phenolic comps.	Biedrzycki et al., 2011
Arabidopsis thaliana	Col Ler	intraspecific intragenotypic	exudates pooled from 3-12 seedlings	exudates	Total biomass PR length LR length Vertical growth index Total number of roots contacts Number of contact among roots per lateral root	na	less root contacts with another ecotype	phenolic comps.	Caffaro et al., 2011
Arabidopsis thaliana	Col-0 Ler	intraspecific intragenotypic intergenotypic	1 plant	exudates	Secreted proteome	na	no difference single *vs*. paired no difference in intergenotypic int.	proteome	Badri et al., 2012
Oryza sativa	Azucena Caiapo IR64	intraspecific intragenotypic intergenotypic	1-2 plants	exudates	Shoot biomass Root biomass Allocation Total root length Root surface area Root system volume Convex area Specific root length Median root number Maximum root number	na	reduced shoot biomass reduced root biomass reduced total root length, surface area, root system volume, convex area, median root number single *vs*. paired no differences in intergenotypic int.	na	Fang et al., 2013

T. aestivum	Monkhead 92-46	Intraspecific interspecific	4 plants	exudates	Seed biomass Root biomass Total biomass	na	Lower seed biomass and higher root biomass in 92-46 in response to Monkhead Higher seed biomass but no change in root biomass in Monkhead in response to 92-46	na	Zhu & Zhang, 2013
D. caespitosa	na	Intraspecific interspecific	1 plant	exudates	Root length density Root mass Root branching intensity Specific root length	na	Higher root length density and specific root length in response to unrelated conspecifics	na	Semchenko et al., 2014
Lactuca sativa *Lactuca serriola*	na	intraspecific intragenotypic	1-2 plants	exudates	PR length	na	no difference	na	Aguilera et al., 2016
Glycine max *Helianthus annuus*	na	intraspecific intragenotypic	2 plants	exudates	Seed biomass Vegetative biomass Root biomass	na	no difference	na	Chen et al., 2021
Pisum sativum	Little marvel	intraspecific intragenotypic	2 plants	belowground	Meta-analysis Pods biomass Leaves biomass Shoot biomass Root biomass	na	no difference	na	Mobley et al. 2022
Pisum sativum	Torsdag / wt, rms1, rms3 L77 / wt, rms1	intraspecific intragenotypic	1-4 plants	exudates	Branches number Shoot biomass Root biomass	na	less branches smaller shoots and roots single *vs.* 4 phenotypes occurring later in intergenotypic int.	SLs	Wheeldon et al., 2022
Oryza sativa	Nipponbare / wt, d10-2, d14-2, d17-2 Shiokari / wt Norin 8 / wt, d53	intraspecific intragenotypic	1-3 plants	exudates	Branches number	na	reduced branches number strigolactones synthesis reduced single vs. 3 strigolactones perception increased single *vs.* 3	SLs	Yoenyama et al., 2022

1.5 Thesis aims

Plants respond to their abiotic environment and neighbouring plants, while neighbouring plants modify the abiotic environment (Figure 1.2). The mechanistic understanding of how plants integrate these complex scenarios is essential for unraveling the adaptive strategies underlying their behaviour. Reflecting on the current knowledge gaps motivates a series of questions regarding better comprehension of plant-plant interactions, which are subsequently examined in this thesis:

1. How do abiotic factors impact plant-plant interactions?
2. How do biotic factors impact plant-plant interactions?
3. How do plants detect neighbouring plant presence?
4. What are the responses to neighbouring plants?
5. What determines the interaction outcome?
6. What role do rhizodeposits play in plant-plant interactions?
7. Can we find novel receptors and signals relevant for plant-plant interactions?

This thesis aimed to evaluate the phenotypic responses and outcomes of intraspecific interactions in *Arabidopsis thaliana* and understand the genetic basis of variation in competitive ability and interactions mechanisms.

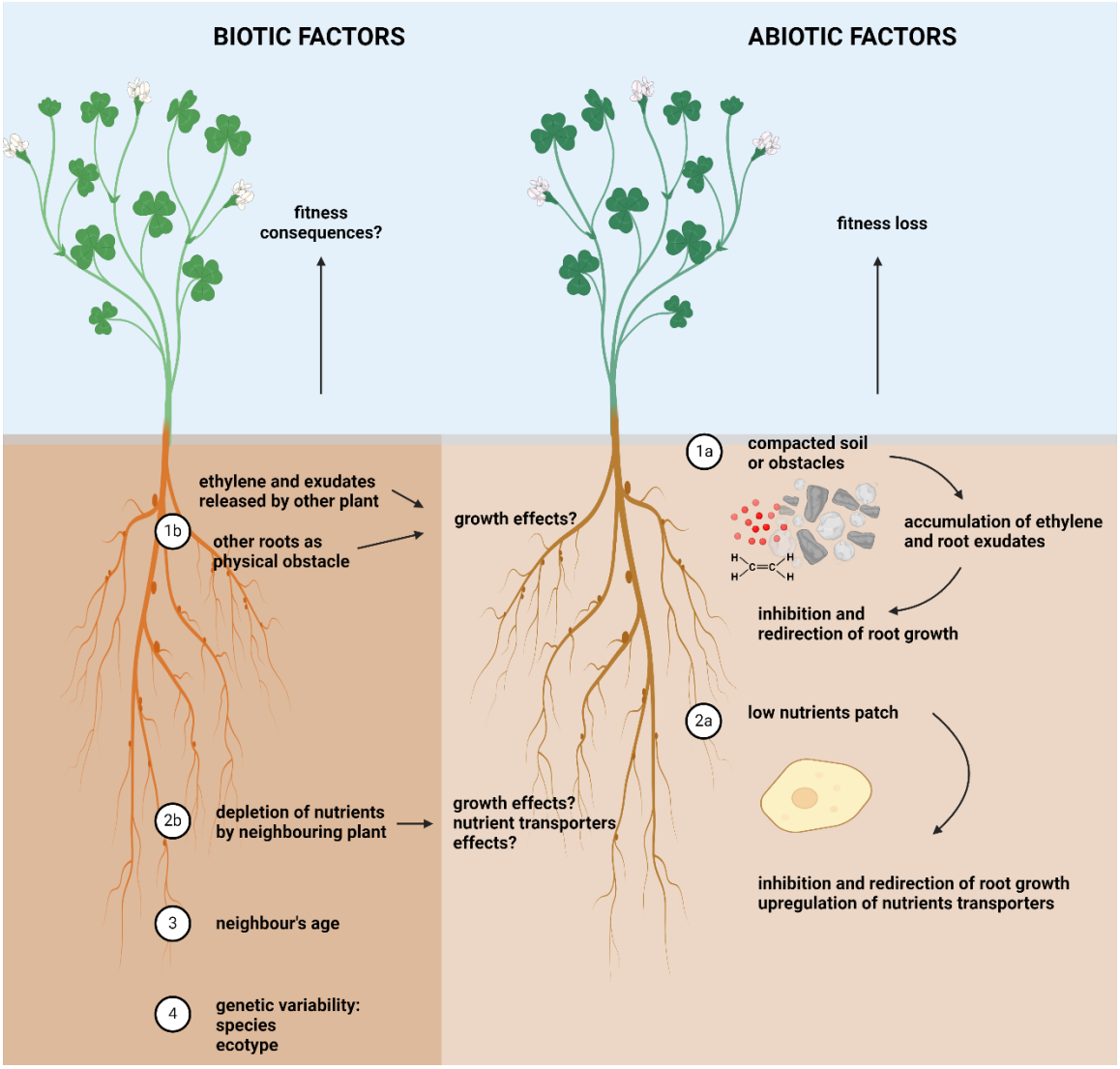


Figure 1.2. An overview of biotic and abiotic factors influencing plant-plant interactions, with a particular emphasis on the rhizosphere.

The abiotic factors relate to the environment i.e. physical space (1a) and nutrients conditions (2a). The biotic factors span from the conditions that are created by neighbouring plants such as physical space occupancy (1b) and nutrient depletion (2b) to inherent characteristics of neighbouring plant such as its age (3) and genotype (4). The biotic and abiotic factors are interconnected and interdependent.

Chapter 2
General Methods

In this dissertation, I have used species from the *Brassicaceae* family. These species, including *A. thaliana*, rely entirely on their root systems for nutrient absorption, as they do not form mycorrhizal or nodulation associations. This characteristic (1) helped minimise the background noise in root signalling by eliminating signals typically involved in listed microbial associations, (2) enhanced the likelihood of observation of plant-plant interactions, as these species depend solely on their own abilities to acquire nutrients therefore are likely very plastic and responsive. *A. thaliana* thrives in a variety of habitats but is predominantly found in disturbed soils as a primary successor (Le Corre, 2005; Thompson, 1994; Wilczek et al., 2009). Despite its widespread presence, *A. thaliana* is not a dominant species and often faces competition from a variety of annual and perennial vegetation. It naturally competes in groups with individuals that are both maternally related and genotypically distinct, even across short microgeographic distances (Bomblies et al., 2010; Brachi et al., 2013; Le Corre, 2005; Frachon et al., 2018).

Whilst data chapters have their own specific methods, this chapter outlines methods common to multiple chapters.

2.1 Plants and growth conditions

For aboveground growth assessment seeds were sown directly on soil and germinated in trays covered with plastic lids under high humidity in controlled environment walk-in chambers (peat-based compost Petersfield No.2 Supreme; 16hrs light, 8 hrs dark; 20°C; 60% humidity). The bottoms of the 30 ml pots were secured with water-permeable tape to prevent roots from escaping. Seedlings were thinned until a single or a pair of healthy seedlings of equal size remained per position. After approximately two weeks, the lids were removed. The pots were watered ad libitum, and to prevent high herbivory pressure by larvae of the dark-winged fungus gnat the biological control agent that contains the insect pathogenic nematode, *Steinernema feltiae* as infective juveniles (ExhibitLine Sf) was applied according to the manufacturer's recommendation. The date of harvesting was

determined through the occurrence of the primary and secondary meristems arrest. All single and one plant chosen at random from each pair were considered focal individuals. Next, as a default in the majority of experiments the aboveground parts were bagged and left to mature and dry. Then shoots and seeds were separated and weighed. In the rare instances where this was not feasible, the total number of siliques was counted. These cases are clearly noted in the methods and results sections of the chapters.

For belowground growth assessment seeds were surface sterilised in 70% ethanol and 7% bleach, resuspended in 0.1% sterile agar, and stratified in 4°C for 48 hours. Afterwards seeds were placed on plates and germinated in controlled environment room (ATS (Wilson, 1988) with 1% sucrose and 1% phytagel (Duchefa); 16hrs light, 8 hrs dark; 20°C). After 7 days seedlings of equal size were transplanted and grown vertically for the next 7 days, single or in pairs. Plates were scanned and analyzed in ImageJ.

2.2 Plant measurements

Direct measurements

For experiments involving measurements at the plant level, the following traits were assessed:

Distribution of traits – the magnitude of given trait on left side of the root which for single grown plants is left side and for paired plant is also the side away from the neighbour as a ratio to the total magnitude of the trait. For example, the proportion of lateral roots number away from the neighbour is the number of roots on the left side of the primary root divided by the total number of lateral roots.

Lateral root density – the number of lateral roots that emerge from the primary root divided by the length of the primary root.

Lateral root number – the count of secondary roots that emerge from the primary root.

Primary root length – the length of the main root.

Relative lateral roots length – the proportion of the length of lateral roots to the length of the primary root.

Reproductive allocation – seed to shoot dry biomass ratio.

Reproductive effort – the proportion of total plant dry biomass allocated to seeds.

Root branching zone – the length of the part of the primary root that has lateral roots emerged.

Seed biomass – dry seed biomass.

Shoot biomass – dry shoot biomass.

Siliques number – the count of the seed pods (fruits) produced during the life cycle. The silique number is considered a good proxy for seed number (Cahill et al., 2005; Weinig et al., 2006).

Derived measurements

For analysis involving assessment of the response or the interaction outcome strength the following metrics were calculated:

The Relative Interaction Index (RII)

The ratio of the performance in competitive environment as compared to the performance in non-competitive environment that measures the intensity of competition (Armas et al., 2004). RII values range from -1 to 1, where it is negative for competition and positive for facilitation.

RII was calculated as follows:

$$RII = B_w - B_o / B_w + B_o$$

where B_o is the trait value of an individual grown in the absence of competition, and B_w is the trait value of an individual grown with competitor.

The mean percentage change

The percentage change in trait value between plants grown in pairs *versus* plants grown single, was calculated as follows:

$$\% \text{ change} = (A_p - A_s) / A_s \times 100\%$$

where A_p is the trait value of an individual grown with competitor, and A_s is the trait value of an individual grown in the absence of competition.

The effect size (Cohen's D)

Cohen's d is a measure of the effect size used when comparing the means of two groups, was calculated as follows:

$$d = M_1 - M_2 / SD_{pooled}$$

where M_1 is the mean of group 1, M_2 is the mean of group 2, SD_{pooled} is pooled standard deviation.

2.3 Experimental design

For the assessment of the effect of being grown singly *vs.* paired plants were grown either as solitary or in pairs. For the assessment of the neighbour genotype effect on interactions the neighbour swap method was used (Figure 2.1). In experiments testing aboveground responses and outcomes, the pot volume was 100 ml. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined earlier in Chapter 2.1 Plants and growth conditions. In experiments testing belowground responses, plants were grown on vertical plates. Plants were co-cultivated for 14 days after which they were treated as described in Chapter 2.1 Plants and growth conditions.

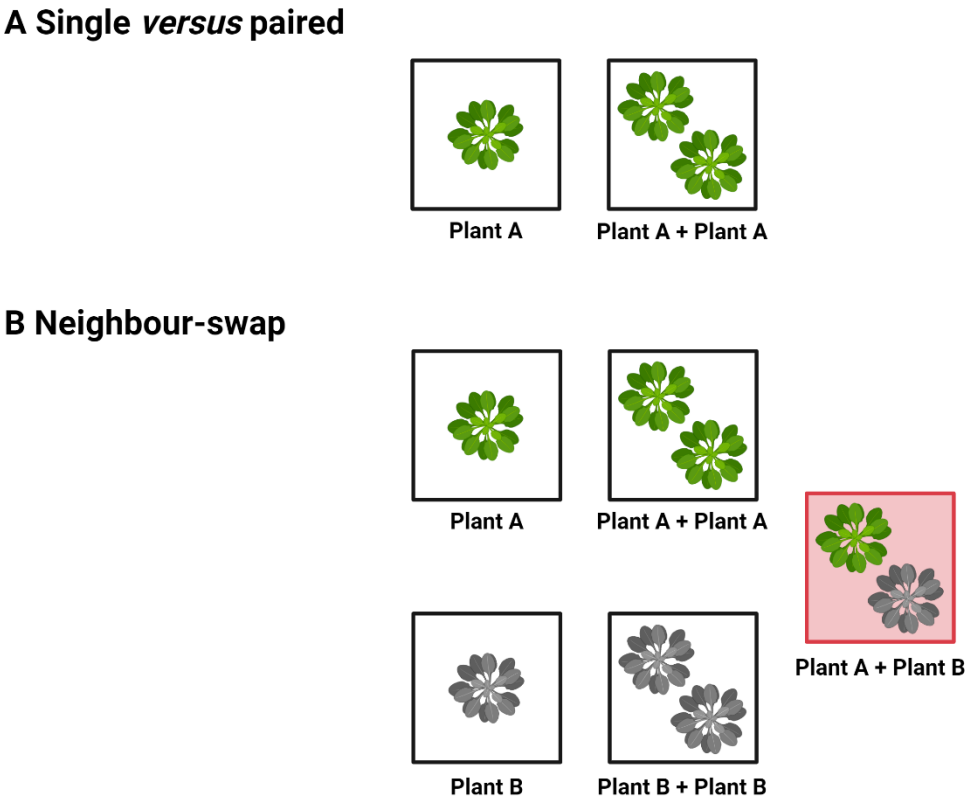


Figure 2.1. The general overview of experimental designs.

(A) An experimental design used to study the effect of neighbour's presence. Plants were grown as single (Plant A) and in intragenotypic pairs (Plant A + Plant A). (B) An experimental design used to study the effect of neighbour's genotype through Neighbour-swap. The method included a single grown plant (Plant A), an intragenotypic pair of plants (Plant A + Plant A), a single plant of interest (Plant B), an intragenotypic pair of plants of interest (Plant B + Plant B), and an intergenotypic pair consisting of one wild type plant and plant of interest (Plant A + Plant B). In intragenotypic pairs consisting of two plants of the same genotype, one random plant from the pair was considered focal plant.

2.4 RNA extraction and gene expression analysis

For expression analysis, Col-0 and chosen other ecotypes or mutants were grown as described in previous section (2.1 Plants and growth conditions). Briefly, plants have been grown either as single or paired for 7 days (this specific timing is following similar method described in Biedrzycki et al. 2011) after which for each genotype and

condition 3 biological samples were collected by pooling ~5 seedlings which were then flash-frozen in liquid nitrogen and stored at –80 °C. Total RNA was extracted using RNeasy Plant Mini kit (Qiagen) and treated with DNase I (Invitrogen) both as per manufacturer instructions. RNA was quantified using Nanodrop 1000 and used as template for cDNA synthesis. For cDNA synthesis, Transcriptor First Strand cDNA Synthesis kit (Roche) was used to reverse transcribe 500 ng of total RNA according to manufacturer's instructions. Quantification of transcript levels was carried using 5 ng of cDNA and PowerUp SYBR Green Master Mix (Applied Biosystems) in reactions of 20 µl volume in CFX Connect Real-time System (BioRad) and the following cycling conditions, according to the manufacturer's guidelines: 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). Three technical replicates were run for each biological replicate and averaged. Two commonly used reference housekeeping genes were tested for their expression stability in single *vs*. paired treatments: ACT2 (ACTIN 2; AT3G18780) and UBQ14 (Ubiquitin 14; AT4G02890) and both genes expression levels did not differ between individually grown and paired plants. Therefore, the relative expression was obtained after normalization to the reference housekeeping gene ACT2 using the ΔΔCt method (Czechowski et al., 2005).

2.5 Statistical analysis and data visualization

All statistical analysis and data visualization were conducted in R version 4.3.1 (R Core Team 2023). Graphs were created with BioRender.com.

Chapter 3
Characterization of abiotic factors that alter neighbour responsiveness and tolerance

Abstract

Plant-plant interactions are strongly influenced by abiotic factors such as rooting volume and nutrient availability, yet their effects on neighbour responsiveness and competition dynamics remain unclear. This study investigates how these factors shape plant interactions by examining *Arabidopsis thaliana* growth under varying rooting volumes and nutrient conditions. Results show that single plants exhibit greater sensitivity to increased space and nutrients than paired plants, challenging the assumption that space and nutrients are interchangeable with neighbour presence. Rooting volume significantly influenced shoot and seed biomass, with competition intensifying as space increased. Similarly, nutrient supply enhanced growth, but paired plants derived fewer benefits than solitary ones. Predictive models based on proportional growth assumptions failed to accurately estimate biomass, suggesting that neighbour effects and abiotic factors interact in complex, non-substitutable ways. These findings support the stress gradient hypothesis, where competition is stronger under optimal conditions, but do not indicate facilitation under resource-limited environments. This study highlights the need for refined experimental designs that account for the interdependence of abiotic and biotic factors in plant interactions. Understanding these dynamics is crucial for predicting plant responses to environmental change and optimizing agricultural resource use.

3.1 Introduction

Plant-plant interactions modulate growth and productivity and are influenced by a complex interplay of abiotic factors, such as the availability of light, nutrients and water resources and environmental stress related to climate (Ballaré & Pierik, 2017; Bilas et al., 2021; Gundel et al., 2014; McNickle et al., 2009; Wang et al., 2021). These factors modulate how plants compete and coexist, shaping their adaptive strategies and interactions (Maestre et al., 2009b; Malkinson & Tielbörger, 2010b; Qi et al., 2018). Understanding these dynamics is crucial for predicting general plant strategies as well as their changes in response to environmental shifts (Aschehoug et al., 2016; Brooker et al., 2008).

3.1.1. Soil physical space as a resource

When a root tip encounters an impenetrable or immovable obstacle, it typically alters its growth direction, bending away from the obstacle to seek an alternative route (Bizet et al., 2016; Fakih et al., 2017; Gong et al., 2021b; Jin et al., 2013; Popova et al., 2013, 2016). The changes of root growth are driven by root perception of space through the increasement of either root exudates or root VOCs concentration rather than through the mechanical force (Falik et al., 2005; Pandey et al., 2021; Semchenko et al., 2007; Wheeldon et al., 2020, 2022). The thigmotropic response is essential for roots to efficiently explore the soil, allowing plants to maximise coverage of areas that contain accessible nutrient and water resources (Joseph et al., 2015; McConnaughay & Bazzaz, 1991). In nature, the most common obstacles comprise rocks, compacted soil or dense rooting systems of neighbouring plants whereas in the experimental settings, the pot volume determines the rooting space (NeSmith & Duval, 1998; Poorter et al., 2012). The effect of rooting volume on plant growth is independent from nutrients availability (Gong et al., 2021b; Poorter et al., 2012; Wheeldon et al., 2020). Previous studies have documented that plant growth is closely related to the availability of soil space (Fry et al., 2021; Gao et al., 2014; Poorter et al., 2012; Schenk, 2006; von Felten & Schmid, 2008). Root growth becomes more restricted

over time as the root density gradually reaches saturation (Casper et al., 2003; Poorter et al., 2012). The decreasing of the rooting volume limits development of aboveground plant organs (NeSmith & Duval, 1988; Poorter et al., 2012). A meta-analysis of various species concluded that, in general, the rooting volume exhibited a linear relationship with reproductive output with doubling the pot volume resulting in a 43% increase in the average mass of plants (Poorter et al., 2012).

The general effect of rooting volume on plant-plant interactions is less clear. Studies on the detection of roots of neighbouring plants are thought to be confounded, among others, by the pot size and it is thought that disentangling the effects of the neighbour from the effects of rooting volume is challenging with the existing experimental methods (Chen et al., 2015, 2020; Hess & De Kroon, 2007; McNickle, 2020; Poorter et al., 2012). A recent meta-analysis concluded that when accounted for the volume-driven increasement in root biomass, a most commonly studied species *P. sativum* showed no additional neighbour response (Mobley et al., 2022). However, this re-analysis did not account for fine root traits and was unable to compare the effects on reproductive output due to the absence of relevant data in the source studies. Further experimentation would be needed to characterise how the relative difference in reproductive output between single plants and paired plants changes as pot volume increases.

3.1.2. Nutrient supply as a stimulus

Plants respond to nutrient availability through a variety of physiological and morphological changes (Khan et al., 2023; Pandey, 2018; Vidal et al., 2020). Some plants also form symbiotic relationships with mycorrhizal fungi or nitrogen-fixing bacteria to enhance nutrient acquisition, especially for phosphorus and nitrogen (Delavaux et al., 2017). Plant strategies for optimizing resource uptake are highly dynamic, complex, and interconnected. In general, root responses can be divided into two main categories: modification of root architecture and regulation of transporter expression (Aibara & Miwa, 2014).

In conditions of abundant nutrients, plants often enhance root growth to take full advantage of the available resources. However, in nutrient-poor environments, plants may also increase root growth in search of more nutrients. The nutrient-related root architecture changes often entail the production of finer or more branching roots aimed to explore larger soil volumes (De Kroon et al., 2009; McNickle et al., 2009). Specific lateral roots chemotropism towards nutrient-rich patches was also observed (Ferrieri et al., 2017). When specific nutrients are scarce, plants upregulate nutrient transport in roots to increase the efficiency of nutrient uptake from the soil (Bhardwaj et al., 2021; Mitra, 2018). This response is sensitive to the location of nutrient-rich patch as well. For example, the CEPR-CEP signalling pathway redirects the upregulation of nitrate transporters NRT1.1, NRT2.1 and NRT3.1 and lateral root growth from root parts located in areas of low nutrient availability to regions with higher nutrient concentrations, optimizing nutrient uptake (Chapman et al., 2020; Tabata et al., 2014; Taleski et al., 2018). The high-affinity phosphate transporters PHT1;1 and PHT1;4 play major roles in its acquisition in both low and high phosphate environments (Misson et al., 2004; Shin et al., 2004).

Much less is known about the specific aspects of nutrient regulation of plant-plant interactions. The mathematical models based on the *ideal free distribution* (IFD) suggest that when soil fertility is low plants produce fewer roots in the presence of neighbours compared to when grown alone. Conversely, in high soil fertility plants invest heavily in excess roots to preempt neighbouring plants, resulting in greater root production compared to solitary growth (McNickle & Brown, 2014). This is in line with the *stress gradient hypothesis* (SGH), according to which plants are anticipated to exhibit cooperative tendencies under conditions of nutrient scarcity (Maestre et al., 2009; Malkinson & Tielbörger, 2010b; Qi et al., 2018). Experimental research also indicates that the adverse effects of root competition on diversity are amplified with the addition of resources through fertilization (Rajaniemi et al., 2003). It was shown that the negative effects of competition on plant survival are particularly potent under resource-poor conditions, as even minor alterations in resource availability can

disproportionately affect the growth and survival of neighbouring plants (Goldberg, 1996b). Investigations directly measuring root competition intensity in varying resource environments consistently reveal that root competition is more intense in resource-poor conditions (Cahill, 1999; Pugnaire & Luque, 2001). This conclusion is further supported by indirect measures of competition intensity (Morris et al., 2003; Peltzer et al., 1998; Rebele, 2000; Wilson & Tilman, 1993). Nutrients may impact interactions at different levels due to variations in their mobility (Raynaud & Leadley, 2004). The uptake of immobile resources like phosphate may be significantly influenced by root overlap between plants compared to more mobile resources such as nitrate or water which are less dependent on root placement (de Vries et al., 2017, 2021). Competition notably affects the uptake of phosphate, suggesting that competition for it, rather than nitrate, more significantly influences interaction outcomes (Chen et al., 2020; Ho et al., 2004; Zhang et al., 2020). Additional research is necessary to determine how the relative differences in reproductive output between individual and paired plants shift as nutrient supply decreases.

3.1.3. Summary

This chapter examines the complex interplay of abiotic factors, including nutrients and physical space, in shaping plant-plant interactions, which are often challenging to separate from other variables in experimental settings. Rooting volume is considered as a non-nutritional resource with a linear effect on individual plant growth, though its broader influence on plant interactions remains unclear. Nutrient supply is also highlighted as a key factor driving plant responses, particularly through its impact on root architecture and transporter regulation. While nutrient availability strongly influences individual growth, its role in plant-plant interactions is less well understood. Overall, this chapter underscores the dynamic strategies plants employ to optimise resource uptake in relation to both rooting volume and nutrient availability.

3.2 Chapter aims

In intraspecific pairs, both interacting plants have matching root sizes and resource acquisition efficiencies. Therefore, the resource uptake in a shared soil volume will be directly proportional to their root sizes and likely equal between plants. Consequently, one can anticipate a hypothetical outcome regarding their fitness outcome as well, namely a relatively balanced distribution of resources allocated to seed biomass between the two plants. This equilibrium could result in comparable shoot and seed biomass for both plants, reflecting a harmonious utilization of available resources without significant competitive advantage for either individual (adapted from Schenk, 2006). That is if the rooting volume or nutrients are the primary drivers of plant interactions and if plants aim to maximise the resources usage then their effects and effect of social setting should be interchangeable. This chapter tested the hypothesis by addressing the following key questions:

1. How do rooting volume and nutrient supply influence competition?
2. Are rooting volume and nutrients supply factors the primary drivers of plant interactions? Are they interchangeable with neighbour's presence?
3. Is competition more intense in optimal conditions than under stress?

This chapter aimed to assess the uncertain effects of abiotic factors and clarify their confounding influence on accurately evaluating plant-plant interactions.

3.3 Methods

3.3.1 Experimental design

3.3.1.1 Assessment of the effect of rooting volume on plant-plant interaction

To examine the impact of rooting volume on plant competition, single and paired plants were grown in pots of varying sizes. To determine whether rooting volume could substitute for the presence of a neighbour, the growth differences of single plants in different pot volumes were compared to those of paired plants. Finally, to assess whether competition intensity varies with rooting volume, the growth of paired plants in small and large pots was analysed. In this experiment *A. thaliana* Col-0 ecotype was used. Plants were grown either singly or in intragenotypic pairs in pots of different volumes: 30, 100 and 500 ml (Figure 3.1). Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The total number pots in this experiment was 42; that comprised 7 replicates per each rooting volume x social setting combination.

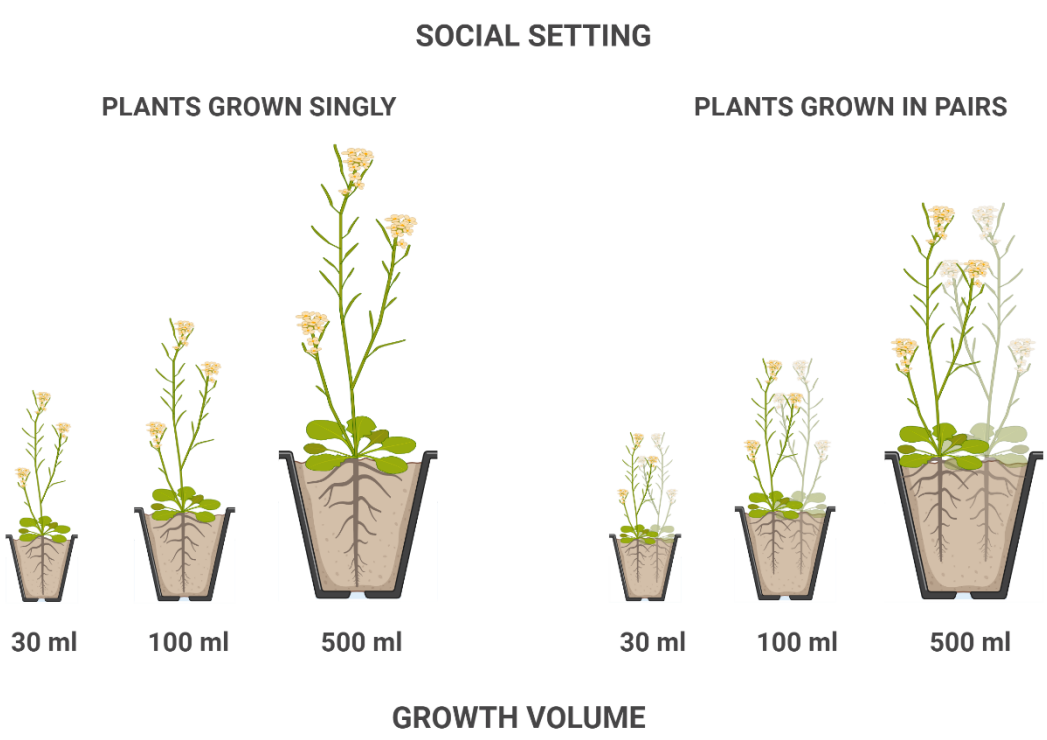


Figure 3.1. The experimental design for assessing the effect of the rooting volume on interactions.

A. thaliana Col-0 plants were grown either singly or in pairs in pots of different volumes: 30, 100 and 500 ml. A one random plant from the paired setting was chosen as focal plant.

3.3.1.2 Assessment of the effect of nutrients on plant-plant interaction

To evaluate the effect of nutrients on plant growth and competition, single and paired plants were cultivated in media with different amounts of nutritious soil mixed with non-nutritious sand to simulate different nutrients concentrations. The growth responses of single plants grown in low and high nutrients were compared to those of paired plants to assess whether nutrients alone influence plant development in paired setting. Additionally, paired plants grown in different nutrients conditions were analysed to determine how nutrients competitive interactions change between optimal an starvation conditions. In this experiment *A. thaliana* Col-0 ecotype was used. Plants were grown either singly or in intragenotypic pairs in pots with different

levels of nutrients: 10% and 100% (Figure 3.2). The 100% nutrients consisted of pure soil and 10% was achieved by proportional dilution of pure soil (100% nutrients) with sand (0% nutrients). The pot volume used was 100 ml. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The total number pots in this experiment was 23; that comprised 5 replicates per single and 5 replicates per paired plants pots in low (10%) nutrients conditions and 6 and 7 pots for single and paired plants pots respectively in high (100%) nutrients conditions.

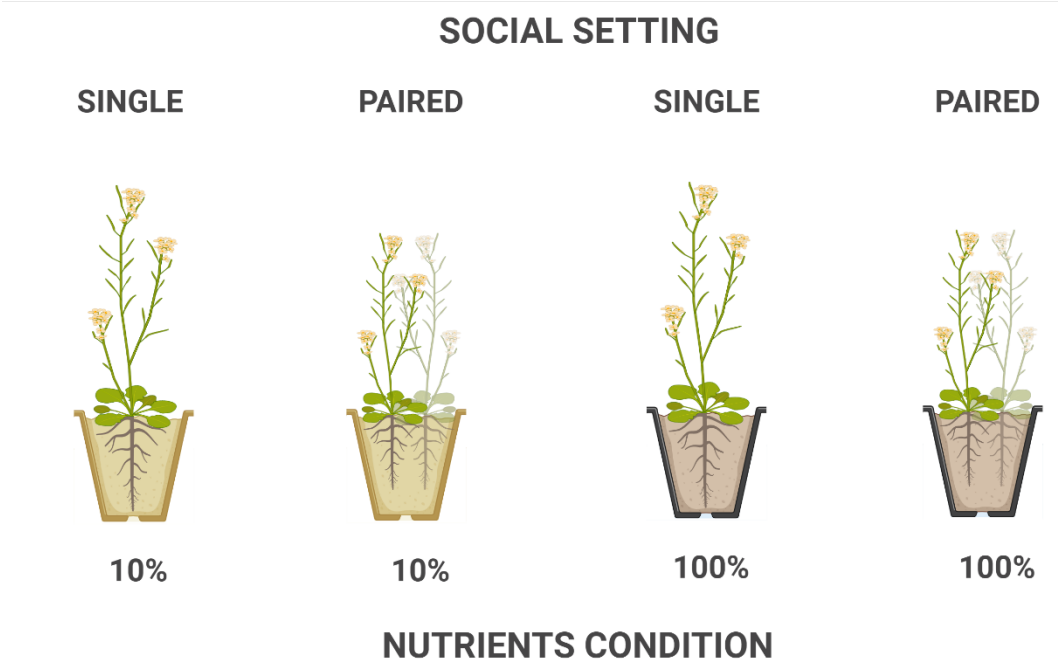


Figure 3.2. The experimental design for assessing the effect of the nutrients condition on interactions.

A. thaliana Col-0 plants were grown either singly or in pairs in pots with different nutrient levels: 10% and 100%. A one random plant from the paired setting was chosen as focal plant.

3.3.3 Statistical analysis

To assess the relationship between abiotic factors and plant-plant interactions, a similar approach was applied to both rooting volume and nutrient levels. Briefly, the first analysis aimed to quantify how changes in rooting volume and nutrients are associated with variation in shoot and seed biomass in single and paired plant social settings separately. Following the published meta-analysis (Poorter et al., 2012), the assumption underlying the model was that the relationship between rooting volume and nutrients (predictor variables) and shoot and seed biomass (response variables) follows a linear pattern. Therefore, Generalized Linear Models (GLMs) with a Gaussian family were employed.

The models were specified as follows:

$$\text{biomass} = \beta_0 + \beta_1 \times \text{predictor} + \epsilon$$

where β_0 is the intercept, β_1 is the coefficient estimating the effect of predictor on biomass, predictor is volume or nutrients, and ϵ denotes the error term, assumed to be normally distributed. The analysis was conducted using the *glm* function from base R (R Core Team 2023). To assess the validity of the model assumptions, diagnostic plots, including residual versus fitted values, Q-Q plots for normality assessment, and homoscedasticity checks, were generated using the performance and ggResidpanel R packages. Additionally, Shapiro-Wilk tests for normality and Levene's tests for homogeneity of variance were performed. To test the significance of the effects of rooting volume or nutrients, social setting type, and their interaction, a two-way Analysis of Variance (2-way ANOVA) was applied. The response variable was biomass, and the predictor variables were either: Rooting volume, with three levels: 30, 100, and 500 mL, or Nutrient condition, with two levels: 10% and 100%, and Social setting type, with two levels: single and paired. The analysis was conducted using the aov function from base R (R Core Team, 2023), and model assumptions were checked as described above.

To investigate the relationships between shoot and seed biomass and explanatory variables, including rooting volume or nutrient levels and social setting type, as well as their interaction, Generalized Linear Models (GLMs) were fitted. Specifically, GLMs with Gaussian error distributions were applied, with the following model specification:

$$\text{biomass} = \beta_0 + \beta_1 \times \text{predictor} + \beta_2 \times \text{social setting} + \beta_3 \times (\text{predictor} \times \text{type}) + \epsilon$$

where β_0 is the intercept, β_1 is the coefficient for the effect of rooting volume or nutrients on biomass, predictor is volume or nutrients, β_2 is the coefficient for the effect of social setting type on biomass, β_3 is the coefficient for the interaction effect between rooting volume or nutrients and social setting type on biomass and ϵ represents the error term assumed to be normally distributed. The analysis was conducted using the *glm* function from base R (R Core Team 2023). Model fit was evaluated using adjusted R^2 and Akaike Information Criterion (AIC). However, since AIC comparisons are not appropriate between models fitted to different datasets (i.e., single and paired plants), model fit for single- and paired-plant models was primarily assessed using adjusted R^2. These values were extracted using the MuMIn package in R (Bartoń, 2023).

To estimate biomass for single and paired plants, predictive models based on established statistical frameworks were employed. Predictions for single plants were derived using the paired model and the predict function from base R (R Core Team, 2023), and the resulting predictions were multiplied by a factor of two. This scaling reflected the theoretical assumption that single plants would exhibit biomass twice the size of paired plants. Conversely, predictions for paired plants were obtained using the single-plant model, with predicted values scaled by a factor of 0.5. This scaling aligned with the theoretical expectation that paired plants would manifest biomass values half the size of single plants in a constant rooting volume or nutrient condition.

3.4 Results

3.4.1 The effect of the rooting volume on interactions

The overall impact of rooting volume, considered as a physical space resource, on plant-plant interactions is widely recognised, however not fully comprehended (Chen et al., 2020; McNickle, 2020). This study tested the hypothesis that if rooting volume is the primary factor influencing interactions, then changes in rooting volume should directly correspond to changes in social settings. Specifically, halving the rooting volume should result in a proportional decrease in growth, with a comparable response anticipated in plants grown in pairs compared to those grown individually in the same volume. To explore this, *A. thaliana* Col-0 plants were grown either individually or in intragenotypic pairs in pots of varying volumes (30, 100, and 500 ml; Figure 3.1). Shoot and seed biomass were then measured as indicators of neighbour responsiveness and tolerance, respectively.

First, separate Generalized Linear Models (GLMs) were fitted for each social setting condition—one model for plants grown individually and another for plants grown in pairs—to assess the overall effect of rooting volume on shoot and seed biomass (Table 3.1). This analysis was conducted to test whether growth responses and outcomes followed a similar pattern in both conditions, and whether single and paired plants responded to changes in rooting volume in a comparable manner. The impact of rooting volume on biomass was positive and consistently significant across all models ($p < 0.001$ for all models), though the strength of this effect differed. The effect of rooting volume on both shoot and seed biomass was stronger in single grown plants compared to plants grown in pairs (for shoot biomass: $\beta_{single} = 0.0061$, SE = 0.0004 *vs.* $\beta_{paired} = 0.0041$, SE = 0.0002; for seed biomass: $\beta_{single} = 0.0027$, SE = 0.0002 *vs.* $\beta_{paired} = 0.0016$, SE = 0.0001). However, in general, the paired models demonstrated better fits to the data than single models (for shoot biomass: $RD_{single} = 2.6336$, $AIC_{single} = 21.995$ *vs.* $RD_{paired} = 0.8748$, $AIC_{paired} = -1.1477$; for seed biomass: $RD_{single} = 0.4346$, $AIC_{single} = -15.842$ *vs.* $RD_{paired} = 0.2259$, $AIC_{paired} = -29.583$). These findings indicated that

while rooting volume positively and significantly impacted both shoot and seed biomass, the relationship was influenced by the social setting, with single-grown plants showing greater sensitivity to changes in rooting volume (Figure 3.3AB, models for single and paired plants labelled as 'observed').

Table 3.1. The summary of GLMs utilised to investigate the effect of independent variable rooting volume (3 levels: 30ml, 100ml and 500ml) on dependent variables shoot and seed biomass. Models were fitted separately for single and paired plants. GLM, n = 7.

Shoot biomass of single plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.1754	0.1157	1.5160	0.1460	
Rooting volume	0.0061	0.0004	15.5400	**2.95E-12**	***

Dispersion parameter for gaussian family taken to be 0.1386	
Null deviance:	36.1071 on 20 degrees of freedom
Residual deviance:	2.6336 on 19 degrees of freedom
AIC	21.995
Fisher Scoring iterations	2

Shoot biomass of paired plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.1074	0.0667	1.6110	0.1240	
Rooting volume	0.0041	0.0002	17.9090	**2.35E-13**	***

Dispersion parameter for gaussian family taken to be 0.0460	
Null deviance:	15.64328 on 20 degrees of freedom
Residual deviance:	0.87483 on 19 degrees of freedom
AIC	-1.1477
Fisher Scoring iterations	2

Seed biomass of single plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.0366	0.0470	0.7790	0.4460	
Rooting volume	0.0027	0.0002	16.9370	**6.39E-13**	***

Dispersion parameter for gaussian family taken to be 0.0229	
Null deviance:	6.99528 on 20 degrees of freedom
Residual deviance:	0.43455 on 19 degrees of freedom

AIC	-15.842
Fisher Scoring iterations	2

Seed biomass of paired plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.0235	0.0339	0.6920	0.4970	
Rooting volume	0.0016	0.0001	13.6320	**2.92E-11**	***

Dispersion parameter for gaussian family taken to be 0.0119	
Null deviance:	2.43505 on 20 degrees of freedom
Residual deviance:	0.22588 on 19 degrees of freedom
AIC	-29.583
Fisher Scoring iterations	2

Further, the combined effects of social setting and rooting volume on shoot and seed biomass were investigated using 2-way ANOVA and GLMs analyses. A significant interaction between social setting and rooting volume was observed for both shoot and seed biomass (for shoot biomass: $\beta = 0.0019$, SD = 0.0004, $p < 0.001$; for seed biomass: $\beta = 0.0011$, SD = 0.0002, $p < 0.001$) (Table 3.2; Appendix A, Table A.1). These results indicated that the effect of rooting volume on both shoot and seed biomass varied depending on whether the plants were grown singly or in pairs.

Table 3.2. The summary of GLMs utilised to investigate the combined effects of explanatory variables: social setting (2 levels: single and paired) and rooting volume (3 levels: 30ml, 100ml and 500ml) on response variables: shoot and seed biomass. The models included interaction terms to assess whether the impact of rooting volume differs between single and paired grown plants. GLM, n = 7.

Shoot biomass

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.1074	0.0959	1.1200	0.2690	
Social setting: single	0.0896	0.1313	0.6830	0.4990	
Rooting volume	0.0041	0.0003	12.4590	**1.59E-15**	***
Social setting: single × Rooting volume	0.0019	0.0004	4.3270	**9.46E-05**	***

Dispersion parameter for gaussian family taken to be 0.0951	
Null deviance:	58.1525 on 44 degrees of freedom

Residual deviance:	3.9005 on 41 degrees of freedom				
AIC	27.655				
Fisher Scoring iterations	2				

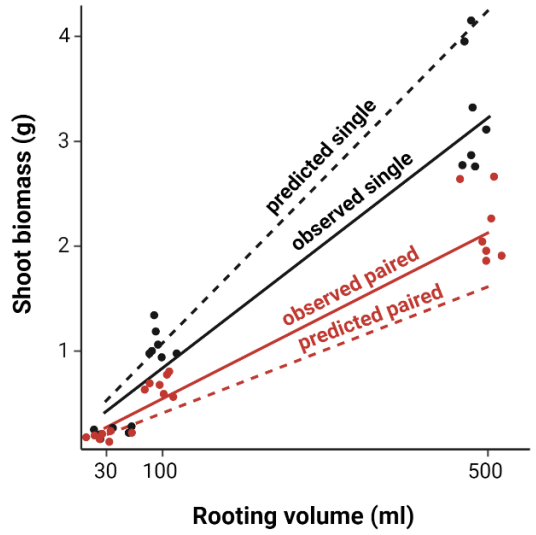
Seed biomass

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.0235	0.0396	0.5930	0.5570	
Social setting: single	0.0195	0.0542	0.3600	0.7210	
Rooting volume	0.0016	0.0001	11.6740	**1.29E-14**	***
Social setting: single × Rooting volume	0.0011	0.0002	6.0730	**3.41E-07**	***

Dispersion parameter for gaussian family taken to be 0.0162	
Null deviance:	10.9978 on 44 degrees of freedom
Residual deviance:	0.6646 on 41 degrees of freedom
AIC	-51.979
Fisher Scoring iterations	2

Next, it was tested whether the effect of rooting volume is interchangeable with the effect of social setting. If space availability was the key driver of plants growth adaptations, then a single grown plant should consistently grow to twice the size of a plant grown in a pair in a given volume. Predictive modeling was employed to estimate biomass outcomes for single and paired plant setting based on separate models established earlier in this chapter (Table 3.1) with adjustments of predictions being multiplied by 2 for single plants based on observed paired plants and by 0.5 for paired plants based on observed single plants (Figure 3.3AB). The predictive models significantly overestimated both shoot and seed biomass for single plants (for seed biomass: $t = -2.67$, $df = 20$, $p = 0.0146$; for shoot biomass: $t = -3.77$, $df = 20$, $p = 0.0012$). In contrary, predictive models significantly underestimated these metrics for paired plants (for seed biomass: $t = 2.01$, $df = 20$, $p = 0.0587$; for shoot biomass: $t = 3.54$, $df = 20$, $p = 0.0021$). These discrepancies were especially notable at higher rooting volumes.

A Neighbour response



B Interaction outcome

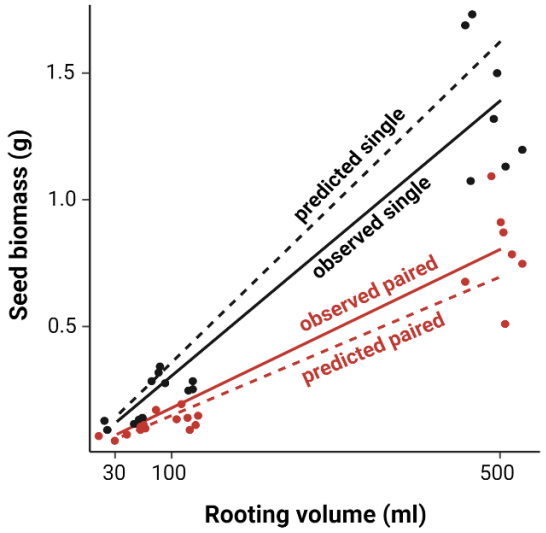


Figure 3.3. The predictive models based on rooting volume overestimate the performance of single plants while underestimating the performance of paired plants.

The observed and predicted relationships between neighbour response measured as shoot biomass (A) and interaction outcome measured as seed biomass (B) and rooting volume for single and paired plants. The observed values are indicated by datapoints and were used to formulate the observed models. Predictive models were derived from these observed data models, based on theoretical assumptions of proportional growth differences between single and paired plants. Specifically, the predicted paired models were derived from observed single plant models assuming

0.5x performance, while the predicted single models were derived from observed paired plant models assuming 2x performance. GLM, n = 7.

3.4.2 The effect of nutrients supply on interactions

While the influence of the nutrient environment on plant-plant interactions is well acknowledged, it is very complex and remains not fully understood (Chen et al., 2020; McNickle, 2020). This study examined the hypothesis that if the nutrient environment is the key factor driving plant interactions, then variations in nutrient supply should directly influence social dynamics. Specifically, reducing the nutrient supply by half should lead to a corresponding reduction in growth, with a similar response expected in plants grown in pairs compared to those grown alone under the same nutrient conditions. To investigate this, *A. thaliana* Col-0 plants were cultivated either individually or in intragenotypic pairs at two nutrient levels: 10% and 100% (Figure 3.2). Shoot and seed biomass were measured to assess neighbour responsiveness and tolerance, respectively.

Initially, individual Generalized Linear Models (GLMs) were applied to each social setting—one model for plants grown individually and another for plants grown in pairs—to evaluate the overall impact of nutrient level on shoot and seed biomass (Table 3.3). This analysis aimed to determine whether growth responses and outcomes exhibited a similar pattern across both settings, and whether single and paired plants reacted comparably to changes in nutrient conditions. The effect of nutrient level on biomass was consistently positive and highly significant across all models ($p < 0.001$ for all), although the magnitude of this effect varied. Nutrient conditions had a stronger influence on both shoot and seed biomass in plants grown individually compared to those grown in pairs (for shoot biomass: $\beta_{single} = 0.0115$, SE = 0.0008 vs. $\beta_{paired} = 0.0075$, SE = 0.0006; for seed biomass: $\beta_{single} = 0.0036$, SE = 0.0002 vs. $\beta_{paired} = 0.0022$, SE = 0.0002). Despite this, the paired models provided a better overall fit to the data than the single models (for shoot biomass: $RD_{single} = 0.1125$, $AIC_{single} = -13.194$ vs. $RD_{paired} = 0.0818$, $AIC_{paired} = -19.81$; for seed biomass: RD_{single} =

0.0078, AIC_{single} = -42.551 vs. RD_{paired} = 0.0103, AIC_{paired} = -44.728). These results suggest that although nutrient level had a significant and positive impact on both shoot and seed biomass, the effect was modulated by social setting, with single-grown plants displaying greater sensitivity to changes in nutrient conditions (Figure 3.4AB, models for single and paired plants labelled as 'observed').

Table 3.3. The summary of GLMs utilised to investigate the effect of independent variable nutrients level (2 levels: 10% and 100%) on dependent variables shoot and seed biomass. Models were fitted separately for single and paired plants. GLM, n = 5-7.

Shoot biomass of single plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	-0.0138	0.0558	-0.2470	0.8100	
Nutrient level	0.0115	0.0008	15.3420	**9.28E-08**	***

Dispersion parameter for gaussian family taken to be 0.0125

Null deviance:	3.05418	on 10	degrees of freedom
Residual deviance:	0.11249	on 9	degrees of freedom
AIC	-13.194		
Fisher Scoring iterations	2		

Shoot biomass of paired plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.0026	0.0451	0.0580	0.9550	
Nutrient level	0.0075	0.0006	12.7130	**1.69E-07**	***

Dispersion parameter for gaussian family taken to be 0.0082

Null deviance:	1.403493	on 11	degrees of freedom
Residual deviance:	0.081777	on 10	degrees of freedom
AIC	-19.809		
Fisher Scoring iterations	2		

Seed biomass of single plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	-0.0057	0.0147	-0.3860	0.7090	
Nutrient level	0.0036	0.0002	18.3110	**1.97E-08**	***

Dispersion parameter for gaussian family taken to be 0.0.0009

Null deviance:	0.2983545	on 10	degrees of freedom		
Residual deviance:	0.0077992	on 9	degrees of freedom		
AIC	-42.551				
Fisher Scoring iterations	2				

Seed biomass of paired plants

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	-0.0035	0.0160	-0.2170	0.8330	
Nutrient level	0.0022	0.0002	10.6860	**8.62E-07**	***

Dispersion parameter for gaussian family taken to be 0.0010			
Null deviance:	0.127319	on 11	degrees of freedom
Residual deviance:	0.010252	on 10	degrees of freedom
AIC	-44.728		
Fisher Scoring iterations	2		

Additionally, the combined effects of social setting and nutrient conditions on shoot and seed biomass were examined using two-way ANOVA and GLM analyses. A significant interaction between social setting and nutrient level was found for both shoot and seed biomass (for shoot biomass: $\beta = 0.0041$, $SD = 0.0009$, $p < 0.001$; for seed biomass: $\beta = 0.0014$, $SD = 0.0003$, $p < 0.001$) (Table 3.4; Appendix A, Table A.2). These findings suggest that the influence of nutrient levels on both shoot and seed biomass differed based on whether the plants were grown individually or in pairs.

Table 3.4. The summary of GLMs utilised to investigate the combined effects of explanatory variables: social setting (2 levels: single and paired) and nutrients (2 levels: 10% and 100%) on response variables: shoot and seed biomass. The models included interaction terms to assess whether the impact of nutrient conditions differs between single and paired grown plants. GLM, n = 5-7.

Shoot biomass

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	0.0026	0.0504	0.0520	0.9590	
Social setting: single	-0.0165	0.0714	-0.2310	0.8196	
Nutrient level	0.0075	0.0007	11.3660	**6.45E-10**	***
Social setting: single × Nutrient level	0.0041	0.0009	4.2900	**0.0004**	***

Dispersion parameter for gaussian family taken to be 0.0102					
Null deviance:	4.68312 on 22 degrees of freedom				
Residual deviance:	0.19439 on 19 degrees of freedom				
AIC	-34.517				
Fisher Scoring iterations	2				

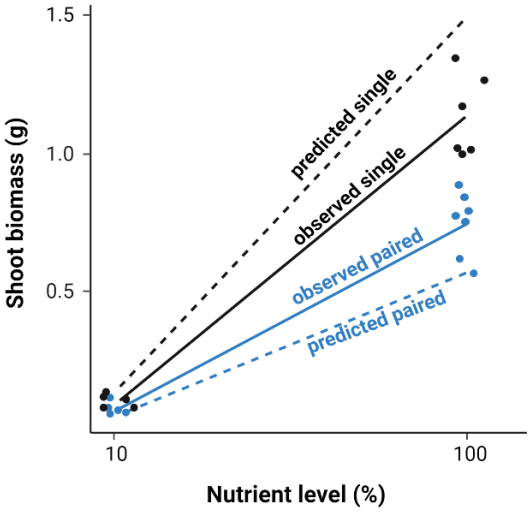
Seed biomass

Coefficients:	Estimate	Std. Error	t value	p value	
Intercept	-0.0037	0.0154	-0.2400	0.8130	
Social setting: single	-0.0020	0.0218	-0.0940	0.9261	
Nutrient level	0.0022	0.0002	11.1220	**9.25E-10**	***
Social setting: single × Nutrient level	0.0014	0.0003	4.8360	**0.0001**	***

Dispersion parameter for gaussian family taken to be 0.0009					
Null deviance:	0.456702 on 22 degrees of freedom				
Residual deviance:	0.018065 on 19 degrees of freedom				
AIC	-89.162				
Fisher Scoring iterations	2				

The next step was to test whether the effect of nutrient level could be considered interchangeable with the effect of social setting. If nutrient supply was the primary driver of plant growth adaptations, a plant grown individually should consistently reach twice the size of a plant grown in pairs at the same nutrient level. Predictive modeling was used to estimate biomass outcomes for single and paired plants, based on separate models developed earlier in the chapter (Table 3.3). Adjustments were made by multiplying the predictions for single plants by 2, based on observed paired plant data, and by 0.5 for paired plants, based on single plant data (Figure 3.4AB). The predictive models significantly overestimated both shoot and seed biomass for single plants (for seed biomass: t = -3.30, df = 10, p = 0.0081; for shoot biomass: t = -3.81, df = 10, p = 0.0034). Conversely, the models significantly underestimated these metrics for paired plants (for seed biomass: t = 2.42, df = 11, p = 0.0340; for shoot biomass: t = 3.44, df = 11, p = 0.0055). These discrepancies were particularly pronounced at higher nutrient levels.

A Neighbour response



B Interaction outcome

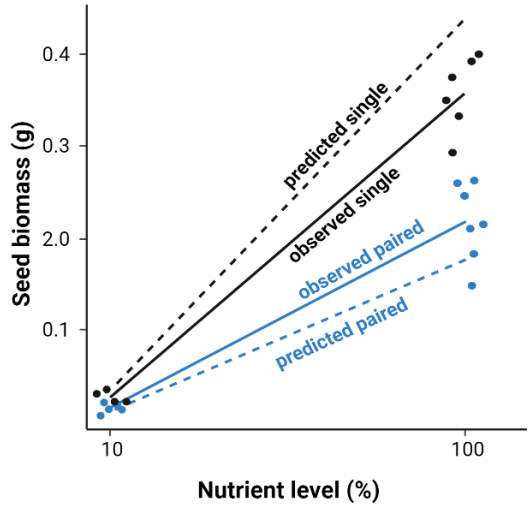


Figure 3.4. The predictive models based on nutrient levels overestimate the performance of single plants while underestimating the performance of paired plants.

The observed and predicted relationships between neighbour response measured as shoot biomass (A) and interaction outcome measured as seed biomass (B) and nutrient level for single and paired plants.. The observed values are indicated by datapoints and were used to formulate the observed models. Predictive models were derived from these observed data models, based on theoretical assumptions of proportional growth differences between single and paired plants. Specifically, the predicted paired models were derived from observed single plant models assuming 0.5x performance, while the predicted single models were derived from observed paired plant models assuming 2x performance. GLM, n = 5-7.

3.5 Discussion

In nature, plant-plant interactions are modulated by multiple abiotic environmental factors (Becker et al., 2023; Cabal et al.,2022; Callaway et al., 2003; Thorpe et al., 2011). The occupation of space is recognised as a key driver of physical niche differentiation, while nutrient conditions shape physiological niches based on nutrient requirements, uptake strategies, and usage efficiency (Levine & Hille Ris Lambers, 2009; Mommer et al., 2010). The significance of these factors has led to doubts about the accuracy of published reports on plant-plant interactions, raising questions about whether experimental designs adequately control for their confounding effects (Chen, 2015; Chen et al., 2020; McNickle, 2020; McNickle et al., 2016; Mobley et al., 2022). This chapter aimed to evaluate the unclear effects of physical space and nutrient supply, and to clarify their impact on plant-plant interactions. To achieve this, neighbour responses and interaction outcomes were examined under varying rooting volumes and nutrient levels, comparing plants grown individually with those grown in pairs.

3.5.1 Plant reactions to space and neighbouring plants are not substitutable

It has long been recognised that physical space functions as a resource for plants and can become a limiting factor for growth (McConnaughay & Bazzaz, 1991; Grams & Luttge 2010). Nevertheless, its effect on plant interactions and the dynamics of competition for space remains unclear (Chen et al., 2020; McNickle & Brown, 2014). The results presented here provide further insight into how rooting volume influences plant growth and reproduction, highlighting potential implications for plant interactions.

The first key finding from this study was that single plants responded differently to changes in rooting volume, showing greater sensitivity and efficiency in utilizing additional space compared to paired plants. Single plants experienced more significant gains in shoot and seed biomass with increased rooting volume, whereas

the benefits for paired plants were diminished—likely due to competition. While the responses of individually grown plants to rooting volume have been widely studied, the specific modulation of these responses by neighbouring plants has received much less attention in the literature. A meta-analysis examining plant responses to pot size across multiple species found that, on average, doubling the pot size leads to approximate doubling of biomass gain (Poorter et al., 2012). Other studies focused on volume sensing in cereals revealed that biomass increases were partly interchangeable between rooting volume and plant density, emphasizing the importance of higher concentrations of root exudates as plant numbers rise and their role in volume sensing (Semchenko et al., 2007; Wheeldon et al., 2020). The conflicting results presented here suggest that these observations may be less common than previously suggested. In the case of *Arabidopsis thaliana*, its sensitivity to rooting volume is distinct from its sensitivity to neighbouring plants. Moreover, this sensitivity intensifies in larger rooting volumes, enabling greater benefits from the additional space. What is more, results presented here challenge the initial hypothesis, revealing discrepancies between observed and predicted growth of single and paired plants under parallel rooting volume conditions. Contrary to expectations, the observed single plants were significantly smaller than predicted, while the observed paired plants were notably larger than anticipated. As a result, the predictive models failed to accurately estimate both single and paired plants based on each other's counterpart's data.

Here, it was also found that, while neighbour effects and rooting responses are distinct, they are highly interactive and interdependent, influencing shifts in the competition strength. In smaller rooting volumes, both solitary plants and those paired with another achieved similar sizes, suggesting limited or weak influence of competition under these conditions. However, at larger rooting volumes, solitary plants grew much larger compared to their paired counterparts, indicating that when space is less limiting, competition becomes more pronounced. These results are somewhat in line with the stress gradient hypothesis (SGH) (Bertness & Callaway

1994; Maestre et al., 2009; Malkinson & Tielbörger, 2010b; Qi et al., 2018). Here, higher levels of competition were observed under the more favourable conditions of larger rooting volumes compared to smaller volumes. However, in low rooting volume conditions, competition—though weaker—was still present, with no evidence of facilitation. So far, SGH was mainly linked to harsh environments characterised by exposure, temperature fluctuations, and drought (Eränen & Kozlov, 2008; Holmgren & Scheffer, 2010; Pugnaire & Luque, 2001). Soil compaction leads to plant stress. However, so far, similar effect from physical obstacles such as high root density or container size have not been explored or linked to the SGH, nor have their impacts been evaluated in this context (Morandage et al., 2021; Pandey et al., 2021). The results presented here suggest that this could be a promising avenue for further research.

3.5.2 Plant responses to nutrients and neighbouring plants are not equivalent

Nutrients are fundamental to plant growth, and the effects of nutrient deficiency—such as inhibited growth, reduced biomass accumulation, and impaired reproductive capacity—have been thoroughly studied (reviewed in Rengel et al., 2022). The relationship between nutrient concentration and distribution has also been widely studied in the context of plant-plant interactions and root foraging (Cahill et al., 2010; Mahal et al., 2023; Padilla et al., 2013; Rehling et al., 2021). Plants have been shown to integrate information about both nutrient availability and the presence of neighbours, generally adopting a broad root foraging strategy in response to resource distribution. However, this strategy was limited by the presence of neighbouring plants (Cahill et al., 2010). Despite extensive research, the impact of nutrients on plant interactions dynamics lacks general conclusions, as separating nutrient depletion caused by neighbouring plants from the overall abiotic effects of the nutrient environment has proven difficult (Chen et al., 2020; McNickle, 2020; Schenk, 2006). The results presented here offer new insights into how the nutrient

environment affects plant growth and reproduction, emphasizing potential consequences for plant interactions.

The primary finding from this study was the differing responses of single and paired plants to changes in nutrient levels, with single plants exhibiting greater sensitivity and efficiency in nutrient absorption compared to paired plants. Single plants experienced more significant increases in shoot and seed biomass as nutrient availability increased, while the benefits for paired plants were less pronounced, likely due to competition. Additionally, this sensitivity intensified at higher nutrient levels, allowing single plants to gain more from the increased nutrient supply. The literature on the effects of nutrients on plant-plant interactions largely emphasises root foraging but oftenly lacks consideration for reproductive output. For example, *H. annuus* was shown to intensely modulate its root occupation in nutrient-rich patches depending on the presence of the neighbouring competitor (Ljubotina & Cahill, 2019), however another study reported that the neighbour presence had no impact on its reproductive output (Chen et al., 2021). Similar conclusions were reported in a recent meta-analysis and parallel experiments focused solely on *P. sativum* that reported no neighbour effect on its reproductive output despite the associated root morphological changes (Mobley et al., 2022). The results presented here contradict the initial hypothesis, uncovering inconsistencies between the observed and predicted growth of single and paired plants under parallel nutrient conditions. Unexpectedly, single plants were significantly smaller than forecasted, while paired plants exceeded predicted growth. Consequently, the predictive models were unable to accurately estimate the growth of both single and paired plants.

3.5.3 Concluding remarks

It is well established that the adoption of competitive strategies by individual plants can depend on contextual factors, with competition being encouraged in some conditions but not in others (Maestre et al., 2009; Malkinson & Tielbörger, 2010b; Qi et al., 2018). This study comprehensively evaluated the effects of rooting volume and nutrient availability on plant-plant interactions. Interestingly, both rooting volume

and nutrient supply had similar impacts on neighbour response, as well as on the interaction outcome—an essential, yet rarely reported, measure of plant-plant relationships. Importantly, the effects of rooting volume and nutrients were found to be interdependent and could not substitute for the influence of neighbouring plants on focal plant growth. The complexity of these relationships made predictive models for interaction outcomes unreliable. Furthermore, intraspecific competition decreased as space and nutrient resources diminished, but unlike other abiotic factors, this did not lead to facilitation in plant-plant interactions. Future research should focus on further disentangling of the complex interplay between rooting volume, nutrient availability, and neighbour presence to improve the accuracy of predictive models. Additionally, further studies are needed to explore how specifically the intraspecific competition dynamics evolve under varying resource constraints and whether these factors could eventually lead to facilitation in certain contexts.

Chapter 4
Evaluation of biotic factors that affect neighbour responsiveness and tolerance

Abstract

Biotic factors such as species identity, genetic variation, and plant age significantly influence neighbour responsiveness and competitive dynamics in plant-plant interactions. This study examines how these factors shape competition and tolerance in *Arabidopsis thaliana* and closely related *Brassicaceae* species. Results reveal that early emergence confers a long-term competitive advantage, with younger plants experiencing reduced fitness when paired with older neighbours. Interspecific competition among *Olimarabidopsis pumila*, *Capsella rubella*, and *Cardamine hirsuta* showed species-specific responses, with *C. hirsuta* demonstrating the highest tolerance to neighbours. Intraspecific variation among *A. thaliana* ecotypes further highlighted differences in neighbour responsiveness and interaction outcomes, with shoot biomass being an unreliable predictor of seed biomass across different social settings. Cluster analysis identified distinct ecotype groups, each exhibiting unique patterns of above- and belowground trait relationships in response to competition. These findings underscore the complex nature of plant-plant interactions and the interplay between genetic background, developmental timing, and competition. Understanding the role of biotic factors in shaping plant competition provides insights into ecological strategies and may contribute to optimizing plant productivity under dynamic environmental conditions.

4.1. Introduction

Biotic factors refer to the living components of an ecosystem, including the characteristics of *plants themselves* that influence their interactions with one another (Callaway, 1995; Kardol et al., 2013; Phillips, 1931; Thorpe et al., 2011). These factors include a variety of plant characteristics that influence their competitive abilities—the capacity to acquire essential resources more efficiently than neighbouring plants. The key factors include species identity, genetic variation, age, and relatedness (Becker et al., 2023; Callaway et al., 2003; Cahill et al., 2005; Subrahmaniam et al., 2018). These biotic factors shape how plants compete for resources or, in some cases, cooperate with neighbouring plants (Dudley, 2015; West et al., 2002, 2021). Consequently, biotic factors determine plant traits such as root architecture, growth rate, and reproductive strategies that as a result play roles in determining the outcome of interactions (Callaway et al., 2003; De Kroon et al., 2005; Turcotte & Levine, 2016). A deeper understanding of the range of biotic factors and their influence on plant-plant interactions is important for identifying both common and specific neighbour responses. This knowledge could help fine-tune the balance between competition and cooperation in response to the demands of environmentally dynamic conditions.

4.1.1. Plant age as a contributor to competitive ability

The relationship between ontogeny and competition in plants is a complex and dynamic interaction that plays an important role in shaping plant communities (Dayrell et al., 2018). Seedling, sibling, and offspring-parent conflicts have large effects on seedling recruitment and has been vastly studied particularly in perennial plants (Cheplick, 1993; File et al., 2012; Milla et al., 2009; Smith et al., 2019). Seedlings may exhibit different growth rates and competitive abilities depending on factors such as seed size, germination timing, and initial resource endowment (Cheplick, 1993; Smith, Atwater & Callaway, 2019). The extent to which the age of the neighbouring plant influences the fitness of the focal plant remains uncertain.

As plants progress through their ontogeny, their competitive abilities may change following a phenomenon of ontogenetic shifts in competitive ability. For example, young plants may invest more resources in rapid growth and resource acquisition to compete effectively with neighbouring plants. However, as plants mature, they may allocate resources differently, prioritizing reproduction or defence mechanisms against herbivores and pathogens (Dayrell et al., 2018; Mason et al., 2013; Schiffers & Tielbörger, 2006). It is unclear whether and how the competitive rank of individuals changes with age.

Ontogenetic shifts in size and resource acquisition can lead to size asymmetry among competing plants, where larger individuals have a competitive advantage over smaller ones. On the other hand, plants of different age may exploit the opportunities for niche differentiation better (File et al., 2012; Kelly & Bowler, 2005; Levine & Hille Ris Lambers, 2009). The size asymmetry between competitors of different age can influence resource partitioning and community structure, with larger plants dominating resource-rich environments and smaller plants persisting in resource-limited habitats (Bassar et al., 2017; Bauer et al., 2004; Cameron et al., 2007; Weiner et al., 1990). Root exudation also differs depending on age and the rate of exudation mirrors plant's metabolic activity being the highest in young roots while decreasing over time as plant matures (Aulakh et al., 2001). In *A. thaliana*, temporal patterns were observed from decrease of sugar and sugar alcohols to increase of amino acids and phenolics over developmental time (Chaparro et al., 2013). Belowground competition is particularly important in plant ontogeny. Early in life, shallow-rooted species may compete more effectively for surface resources, while deep-rooted species can access deeper soil layers. However, as plants mature, the development of deep root systems may confer a competitive advantage in resource-poor environments. While competition between plants of different ages often favours older individuals, the extent of asymmetry can vary depending on various factors. The importance and nature of asymmetric competition remain uncertain, as it is unclear whether competition will be less intense with a younger, smaller neighbour or more

intense due to the neighbour's higher metabolic activity. In summary, the effect of neighbouring plant age on the competitor's fitness and the nature of asymmetric competition remains uncertain, particularly regarding the intensity of competition with younger, smaller neighbours.

4.1.2. Plant genotype as a determinant of competitive ability

Plant genotype defines genetic variation between and within species, influencing traits such as life strategy and relatedness to neighbouring plants (Wuest et al., 2023; Yamawo & Mukai, 2020). Genetic variation, especially among ecotypes adapted to different environments, influences traits like growth rate, root architecture, and nutrient uptake (Byrne & Warren, 2024; Krannitz et al., 1991; Nordborg et al., 2005; Shindo et al., 2007). These variations affect how plants compete and interact, with some species being stronger competitors while others coexist (Callaway et al., 2003; Turcotte & Levine, 2016). Despite an extensive research on plant competition, no universal neighbour responses have been identified, nor have these responses been clearly linked to specific interaction outcomes.

Species

Studies have shown a considerable variation in neighbour responsiveness and tolerance between plant species, with some species exhibiting strong responses to competition, while others being less affected by the presence of neighbours (Smyčka & Herben, 2017). Some species such as *A. sativa*, *V. tricolor*, *A. gerardii* or *B. rapa* maximise their resource acquisition as a response to competition, leading to negative consequences for neighbouring plants (Lankinen, 2008; Markham & Halwas, 2011; Mcnickle & Brown, 2014; Semchenko et al., 2007). Whereas other species i.e. *P. sativum*, *P. vulgaris* or *G. max* do not show those responses (Mobley et al., 2022; Nord et al., 2011; O'Brien et al., 2005). The reason behind such different approaches towards competition between species is not fully comprehended yet. While it is clear that species vary in their competitive abilities, it remains unclear whether those with *similar* phenotypic plasticity exhibit *similar* responses to neighbouring plants.

Species differ in their life strategies regarding survival and reproduction that have consequences on their response to competition. According to Grime (1977; 1979): r-strategists thrive in disturbed environments by growing quickly and reproducing prolifically, K-strategists focus on long-term competition and stability in resource-rich environments, while S-strategists are adapted to tolerate stress, surviving in harsh, resource-limited conditions. Most annual herbaceous plants employ an r-strategy (Bonser & Ladd, 2011; Liao et al., 2021; Šerá & Šery, 2004). The competition between r-strategists is typically short-lived and intense, as both species have fast life cycles (Silvertown & Dale, 1991). It can be hypothesised that different species following the r-strategy are *similarly* highly vulnerable to competition and may respond *similarly* and experience *similar* significant reductions in reproductive output as a result. However, the scarce literature on this topic does not allow such generalizations yet.

Intraspecific diversity

Within a species, subspecies can be distinguished, and these may include ecotypes and varieties that have adapted to different environmental conditions (Hufford & Mazer, 2003). Additionally, in cultivated plant species, cultivars and landraces represent groups shaped by selective breeding and local adaptation (Korir et al., 2013; Villa et al., 2005). A within-species variation in neighbour responsiveness and tolerance has been suggested but the evidence remains ambiguous (Subrahmaniam et al., 2018).

Kin selection theory posits that individuals enhance their fitness by assisting the survival and reproduction of their *genetic relatives*, even when such actions incur costs (Gardner & West, 2014; West et al., 2007). Developed by Hamilton (1964), the theory highlights that the benefits of helping kin can outweigh the costs of reduced individual reproductive success and coined the term *inclusive fitness* (West et al., 2002). However, kin selection probably has limited relevance for wild herbaceous annual species like *A. thaliana*, which are predominantly self-pollinating. As a result,

most intraspecific neighbours are likely to be highly related. Indeed, it was experimentally refuted (Masclaux et al., 2010). In fact, some species, such as i.e. *C. hirsuta*, reduce the likelihood of close genetic encounters through specific seed dispersal strategies (Hay & Tsiantis, 2016; Neumann & Hay, 2020). Additionally, it is known that facilitative and cooperative responses in plants often require a history of co-existence (Schmutz & Schöb, 2024). However, the impact of these reproductive strategies on intraspecific interactions remains unclear.

There are only a few examples of studies on intraspecific interactions in *A. thaliana*. The study of Willis and colleagues (2010) reported significant genetic variation amongst different recombinant inbred lines (RILs) originating from Col-0 × unknown genotype in neighbour tolerance. However, this study probably represents only a subset of the possible variation present in natural ecotypes. Another study focused on *A. thaliana* ecotypes emphasised that interaction outcomes are largely driven by the specific competitive abilities of the accessions (Masclaux et al., 2010). While ecotypes are genetically similar, they display considerable variation in traits shaped by adaptation to their local environments, making them a promising source for exploring common and specific determinants of neighbour responses and interaction outcomes. However, our understanding of ecotypes' capacity in plant-plant interactions remains incomplete.

4.1.3. Summary

This chapter examines key biotic factors shaping plant-plant interactions, including neighbours' age and natural variation between and within species. Plant age influences competitive ability, but the impact of developmental stage and time of neighbour exposure on competition requires further investigation. The genotypic variation affects competitive responses, though it remains unclear why some species are more competitive than others and whether differences exist as well within species between ecotypes. Overall, this chapter underscores the influence of plant age and genetic diversity to better understand plant-plant interactions dynamics.

4.2 Chapter aims

Considering the gaps in our current knowledge prompts several questions about the mechanisms of plant-plant interactions, which are addressed in this chapter:

1. How does competitive rank change with individuals age? Does the age of the neighbour have fitness consequences? What is the importance of asymmetric competition, will competition be less intense with younger neighbours?
2. How are primary neighbour response and tolerance executed?
3. How common are neighbour responses between species?
4. How common are neighbour responses between ecotypes?

This chapter aimed to evaluate the phenotypic responses and outcomes of intraspecific interactions in *Brassicae* and understand the natural variation in competitive ability and interactions mechanisms.

4.3 Methods

4.3.1 Germplasm

Species from *Brassicaceae* family

The *Brassicaceae* family is a large and diverse group of flowering plants that includes economically important species as well as numerous wild species known for their adaptability and ecological significance. All species studied here — *Olimarabidopsis pumila, Capsella rubella*, *Cardamine hirsuta* and a model species *Arabidopsis thaliana* — are wild herbaceous annuals and share a common evolutionary lineage (Figure 4.1). They are characterised as invasive with the ability to colonise disturbed habitats and *C. rubella*, *C. hirsuta* and *A. thaliana* often co-occur. Therefore their competitive abilities in these environments are likely similar. Apart from, *O. pumila* which adapted to high-altitude environments, rarely overlaps with those species in nature. Perhaps different evolutionary paths within the same family resulted in varied ecological roles and sensitivity to plant-plant interactions. What is more, *C. hirsuta* is an example of a ballistic seed disperser, therefore spreads seeds over a wider area, decreasing the likelihood of intraspecific interactions and perhaps due to this also has different neighbour responsiveness and tolerance.

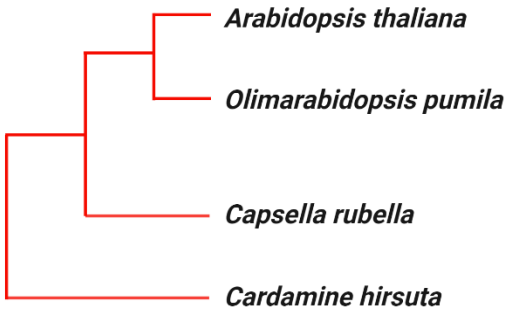


Figure 4.1. The phylogenetic relationship between *Brassicaceae* species.

Arabidopsis thaliana and *Olimarabidopsis pumila* are the most closely related species, *Capsella rubella* is less closely related to *Arabidopsis thaliana* and

Olimarabidopsis pumila. *Cardamine hirsuta* is the most distantly related species among the four.

Arabidopsis thaliana ecotypes

A. thaliana is native to Europe, Asia, and northwestern Africa, but has expanded and is now also found throughout North America and Australia, demonstrating its broad natural distribution across diverse climates and environments (Le Corre, 2005; Thompson, 1994; Wilczek et al., 2009). The 15 *A. thaliana* ecotypes used in this study span a wide geographical range (Figure 4.2). Ecotype seeds were obtained as a courtesy from Prof. Alison Baker (University of Leeds) and further details regarding the germplasm are available (Paudyal et al., 2014), ecotype Ts-0 was purchased from NASC (Table 4.1).

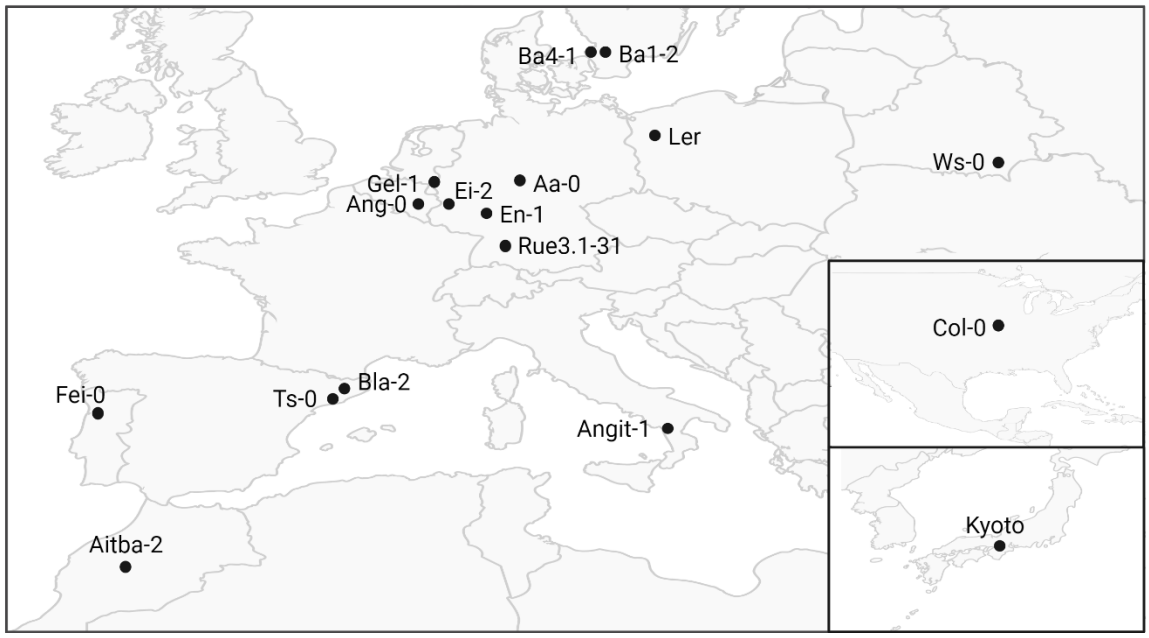


Figure 4.2. The geographical distribution of *A. thaliana* ecotypes used in this study.

Table 4.1. The details of *A. thaliana* ecotypes used in this study.

Code	NASC ID	Country	Location	Latitude	Longitude	Source
Aa-0	N900	Germany	Aua/Rhon	50.9	9.6	Baker
Aitba-2	N76347	Morocco	Ait Barka	31.5	-7.4	Baker
Ang-0	N948	Belgium	Angleur	50.3	5.3	Baker
Angit-1	N76366	Italy	Ponte Angitola	38.8	16.2	Baker
Ba1-2	N76676	Sweden	Ba	56.4	12.9	Baker
Ba4-1	N76677	Sweden	Ba	56.4	12.9	Baker
Bla-2	N28080	Spain	Blanes, Gerona	41.7	2.8	Baker
Col-0	N1092	USA	Columbia	38.3	-92.3	Bennett T
Ei-2	N76478	Germany	Eifel	50.3	6.3	Baker
En-1	N76841	Germany	Enkheim	50.0	8.5	Baker
Fei-0	N76412	Portugal	St Maria da Feira	40.9	-8.5	Baker
Gel-1	N76492	Netherlands	Geleen	51.0	5.9	Baker
Kyoto	N76535	Japan	Kyoto city	35.0	135.8	Baker
Rue3.1-31	N76406	Germany	Rubgarten - 3	48.6	9.2	Baker
Ws-0	N78857	Russia	Wassilewskija	52.3	30.0	Baker
Ler	N6928	Germany	Landsberg	47.9	10.9	Bennett T
Ts-0	N76268	Spain	Tossa de Mar	41.7	2.9	NASC

4.3.2 Experimental design

4.3.2.1 Assessment of the effect of plant age on interaction

To test how does competitive rank change with individuals age plants differing in age were grown together in paired conditions and compared to plants grown in pairs of

equal age. To determine whether asymmetric competition has effect on competition plants paired with younger and smaller neighbour were compared to plants grown with equal neighbour and neighbour that was older and bigger. To examine whether the age of the neighbouring plant has long-term effects on the focal plants fitness plants were grown in pairs till the growth arrest of the focal plant after which its reproductive yield was measured. In this experiment A. thaliana Col-0 ecotype was used. To evaluate the effect of plant age on interaction, neighbouring plants were sown at 5-day intervals, in relation to the focal plant's development (Figure 4.3). The pot volume used was 100 ml. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. In this experiment, the total number of siliques was measured as an indicator of the reproductive output and interaction outcome. The total number pots in this experiment was 28; that comprised 7 replicates per treatment with equal age neighbour, 4 replicates in which the neighbour was 5 days older, 7 replicates where neighbour was 10 days older, 4 replicates where neighbour was 5 days younger and 6 replicates where neighbour was 10 days younger.

4.3.2.2 Assessment of the effect of species on interaction

In order to characterize how neighbour response and tolerance are executed in different but closely related species and to verify the existence of common neighbour responses model plants from *Brassicae* family were grown either single or in pairs and compared. Plants were grown in pots to assess the reproductive output and on plates to measure root responses. In this experiment *O. pumila*, *C. rubella* and *C. hirsuta* were used. To assess the intraspecific neighbour responsiveness and tolerance between different species, plants were grown singly or in intragenotypic pairs. The pot volume used was 100 ml. In paired condition one random plant was chosen as focal plant. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The total number of pots in this experiment was 66;

that comprised 12 replicates of *C. rubella* single, 16 replicates of *C. rubella* paired, 13 and 15 pots of *C. hirsuta* single and paired respectively, 3 and 7 pots of *O. pumila* single and paired respectively. The total number of plates in this experiment was 66; that comprised 10 replicates of *C. rubella* single, 6 replicates of *C. rubella* paired, 19 and 6 replicates of *C. hirsuta* single and paired respectively, 9 and 12 replicates of *O. pumila* single and paired respectively.

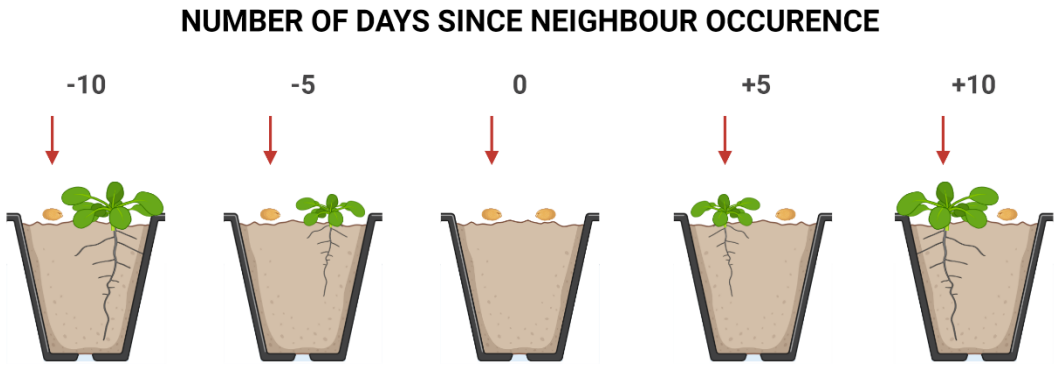


Figure 4.3. The experimental design for assessing the effect of plants age on interactions.

The focal *A. thaliana* Col-0 plants (indicated with red arrow) were grown either alongside the neighbour (Day 0) or with differential neighbour occurrence timing: focal plant was planted when the neighbour was 10 days old (-10 days), 5 days old (-5 days) or neighbour was planted when focal plant was 5 days old (+5 days) or 10 days old (+10 days).

4.3.2.3 Assessment of the effect of ecotype on interaction

In order to characterize how neighbour response and tolerance are executed in different but closely related ecotypes within single species and to verify the existence of common neighbour responses different Arabidopsis thaliana ecotypes were grown either single or in pairs and compared. Plants were grown in pots to assess the reproductive output and on plates to measure root responses. In this experiment the *15 A. thaliana* ecotypes were used (Figure 4.2, Table 4.1). To assess the intraspecific

neighbour responsiveness and tolerance between different ecotypes, plants were grown singly or in intragenotypic pairs. The pot volume used was 100 ml. In paired condition one random plant was chosen as focal plant. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The number of replicates varied but each sample of ecotype in single or paired condition was > 3 replicates.

4.3.3 Statistical analysis

4.3.3.1 Assessment of the effect of plant age on interaction

In order to assess the relationship between the plant's age and the outcome of interactions a linear regression analysis was applied. Briefly, the *lm* function from base R (R Core Team 2023) was used to fit linear model using the neighbouring plants age and reproductive output measured as the total number of siliques. The *residuals* function was used to extract residuals from the fitted model, with *df.residual* used to extract the degrees of freedom of residuals. The *predict* function was used to predict values at regular linear intervals based on the fitted model (R Core Team 2023).

4.3.3.2 Assessment of the effect of ecotype on interaction

In order to assess the relationship between the ecotype and the response and outcome of interactions the following method was applied:

First, to identify trait associations with single and paired growth conditions separate linear regression models were fitted for each ecotype cluster using the *lm* function. Specifically, the relationship between seed biomass (as the dependent variable) and shoot biomass (as the independent variable) was modeled for each cluster. After fitting the models, predictions were generated for each data point within each cluster using the corresponding model. The *predict* function was used to generate predictions (R Core Team 2023). Second, to identify similar response and outcome patterns among the ecotypes and classify them into clusters the *cohens_d* function was defined to calculate effect sizes between paired and single plants for all

traits in all ecotypes using *Cohen.d* function from *effsize* package (Torchiano, 2020). The effect sizes were subsequently z-score normalised with *scale* function. The Principal Component analysis (PCA) was performed using *prcomp* function. Components that explained at least 90% of the variance and their loadings were retained and the top contributing traits were selected. The hierarchical clustering was performed using Ward's method and Euclidean distance matrix with *hclust* function from base R (R Core Team 2023). The Elbow method was used to determine the optimal number of clusters. The resulting dendrogram and heatmap were visualised using *pheatmap* function from *pheatmap* package (Kolde, 2019). Next, the correlation matrices were calculated for each cluster using function *cor* to analyze how different traits correlate with seed biomass within each cluster. Lastly, a linear mixed-effects model was specified to investigate how different traits affect seed biomass while accounting for the variability across clusters. The fixed effects in the model included the selected traits (PR length, Shoot biomass, Reproductive allocation, LR density) as predictors of seed biomass. The random effect's structure included a random intercept for Cluster, allowing the model to account for differences between clusters that are not explained by the fixed effects. The model was fitted using *lmer* function from the *lme4* package. The model was fitted using the Restricted Maximum Likelihood (REML) method, because it provides unbiased estimates of variance components. The model estimated both fixed effects (the influence of each trait on seed biomass) and random effects (the variability in seed biomass due to differences between clusters). All p values for the fixed effects were calculated using Satterthwaite's method, which adjusts the degrees of freedom for the t-tests of the fixed effects. The model was specified as follows:

$$\text{Seed biomass}_{ij} = \beta_0 + \beta_1 \text{PR length}_{ij} + \beta_2 \text{shoot biomass}_{ij} + \beta_3 \text{reproductive allocation}_{ij} + \beta_4 \text{LR density}_{ij} + u_j + \epsilon_{ij}$$

where: β_0 is the overall intercept; $\beta_1, \beta_2, \beta_3, \beta_4$ are the fixed effect coefficients for the predictors PR length, shoot biomass, reproductive allocation, and LR density,

respectively; u_j represents the random intercept for cluster j, with $u_j \sim N(0,\sigma_u^2)$; ϵ_{ij} is the residual error for observation i within cluster j, with $\epsilon_{ij} \sim N(0,\sigma_\epsilon^2)$.

4.4 Results

4.4.1 Assessment of the effect of plant age on interaction

The relationship between ontogeny and competition in plants is complex and varies across space and time, significantly influencing niche partitioning (Bassar et al., 2017; Dayrell et al., 2018; Schiffers & Tielbörger, 2006). However, the degree to which the age of neighbouring plants impacts the fitness of the focal plant is still unclear. To investigate the impact of the neighbour age on plant-plant interactions, the timing of neighbour occurrence was manipulated. Neighbours were planted at varying times relative to the focal plants: earlier, simultaneously, or later (Figure 4.3). Subsequently, the reproductive output measured as the siliques number of the focal plants was assessed to determine the outcome of the plant-plant interactions.

The timing of the neighbour occurrence significantly affected the focal plant, with variations in the strength of effect observed (Figure 4.4A). The presence of a neighbour plant that was already 10 days older significantly undermined the fitness of the newly emerging focal plant. Focal plants that have been grown alongside a neighbour from day 0 exhibited fitness that was not significantly different from focal plants with neighbours 5 days older or younger. The analysis of the relationship between the number of siliques and the timing of neighbour occurrence provided statistically significant, but moderate correlation between the neighbour' age and the number of siliques produced by the focal plant ($r = 0.665$, $p < 0.0001$) (Figure 4.4B).

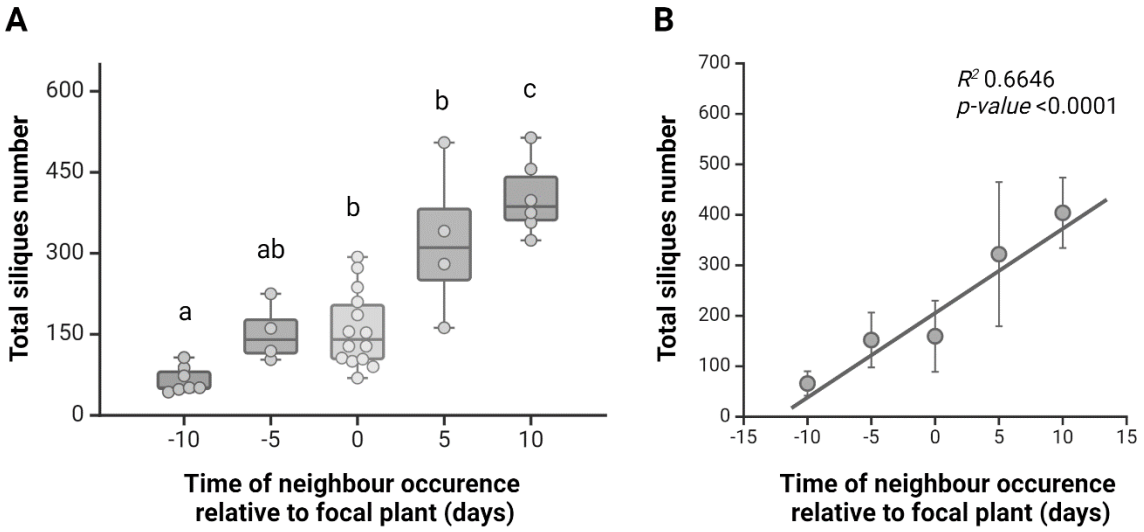


Figure 4.4. The negative effect of neighbour presence scales with neighbour age and the time of focal plant exposure.

(A) The silique number of focal plants from pairs of different age difference (neighbour planted 10 days in advance (-10), 5 days in advance (-5), at the same day as focal plant (0), 5 days older neighbour (5) and 10 days older neighbour (10)). One-way ANOVA with Tukey HSD, $p < 0.01$, $n = 4\text{-}14$. (B) The correlation between neighbour occurrence timing and siliques number. Pearson correlation coefficient, $p < 0.0001$.

4.4.2 Assessment of the effect of species on interaction

Plant species are distinct groups defined by variations in their genotypes, morphology, physiology, and reproductive strategies. They display a wide range of responses to competition (Subrahmaniam et al., 2018, 2021). The *Brassicaceae* family comprises numerous annual herbaceous wild species known for their adaptability and ecological diversity (Raza et al., 2020). In this study, several *Brassicaceae* members — *O. pumila*, *C. rubella*, and *C. hirsuta* — were chosen for investigation of the interactions on the species level. The plants were grown either individually or in intragenotypic pairs, and multiple traits were assessed to identify potential common patterns or relationships between their above- and belowground responses.

A significant impact of both the species and social setting type was observed on both shoot and seed biomass, but their interaction was significant only for seed biomass (Table 4.2). In other words, in terms of shoot biomass the species and social setting effect are independent of each other therefore, the effect of growing a plant alone or with another plant was the same across all species. The detailed analysis of the *Brassicae* species illustrated the similar shoot growth and varying reproductive outputs when grown singly (Figure 4.5AB). The presence of the neighbour caused a significant decrease in shoot biomass in all species but decrease in seed biomass was significant only in case of *O. pumila* and *C. rubella*, whereas *C. hirsuta* maintained relatively high seed output. The variation could have also been observed in the levels of seed biomass loss due to being grown in pair. The *O. pumila* was proven to be the least and C. hirsuta the most tolerant species (the mean percentage change between single *vs.* paired plants being ~ -20% and ~ -50% respectively) (Figure 4.5C). The RII indicated varying strengths of competitive interaction (Figure 4.5D). Overall, *O. pumila* showed the highest seed production without competitor and experienced the highest loss in the presence of the competitor. However, the same conclusion does not apply to the other two species. *C. hirsuta* produced similar seed biomass as *C. rubella* but both species differed in the level of tolerance to the neighbour.

Table 4.2. The summary of 2-way ANOVA utilised to investigate the effects of explanatory variables: social setting (2 levels: single and paired) and species (3 levels: *O. pumila, C. rubella*, *C. hirsuta*) on response variables: shoot and seed biomass. n = 3-13.

Shoot biomass

	Df	F value	p value	
Species	2	3.408	**0.0400**	*
Social setting	1	18.136	**7.35E-05**	*
Species × Social setting	2	0.236	0.7910	

Seed biomass

	Df	F value	p value

Species	2	9.727	**0.0002**	*
Social setting	1	21.879	**1.70E-05**	*
Species × Social setting	2	4.293	**0.0180**	*

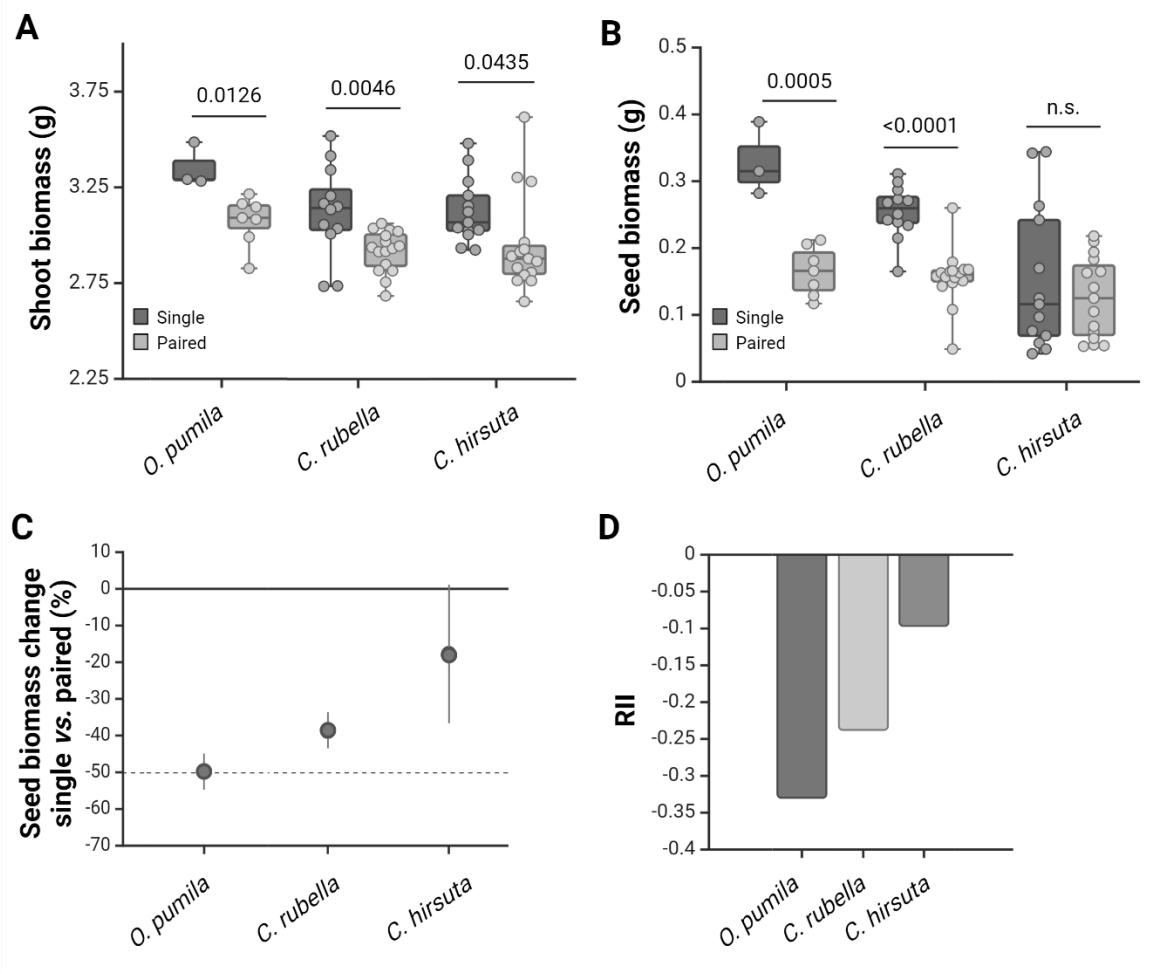


Figure 4.5. Among *Brassica* species, the neighbour tolerance does not scale with seed biomass of plants grown singly.

The variation in aboveground responses to neighbours and interaction outcomes of *Brassicae* species. The species in the graph are arranged in phylogenetic order, from the closest to the most distantly related to *A. thaliana*. The shoot (A) and seed (B) biomass of plants grown singly and paired. Student's t-test, n= 3-13, p-values indicated. (C) The mean percentage change in seed biomass between paired and singly grown plants. -50% decrease threshold indicated with dashed line. (D) RII as a function of seed biomass.

Additionally, it was examined whether the differences observed among species in aboveground responses and interaction outcomes were related to belowground growth. Social setting did not influence any of the root traits tested. Species alone had a significant effect on root morphology changes (Table 4.3). Consequently, only a few significant differences between social settings were found in analyzed root traits (Figure 4.6ABC). A minor and non-significant increase in primary root length in response to neighbouring plant was observed across all species (Figure 4.6A). However, the only statistically significant difference was observed in the increase of lateral root number and length in response to neighbour in *C. rubella*.

Table 4.3. The summary of 2-way ANOVA utilised to investigate the effects of explanatory variables: social setting (2 levels: single and paired) and species (3 levels: *O. pumila, C. rubella*, *C. hirsuta*) on response variables: primary root length and lateral roots number and length. n = 6-13.

Primary root length

	Df	F value	p value	
Species	2	15.8990	**5.32E-06**	*
Social setting	1	2.7130	0.1060	
Species × Social setting	2	0.0480	0.9540	

Lateral root number

	Df	F value	p value	
Species	2	120.927	**2.94E-19**	*
Social setting	1	0.1350	0.7150	
Species × Social setting	2	0.3090	0.7360	

Relative lateral root length

	Df	F value	p value	
Species	2	115.147	**7.68E-19**	*
Social setting	1	0.0001	0.9900	
Species × Social setting	2	0.0910	0.9130	

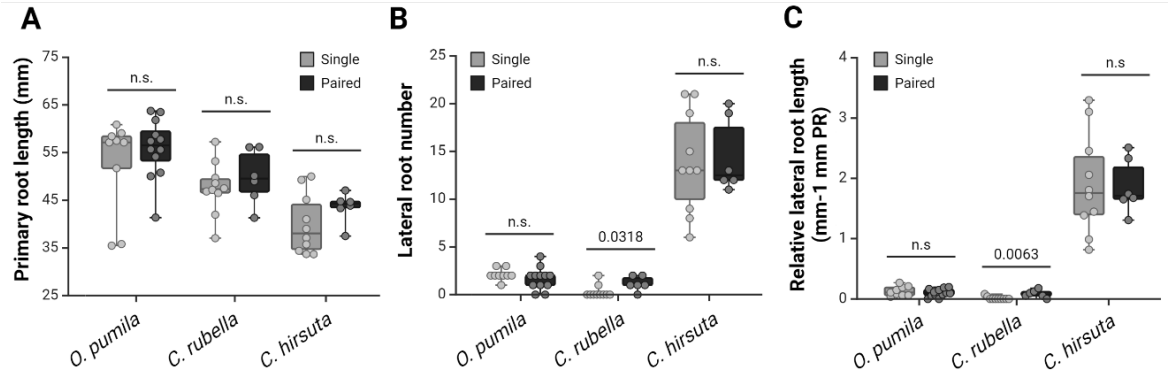


Figure 4.6. Among *Brassica* species, the presence of neighbour does not evoke morphological adjustments in the root system.

The variation in belowground responses to neighbours and interaction outcomes in *Brassicae* species. The species in the graph are arranged in phylogenetic order, from the closest to the most distantly related to *A. thaliana*. The primary root length (A), lateral root number (B) and relative lateral roots length (C) of plants grown singly and paired. Student's t-test, n= 6-13, p-values indicated.

Overall, no relationship was observed between aboveground responses, belowground responses, and interaction outcomes among the studied species, despite their close genetic relationship. *C. hirsuta* demonstrated to be the most neighbour-tolerant species, showing minimal reduction in seed biomass when grown in pairs. In contrast, *O. pumila*, identified as the least tolerant species, exhibited a similar lack of belowground response. Interestingly, *C. rubella*, categorised as moderately tolerant, was the only species to exhibit significant belowground responses, showing root proliferation in reaction to the presence of a neighbour.

4.4.3 Assessment of the effect of ecotype on interaction

Ecotypes are genetically distinct populations within a species that have adapted to specific environmental conditions. These adaptations result from natural selection, allowing ecotypes to thrive in particular climates, soils, altitudes, or other ecological settings (Fletcher et al., 2022). A wide variety of *A. thaliana* ecotypes was tested to provide a thorough evaluation of how genetic variation affects competition

responses and interaction outcomes at the ecotype level (Figure 4.1; Table 4.1). The plants were grown either individually or in intragenotypic pairs, and multiple traits were assessed to identify potential common patterns or relationships between their above- and belowground responses. In general, the paired growth condition led to decrease in seed and shoot biomass but increased reproductive allocation, primary root length, lateral root density, and relative lateral root length. There was a considerable variation among ecotypes, indicating that genetic factors related to environmental adaptations play a significant role in how plants respond to neighbours (Figure 4.7A-F).

The effects of ecotype and social setting on various above- and belowground traits demonstrated significant influences across multiple response traits, with notable interactions between ecotype and social setting for several traits (Table 4.4). Shoot and seed biomass, reproductive allocation and effort were significantly influenced by a significant interaction between the two factors, indicating that the impact of social setting varied across ecotypes. Significant interactions between ecotype and social setting were also observed for several belowground traits, including LR density, total RS length, and relative LR length. Among the traits analyzed, a few were notably affected solely by the social setting. These included the proportion of LR number, proportion of LR length and proportion of LR angle away from neighbour. The absence of significant ecotype effects or interactions for these traits suggests that this response to social setting was consistent across different ecotypes, highlighting a general adaptive strategy in root behaviour influenced primarily by the presence of neighbouring plants.

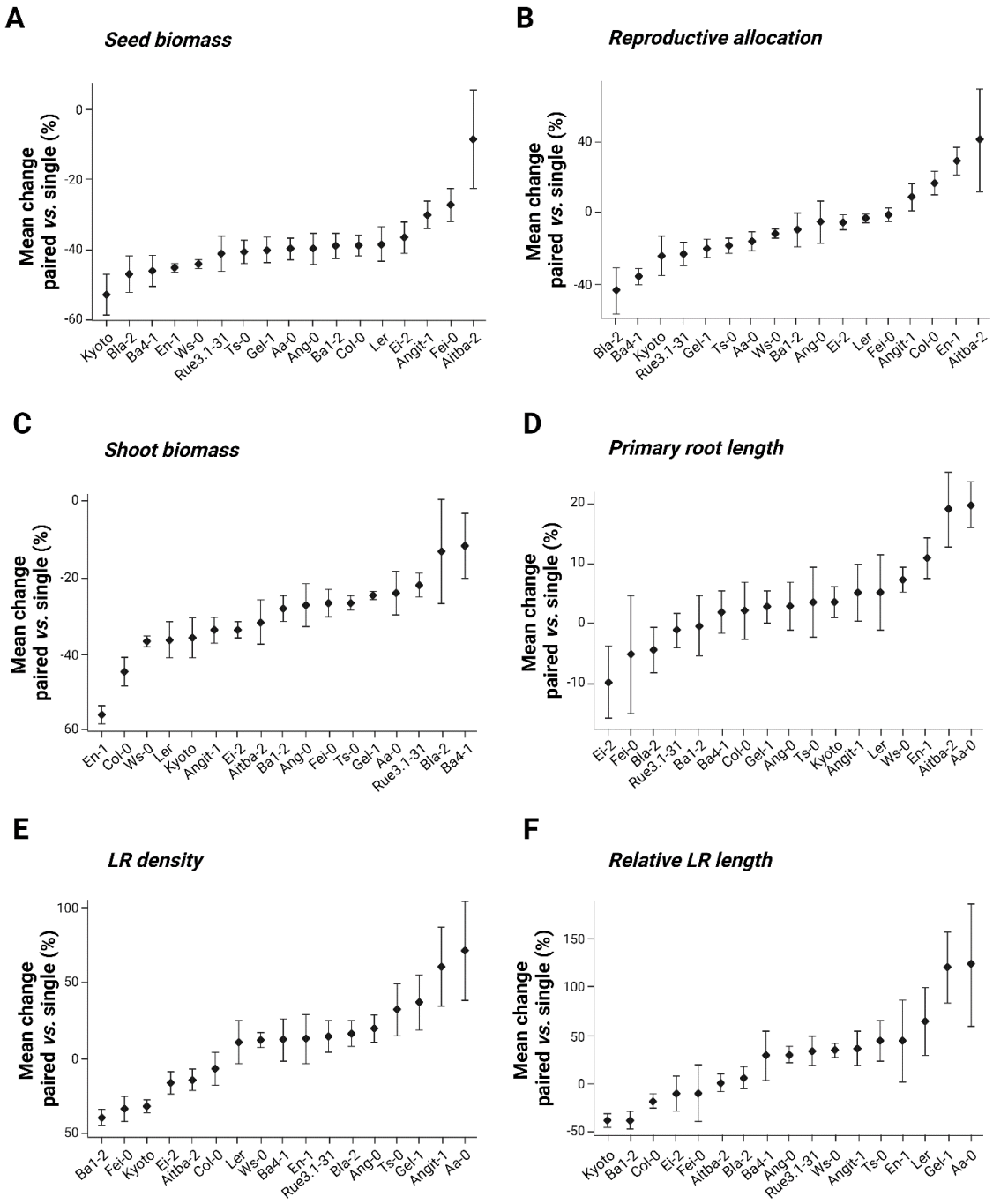


Figure 4.7. The ecotypes of *A. thaliana* are characterised by significant natural variation in neighbour responsiveness and tolerance.

The mean percentage change in traits expression between paired plants and single plants across the ecotypes panel. LR Lateral roots. Mean difference ± SEM, n = 3-20.

Table 4.4. The summary of 2-way ANOVAs utilised to investigate the effects of explanatory variables: social setting (2 levels: single and paired) and ecotype (17 levels: ecotypes panel) on response variables: above- and belowground traits. LR Lateral roots, PR Primary root, RS Root system. 2-way ANOVA, n = 3-20.

		Df	F value	p value	
Shoot biomass	Ecotype	16	20.7	**< 2e-16**	***
	Social setting	1	246.3	**< 2e-16**	***
	Ecotype × Social setting	16	2.8	**0.0003**	***
	Residuals	235			
Seed biomass	Ecotype	16	50.8	**< 2e-16**	***
	Social setting	1	395.6	**< 2e-16**	***
	Ecotype × Social setting	16	5.2	**< 0.0001**	***
	Residuals	238			
Reproductive allocation	Ecotype	16	31.6	**< 2e-16**	***
	Social setting	1	10.2	**0.0016**	**
	Ecotype × Social setting	16	3.3	**< 0.0001**	***
	Residuals	235			
Reproductive effort	Ecotype	16	24.5	**< 2e-16**	***
	Social setting	1	17.6	**< 0.0001**	***
	Ecotype × Social setting	16	3.8	**< 0.0001**	***
	Residuals	235			
Primary root length	Ecotype	16	42.6	**< 2e-16**	***
	Social setting	1	4.2	**0.0419**	*
	Ecotype × Social setting	16	1.3	0.2137	
	Residuals	276			
RS branching zone	Ecotype	16	**32.3**	**< 2e-16**	***
	Social setting	1	0.0	0.9222	
	Ecotype × Social setting	16	**2.1**	**0.0084**	**
	Residuals	276			
LR density	Ecotype	16	**6.4**	**< 0.0001**	***
	Social setting	1	1.1	0.2874	
	Ecotype × Social setting	16	**2.9**	**0.0002**	***
	Residuals	276			
Relative LR length	Ecotype	16	**26.8**	**< 2e-16**	***
	Social setting	1	1.3	0.2560	
	Ecotype × Social setting	16	**2.4**	**0.0028**	**

	Residuals	276			
LR number	Ecotype	16	10.6	**< 2e-16**	***
	Social setting	1	0.0	0.8848	
	Ecotype × Social setting	16	2.5	**0.0012**	**
	Residuals	276			
Total RS length	Ecotype	16	23.6	**< 2e-16**	***
	Social setting	1	0.6	0.4516	
	Ecotype × Social setting	16	2.8	**0.0004**	***
	Residuals	276			
Proportion of LR number away neighbour	Ecotype	16	0.7	0.7625	
	Social setting	1	15.1	**0.0001**	***
	Ecotype × Social setting	16	0.6	0.8980	
	Residuals	276			
Proportion of LR length away neighbour	Ecotype	16	1.1	0.3735	
	Social setting	1	14.5	**0.0002**	***
	Ecotype × Social setting	16	0.5	0.9309	
	Residuals	276			
Proportion of LR angle away neighbour	Ecotype	16	0.6	0.8700	
	Social setting	1	17.8	**< 0.0001**	***
	Ecotype × Social setting	16	1.1	0.3630	
	Residuals	276			
RS depth	Ecotype	16	26.1	**< 2e-16**	***
	Social setting	1	3.6	0.0581	
	Ecotype × Social setting	16	1.3	0.1856	
	Residuals	276			
RS width	Ecotype	16	12.9	**< 2e-16**	***
	Social setting	1	3.8	0.0508	
	Ecotype × Social setting	16	1.5	0.1055	
	Residuals	276			
RS angle	Ecotype	16	9.9	**< 2e-16**	***
	Social setting	1	0.3	0.6150	
	Ecotype × Social setting	16	0.7	0.8290	
	Residuals	276			

Social setting-specific analysis

Further, it was tested whether the relationship between seed biomass and other traits differed between the social settings (single *vs.* paired) by examining if the effect of traits on seed biomass changed depending on the social setting (Table 4.5). The model that included interaction term between each trait and the social setting showed significantly better fit therefore the effect of each trait on the seed biomass differed depending on whether the plants were grown singly or in pairs (Appendix B, Table B.1). The follow-up analysis that used a Mixed-Effects Model approach investigated in detail how various plant traits, along with social setting influenced seed biomass (Table 4.5). The significant interactions between social setting and key traits were observed only in the case of shoot biomass and reproductive allocation suggesting that these traits impact seed biomass differently depending on whether plants are grown alone or in pairs.

Therefore, the linear regression analysis was applied to model a predictive relationship between seed biomass and shoot biomass. A detailed direct examination of the shoot and seed biomass relationships across ecotypes and social settings revealed a highly variable response in terms of both direction and magnitude (Figure 4.8). Of the 17 ecotypes studied, the majority did not exhibit a significant linear relationship between seed and shoot biomass in either social setting. This included 9 ecotypes: Aa-0, Ang-0, Angit-1, Ba1-2, Ba4-1, Bla-2, Col-0, Ei-2, and Kyoto. Only 5 ecotypes (Fei-0, Gel-1, Ler, Ts-0 and Ws-0) showed significant and positive linear relationships between shoot and seed biomass *in both* single and paired settings. However, the strength of the relationship differed between the social settings, being generally stronger in paired plants. In the case of 2 ecotypes (En-1 and Rue3.1-31) shoot biomass was a good predictor of seed biomass only in single plants, not in paired condition. Conversely, in only 1 ecotype (Aitba-2) this relationship was significant only in the case of paired plants.

In summary, this analysis showed that shoot biomass is not a reliable predictor of seed biomass across ecotypes, especially in plant-plant interactions. Most ecotypes did not exhibit a significant linear relationship between shoot and seed biomass, with only a few showing consistent positive relationships in both single and paired settings. The relationship was generally stronger in paired plants, but this varied across ecotypes. Overall, plant-plant interactions introduce variability, making shoot biomass an inconsistent estimator of seed biomass.

Table 4.5. The summary of Mixed-Effects Model utilised to investigate how various plant traits and their interactions with social setting influence seed biomass. Linear mixed model fitted by REML. T-tests used Satterthwaite's method. Number of observations: 233, groups: Social setting × (Ecotype × Cluster), 34; Ecotype × Cluster, 17; Cluster, 4.

Linear mixed model fit by REML

REML criterion at convergence: -908.3

Scaled residuals:

Min	1Q	Median	3Q	Max
-5.9141	-0.2402	0.046	0.4303	3.08

Random effects:

Groups	Name	Variance	Std.Dev.
Social setting × (Ecotype × Cluster)	Intercept	1.00E-04	0.010021
Ecotype × Cluster	Intercept	3.27E-04	0.018081
Cluster	Intercept	5.29E-05	0.007272
Residual		6.78E-04	0.026036

Fixed effects:

	Estimate	Std. Error	df	t value	p value	
Intercept	-1.04E-01	2.13E-02	98	-4.904	**3.72E-06**	***
PR length	-9.13E-05	2.55E-04	174	-0.358	0.720531	
Social setting × single	-1.83E-01	2.94E-02	36	-6.209	**3.62E-07**	***
Shoot biomass	2.71E-01	1.80E-02	193	15.022	**< 2e-16**	***

Repr. allocation	4.15E-01	2.31E-02	135	17.986	**< 2e-16**	***
LR density	-5.19E-02	5.46E-02	214	-0.952	0.342261	
Relative LR length	5.83E-03	8.95E-03	182	0.652	0.515371	
PR length × Social setting × single	8.70E-05	3.73E-04	46	0.233	0.816455	
Social setting × single × Shoot biomass	7.87E-02	2.08E-02	60	3.777	**0.000367**	***
Social setting × single × Repr. allocation	3.79E-01	3.48E-02	39	10.861	**2.20E-13**	***
Social setting × single × LR density	-2.90E-03	8.36E-02	181	-0.035	0.972328	
Social setting × single × Rel. LR length	1.50E-02	1.26E-02	74	1.192	0.237194	

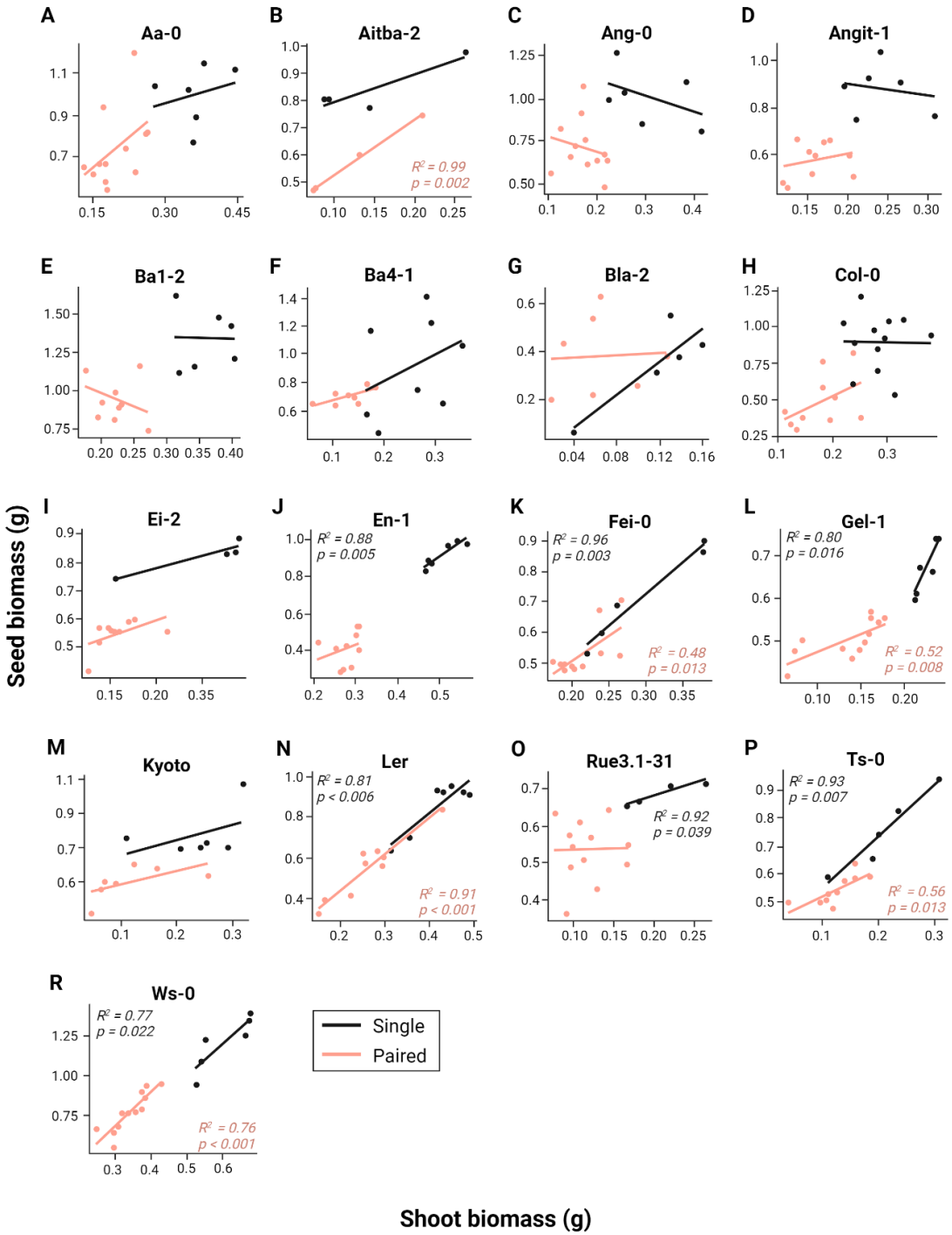


Figure 4.8. The interactive relationship between seed and shoot biomass spans various directions and magnitudes depending on the ecotype.

The relationships between shoot biomass and seed biomass for ecotypes with the focus on social setting. Linear regression, R^2 related to significant p values are indicated on graphs.

Cluster-specific analysis

Since measured traits responses showed variable modes of determination by ecotype and social setting, and the majority of traits showed similar patterns of expression in pairing setting, from now on the rest of the analysis focused on *differences* between plants performance when grown single *vs*. paired with the assumption that the magnitude of the response is a more informative indicator of interaction effect than the straightforward individual comparison of single *vs*. paired plants (Appendix B, Figure B.1abc; Table B.4). Therefore, for each replicate the effect size (Cohen's D) was calculated and subjected to further analysis.

The Principal Component Analysis (PCA) was performed to identify traits which effect sizes contributed significantly to the observed variation in ecotypes responses (Figure 4.9A). PC1 (48.3%) was primarily influenced by changes in traits related to root structure, such as LR density, LR number, relative LR length, root branching zone, total RS length, and primary root length. Seed biomass and shoot biomass had negative contributions to PC1, indicating an inverse relationship with the traits positively loading on this component. PC2 (27.3%) was dominated by traits associated with reproductive characteristics, namely reproductive effort and reproductive allocation, which exhibited strong positive contributions. Primary root length also contributed positively to PC2 but to a lesser extent.

Therefore, the traits that contributed significantly to PC1 and PC2 — capturing the most variance in the dataset, including seed biomass, shoot biomass, reproductive allocation, LR density, relative LR length, and PR length — were included in further analysis. The hierarchical clustering revealed four distinct ecotype groups characterised by patterns of similarities and differences in trait expression between paired and single grown plants (Figure 4.9B). For example, ecotypes in Cluster 1 (Rue3.1-31, Ba4-1, Bla-2) had similar trait changes values across the selected traits, distinguishing them from other clusters whereas En-1 formed its own cluster, highlighting its unique traits change profile compared to other ecotypes.

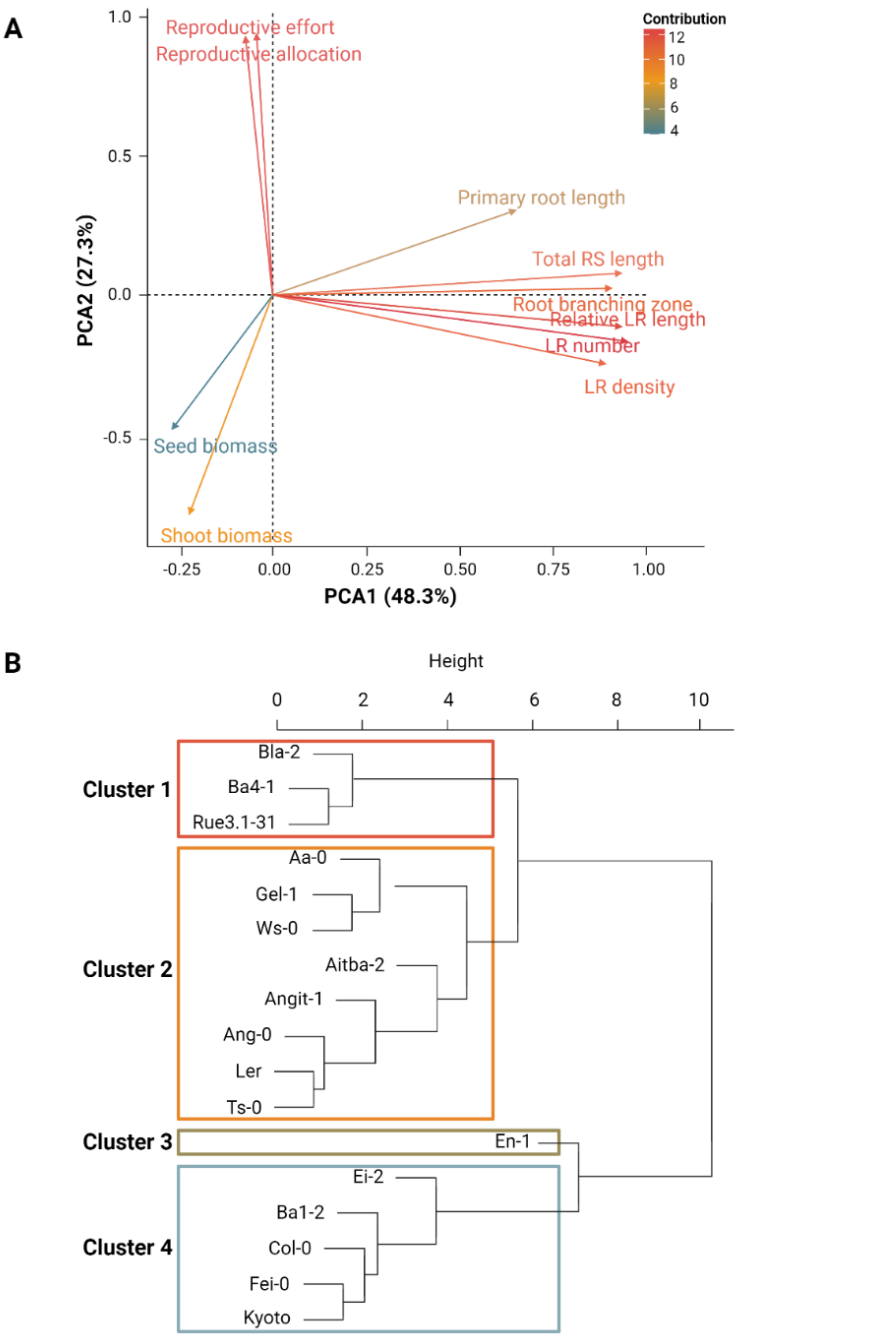


Figure 4.9. The multitrait neighbour responses in *A. thaliana* follow cluster-specific patterns.

(A) The PCA biplot of plant traits effect sizes illustrates the contributions of different traits to the first two principal components (PC1 and PC2), which together explain 75.6% of the total variance in the dataset. Traits relevant for clustering were identified. (B) The dendrogram of ecotypes based on traits effect sizes between paired and single plants. The dendrogram represents the hierarchical clustering of

various ecotypes based on the selected plant traits: seed biomass, shoot biomass, reproductive allocation, PR length, LR density, and relative LR length. The hierarchical clustering was performed on effect sizes using Ward's method, and the distance matrix was computed using the Euclidean distance. The dendrogram is divided into four main clusters.

The rest of the analysis focused on the characterization of the identified clusters. Cluster 3 was excluded from further analysis because it consisted of only one ecotype – En-1 and this low sample size prevented robust analysis.

The correlation analysis within the remaining clusters was performed in order to identify similarities and differences between traits relationships and to highlight which effect sizes correlated with seed biomass changes *within* clusters. The correlation matrices of effect sizes revealed important cluster-specific relationships between seed biomass and other traits (Figure 4.10ABC). In Cluster 1, all correlations between seed biomass and other traits were considerably strong. Seed biomass change was significantly positively associated with shoot biomass and LR density effect sizes ($r = 0.99$ and $r = 0.97$ respectively). It was also significantly negatively correlated with reproductive allocation ($r = -0.88$) and relative LR length ($r = -0.99$). This suggests that in this cluster, those traits tended to respond similarly when plants were grown in pairs, with increases in one trait effect size being strongly associated with increases in the others and *vice versa* (Figure 4.10A). In Cluster 2 the correlations were weaker and mostly negative. Seed biomass changes were positively correlated with reproductive allocation ($r = 0.82$), and negatively with the rest of traits especially with LR density ($r = -0.63$) and LR length ($r = -0.75$) (Figure 4.10B). Cluster 4 presented a pattern similar to Cluster 1 with majority, albeit weaker, positive correlations. Seed biomass changes were moderately positively correlated with LR density ($r = 0.38$) (Figure 4.10C). These results highlighted distinct internal dynamics within each cluster, showing that seed biomass was driven by different trait interactions across clusters. The contrasting patterns between clusters suggested that plant-plant

interactions and their outcomes are highly dependent on the specific trait combinations within each group.

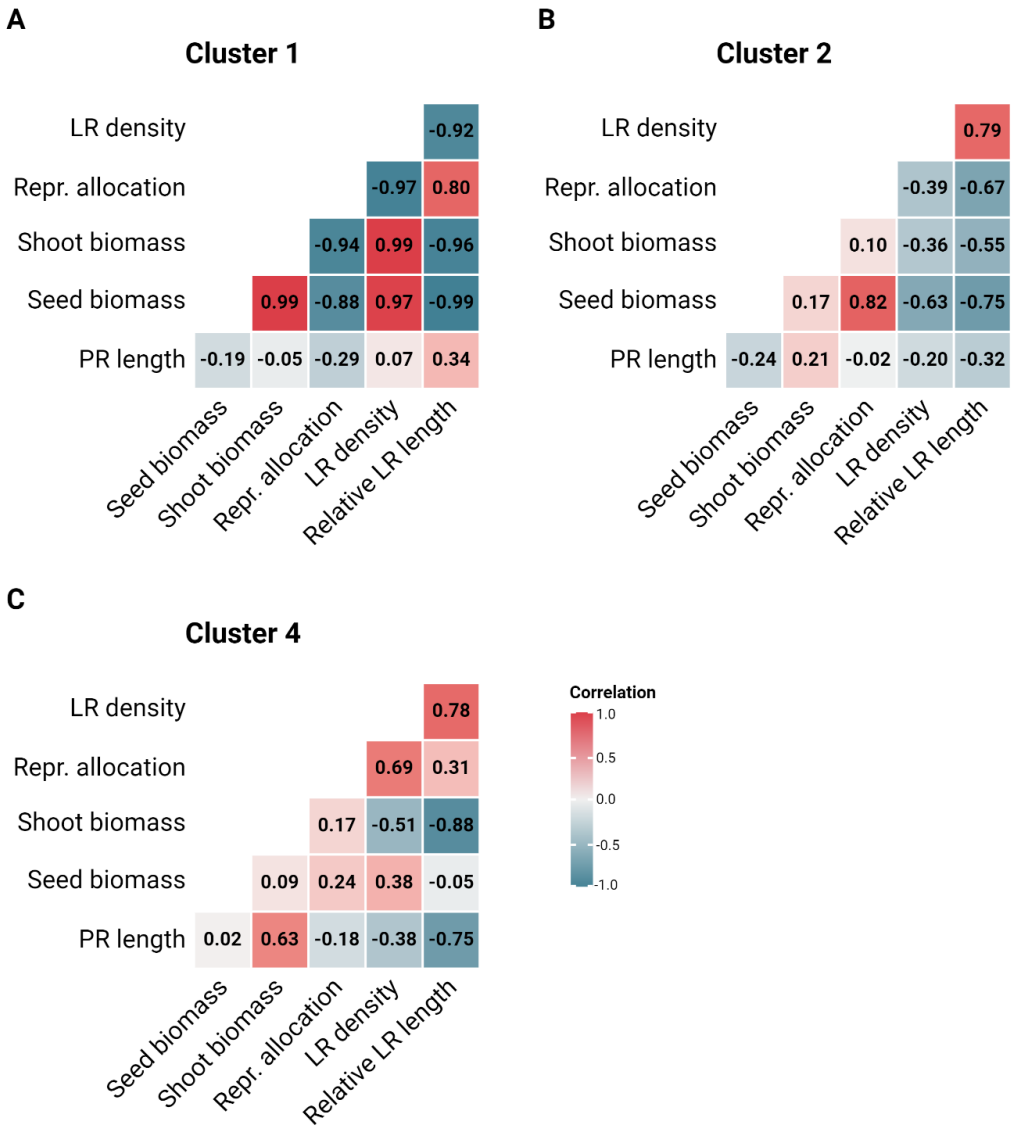


Figure 4.10. The clusters reveal specific relationships between seed biomass effect sizes and other traits.

A correlation analysis within clusters identified similarities and differences in trait relationships and determined which effect sizes were associated with changes in seed biomass within each cluster. Cluster 3 was excluded from further analysis because it consisted of only one ecotype – En-1. The correlations reflect how effect sizes of traits are associated with each other across clusters. Pearson correlation.

It was also investigated how traits that were correlated with seed biomass change affect seed biomass while accounting for the variability *between* clusters in order to identify the traits that generally contribute to seed biomass but to different extent between clusters (Appendix B, Table B.2). First, the shoot biomass was the only trait which change had a significant effect on seed biomass across all clusters ($p = 0.0469$). Therefore, the relationship between seed biomass effect sizes and shoot biomass effect sizes between Cluster 1, Cluster 2, and Cluster 4 was assessed in detail. It was first tested whether the relationship between changes in shoot and seed biomass varied significantly between different clusters. Adding the interaction term between shoot biomass and cluster did not improve the model fit significantly ($p = 0.1008$) (Table 4.6). This suggested that the strength of the shoot biomass effect on seed biomass did not vary significantly between clusters.

Table 4.6. The summary of 2-way ANOVA comparison between two linear mixed models: a baseline model and an interaction model were utilised to investigate whether adding the interaction term between shoot biomass and cluster does improve the model fit.

	npar	AIC	BIC	logLik	deviance	Chisq	Df	p value
baseline model	7	50.692	56.524	-18.346	36.692			
interaction model	9	54.014	61.513	-18.007	36.014	0.6776	2	0.7126

Therefore, the analysis proceeded with cluster characterization using baseline models (Figure 4.11). In Cluster 1 the slope of the model was steep and positive ($\beta_1 = 0.62$), indicating that as the shoot biomass effect size increases, the seed biomass effect size also increases sharply. In Cluster 2 the slope was less steep but still positive ($\beta_1 = 0.29$). This indicated a positive relationship between shoot biomass and seed biomass effect sizes, albeit weaker than in Cluster 1. In this cluster, an increase in shoot biomass effect size is associated with a moderate increase in seed biomass effect size. The slope of the model for Cluster 4 was also positive but quite flat compared to the other clusters, suggesting a weak positive relationship where

changes in shoot biomass effect sizes were associated with small changes in seed biomass effect sizes ($\beta_1 = 0.07$).

In summary, the strength of the relationship between shoot biomass effect sizes and seed biomass effect sizes appeared to vary by cluster. Cluster 1 exhibited the strongest positive relationship, indicating that in this cluster, the two traits were more closely linked. Cluster 2 showed a moderate positive relationship, suggesting some level of linkage between these traits, but not as strong as in Cluster 1. Cluster 4 had the weakest positive relationship, indicating that in this cluster, shoot biomass changes have less impact on seed biomass. However, statistical analysis of models did not reveal significant differences between these models ($p = 0.6076$).

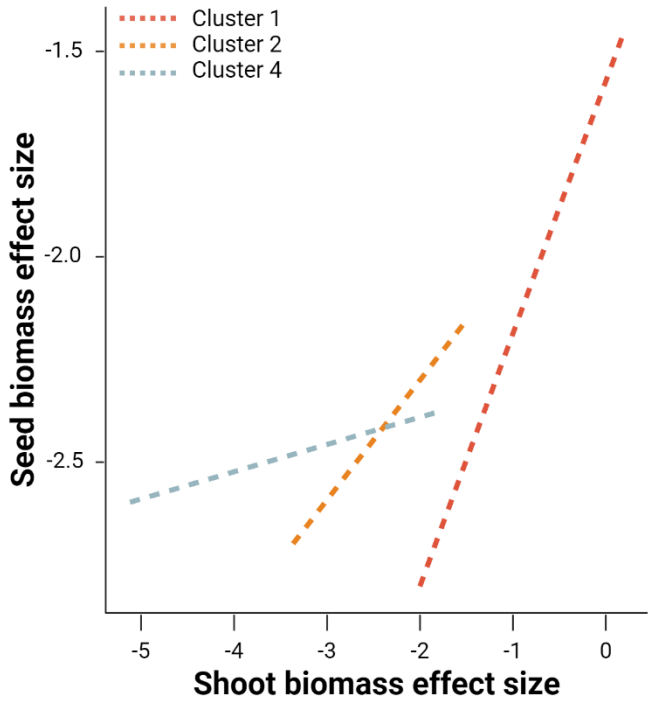


Figure 4.11. The magnitude of the relationship between seed and shoot biomass changes in response to pairing differs between clusters.

The separate linear regression models were fitted to each cluster's data, predicting seed biomass effect size based on shoot biomass effect sizes. Graph presents how the relationship between these two variables differs by cluster, as visualised by the distinct slopes and intercepts in the graph.

4.5 Discussion

Biotic factors are the living components within an ecosystem, including the inherent traits of plants that affect their interactions with one another. These factors encompass various plant characteristics that determine their competitive abilities, such as the efficiency with which they acquire resources compared to neighbouring plants. Key elements influencing these abilities include species, genetic diversity, and age (Dayrell et al., 2018; Subrahmaniam et al., 2018). These biotic influences shape plant competition or, at times, cooperation, impacting traits like root structure, growth rates, and reproductive strategies (Callaway, 1995; Gardner & West, 2014; West et al., 2002). Intraspecific interactions, likely very frequent even in natural environments, play a critical role in species coexistence, as stronger competition within species compared to between species serves as a key balancing mechanism (Grime 1979; Harper 1977; Silvertown & Law 1987). Understanding the role of biotic factors is essential for recognizing both common and unique responses to neighbours and could enhance our ability to balance competition and cooperation in fluctuating environments.

4.5.1. Early emergence confers long-term competitive advantage

The findings presented in this section indicate that the timing of neighbour occurrence has a role in plant-plant interaction outcomes. Seeds germination has been shown to be sensitive to neighbour cues which can both accelerate seedling emergence like in the case of nurse plants (Orrock & Christopher, 2010; Fenesi et al., 2014) delay, or even prevent it (Jutila & Grace, 2002; Fenesi et al., 2014; Leverett et al., 2016; Leverett et al., 2017; Zhang et al., 2014). Accelerated emergence allows individuals to limit their exposure to competition and may pose a fitness advantage especially in annuals (Miller et al., 1994; Dyer et al., 2000; Geber & Griffen, 2003; Mercer et al., 2011).

Here, it was observed that the longer focal plants grew alone, the lesser the impact of a newly introduced neighbour was on their fitness. Likewise, the presence

of a neighbour plant that was older significantly undermined the fitness of the newly emerging focal plant, however no significant difference was evident when the age difference was between 0-5 days. The relationship between the number of siliques and the timing of neighbour occurrence was moderately correlated. Other studies also reported that competition between annual species intensifies from early to late life stages pointing to older neighbours being more competitive and seedlings more sensitive to competition (Foster 1999; Goldberg et al. 2001; Nash-Suding & Goldberg 1999). Schiffers and Tielbörger (2006) described temporal changes with moderate facilitation soon after germination shifting to strong competition at the end of life in annual species in *B. didyma* and *H. circinnatus*.

A plant through early germination extends its growing in isolation period in which it can utilise all available above- and belowground resources (Miller et al., 1994; Mercer et al., 2011). This competitive advantage can be substantial. In this study *A. thaliana* seedlings that emerged 5 days before the neighbour produced ~ 2 times more fruits, and seedlings that emerged 10 days before the neighbour produced ~ 2.5 times more fruits than plants grown with neighbour from day 1. Similar results were observed in seedlings of *Brassica rapa* where 1 week advance in emergence to the neighbour allowed increase of up to 38x more fruits than their neighbours (Weis et al., 2015).

It seems that due to the size-asymmetrical competition larger neighbour limits the resources for the smaller one. This study stands out by evaluating competition through reproductive outputs, contrasting with many previous studies that focused on changes in shoot-to-root allocation. It is hypothesised that these changes primarily reflect allometric scaling—the proportional growth changes in plant traits—as opposed to direct responses to environmental conditions (Evans, 1972; Gedroc et al., 1996; Mueller et al., 2000; Cahill et al., 2003; Litton et al., 2003). Research supports this, showing that factors like nutrient availability or root competition primarily influence overall plant size, which then indirectly affects the shoot-to-root

ratio. This suggests that observed allocation changes are more related to general growth patterns than to specific environmental pressures.

4.5.2. The genotype superiority in determination of neighbour responsiveness and tolerance

Intraspecific interactions between plants of the same genotype have been clearly demonstrated to vary significantly across species, with some studies suggesting that this variability also extends to ecotypes within a species (Masclaux et al., 2010; Subrahmaniam et al., 2018; Willis et al., 2010). Density response, particularly at high levels, has been well-explored, however, studies on plant-plant interactions, crucial for understanding individual growth and ecological impacts, are rare (Postma et al., 2021). Here, a high variability in neighbour responsiveness and tolerance was found both between closely phylogenetically related species from *Brassicae* family and within *A. thaliana* species between ecotypes.

A study focusing on several *Brassicaceae* members: *O. pumila*, *C. rubella*, and *C. hirsuta* demonstrated a significant impact of species and social setting on shoot and seed biomass, though the species-specific responses varied. While all species experienced a reduction in shoot biomass when paired with a neighbour, only *O. pumila* and *C. rubella* showed significant decreases in seed biomass, with *C. hirsuta* demonstrating the highest tolerance to competition. Indeed, the *Brassicaceae* family is recognised for its diversity even between its wild species (Raza et al., 2020). Competitive ranks are commonly applied to species though are usually derived from interspecific interactions studies (Howard & Goldberg, 2001; Soliveres et al., 2018; Wang et al., 2010). Interestingly, root responses were less pronounced, with no significant changes in primary root length or lateral root growth in most cases, except for *C. rubella*, which showed an increase in lateral roots in response to a neighbouring plant. Previous work also has shown that different plant species invest more or less into their roots as a response to the presence of other roots in the soil (Belter & Cahill, 2015; Postma et al., 2021). Overall, this study highlights that despite the close genetic relationships among these species, their aboveground and belowground responses

to neighbours differed, with no clear correlation between root adjustments and competitive outcomes.

The natural variation in neighbour responsiveness and tolerance was also observed between *A. thaliana* ecotypes. The social setting-specific analysis revealed that shoot biomass relationship with seed biomass differed depending on whether plants were grown alone or with neighbours. However, this relationship was highly variable across the 17 *A. thaliana* ecotypes. Only five ecotypes showed positive relationships in both social settings, and stronger shoot and seed biomass relationship in paired plants. Overall, this analysis confirmed that shoot biomass albeit highly influential, is not a reliable predictor of seed biomass across ecotypes, particularly in plant-plant interactions (McNickle, 2020; Mobley et al., 2022). The cluster-specific analysis revealed distinct patterns in how groups of *A. thaliana* ecotypes responded to being grown single or paired. Four clusters of ecotypes were identified, each showing unique relationships between seed biomass and other traits. In Cluster 1, strong positive correlations were observed between seed biomass, shoot biomass, and lateral root (LR) density, while reproductive allocation and LR length were negatively correlated. Cluster 2 exhibited weaker and mostly negative correlations, with seed biomass positively linked to reproductive allocation but negatively associated with root traits. Cluster 4 showed moderate positive correlations, similar to Cluster 1, though weaker overall. The analysis further explored the relationship between shoot and seed biomass across clusters, finding that the strength of this relationship varied. Cluster 1 had the strongest positive relationship, Cluster 2 showed a moderate correlation, and Cluster 4 had the weakest. Despite these differences, statistical analysis did not reveal significant differences between the clusters in how shoot biomass influenced seed biomass. It is difficult to directly relate these results to broader literature, as this is likely the first study that applied such a comprehensive methodology to one species and multiple ecotypes within. Previous research showed that ecotypes within *A. thaliana* generally differed in their

shoot biomass and siliques responses to neighbour and associated this with differences in their competitive abilities (Masclaux et al., 2010).

4.5.4. Concluding remarks

This chapter highlights the significant impact of biotic factors, such as age and species and genetic variation, on plant-plant interactions. It revealed that early emergence provides a long-term competitive advantage, with plants that germinate earlier benefiting from extended access to resources, resulting in higher reproductive output. The findings also emphasised that intraspecific interactions, particularly among closely related species and ecotypes, vary greatly in terms of neighbour responsiveness and tolerance. Among the *Brassicaceae* species studied, *C. hirsuta* demonstrated the highest tolerance to competition, while *O. pumila* and *C. rubella* showed more pronounced reductions in seed biomass when paired with a neighbour. Interestingly, root responses were less consistent, suggesting that aboveground traits may be more closely linked to competitive outcomes. Similarly, *A. thaliana* ecotypes exhibited variable responses, with shoot biomass being an unreliable predictor of seed biomass across different social settings. The cluster analysis further revealed that different ecotype groups displayed unique patterns in how traits such as seed biomass, shoot biomass, and root traits were related, underscoring the complex nature of plant-plant interactions at the ecotype level. While this study introduces a comprehensive methodology for assessing neighbour interactions within species, it also highlights the need for further research to explore the mechanisms behind these varying responses.

Chapter 5
Review of the role of rhizodeposits in plant-plant interactions

Abstract

Rhizodeposits, including root exudates and volatile organic compounds (VOCs), play a critical role in belowground plant-plant interactions, yet their precise function in neighbour detection remains unclear. This study investigates the role of rhizodeposits in *Arabidopsis thaliana*, assessing their impact on competition and plant fitness. Experimental approaches differentiating between root contact, rhizodeposits exchange, and root exudate application revealed that *A. thaliana* does not respond to its own rhizodeposits but can be influenced by those of other species in a context-dependent manner. Specifically, rhizodeposits from *Olimarabidopsis pumila* and root exudates from *Cardamine hirsuta* (grown under high nutrient conditions) enhanced *A. thaliana* reproductive output. Additionally, the widely proposed involvement of ABC transporters in root exudate-mediated interactions was not supported. Ethylene, a VOC known for its regulatory role in root development, was identified as a key mediator of neighbour detection. Mutants impaired in ethylene biosynthesis (*aco1*) and perception (*ein2-1*) displayed altered competition intensities, with *ein2-1* allowing a fitness advantage to neighbouring wild type plants. These findings suggest that ethylene serves as a signal for neighbour presence, providing new insights into plant-plant communication mechanisms. Understanding these processes has important ecological and agricultural implications.

5.1 Introduction

Plants can perceive and respond to both their above- and belowground environment by detecting spatial and temporal variations in far-red light reflection, nutrient availability and various chemical compounds produced by living organisms (Bilas et al., 2021; Callaway, 2002; Callaway & Li, 2020; Novoplansky, 2019). Chemical signalling in plants is particularly effective, with belowground rhizodeposits—including root exudates and volatile organic compounds (VOCs)—playing a key role (Delory et al., 2016; Rahman et al., 2019; Vives-Peris et al., 2020; Wang et al., 2021). The variety of compounds present in rhizodeposits is reflected in multiplicity of their release mechanisms (Figure 5.1) (Canarini et al., 2019). Rhizodeposits perform multiple functions, such as shaping soil microbial communities, modifying nutrient availability, and facilitating plant-plant interactions (Vives-Peris et al., 2020; York et al., 2016). It has to be acknowledged, that while rhizodeposits by definition entail a variety of substances released by plant roots including border cells, mucilage, root exudates and volatile organic compounds (Kazakov & Schneckenberger, 2004; Tian et al., 2020), in this work this term is used mainly with reference to root exudates and VOCs. While rhizodeposits are thought to be central to belowground plant interactions, their exact composition and signalling mechanisms remain underexplored. This presents an intriguing area for further research to better understand how plants interact with each other in the soil.

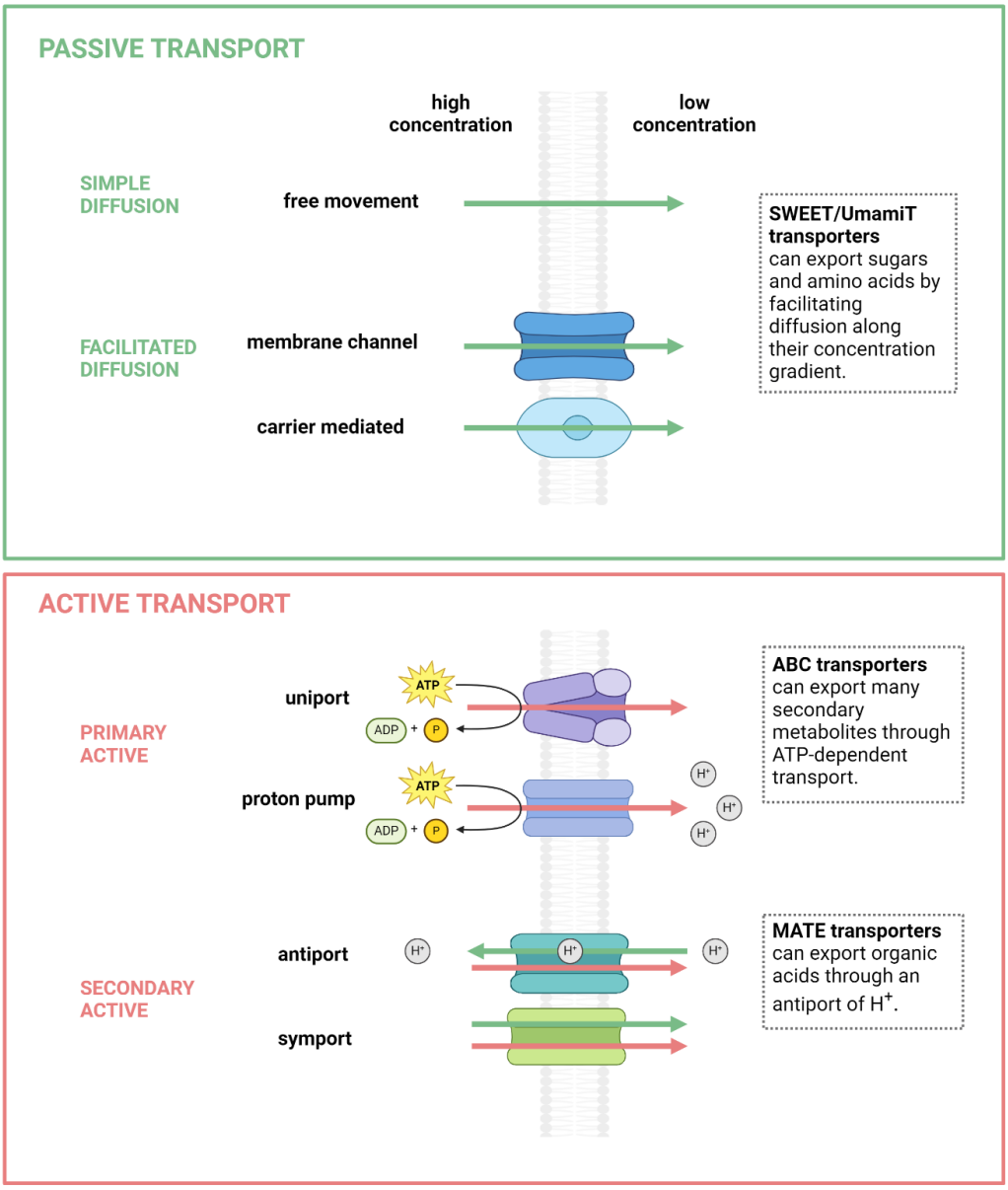


Figure 5.1. An overview of rhizodeposition mechanisms.

The top panel illustrates passive transport mechanisms, enabling diffusion along electrochemical gradients. In contrast, the bottom panel depicts active transport mechanisms, categorised as either primary active (requiring direct ATP consumption) or secondary active (coupled to ATP-consuming H+ pumps). Green arrows signify solute movement along electrochemical gradients, while red arrows indicate movement against such gradients. The right side of the figure presents examples of membrane transporters facilitating the movement of primary metabolites. Figure was adapted from Canarini et al., 2019.

5.1.1 Root exudates as mediators of interaction

Root exudates are primarily non-volatile, water-soluble compounds such as sugars, amino acids, organic acids, phenolics, and secondary metabolites (Canarini et al., 2019; Karlovsky, 2008). The numerous reports concerning description of novel plant-plant signals in the rhizosphere corroborate the hypothesis that, belowground root exudates cocktails participate in plant-plant communication and contain relevant plant-plant signals (Vives-Peris et al., 2020; Wang et al., 2021). The major line of evidence comes from studies on the general effect of root exudates presence on plant-plant interactions.

The main approaches involved general inhibition of root secretion (Biedrzycki, 2010; Biedrzycki et al., 2011; Yang et al., 2018), addition of activated carbon to capture phenolic compounds in exudates (Caffaro et al., 2011, 2013; Lau et al., 2008; Semchenko et al., 2007, 2014; Wurst et al., 2010)and the use of mesh-separated pots (reviewed in Chen et al., 2020; McNickle, 2020). In plant-plant interactions root exudates have been shown to regulate processes like root detection, kin recognition, flowering, and productivity, promoting both inter- and intra-specific facilitation in cropping systems (Sun et al., 2021; Wang et al., 2021). Exudates compounds are secreted passively or actively through root cells into the rhizosphere, depending on their structural complexity (Figure 5.1) (Canarini et al., 2019; Weston et al., 2012). The ATP-binding cassette (ABC) transporter family was suggested to play a particular role in the exudation of compounds involved in plant-plant signalling. Biedrzycki et al. (2011) observed that *A. thaliana* roots showed a general lack of response to neighbouring plants when ABC transporters were inhibited, although the specific transporter and its associated cargo were not identified. Other examples are an unidentified ABC transporter responsible for the secretion of root growth stimulating allantoin in *O. sativa* (Kaur et al., 2021; Yang et al., 2018) and the PLEIOTROPIC DRUG RESISTANCE 1 (PDR1) shown to be involved in exudation of strigolactones in *P. hybrida* (Kretzschmar et al., 2012). However, it is not clear whether root exudates are indeed involved in plant-plant interactions long-term that would lead to neighbouring

plants fitness consequences. Nor is it resolved whether in *A. thaliana* such a mechanism exists in soil growth condition.

Several studies have expanded our understanding using root exudate metabolome profiling to provide detailed evidence of the specific roles certain compounds play in plant-plant interactions. Namely, strigolactones have been shown to mediate plant-plant interactions in *P. sativum* and *O. sativa* (Wheeldon et al., 2022; Yoneyama et al., 2022), jasmonic acid release from roots induced allelopathy in *T. aestivum* (Kong et al., 2018), (-)-loliolide induced allelopathy in *O. sativa* (Li et al., 2020), whereas allantoin was suggested to drive neighbour response in *O. sativa* (Yang et al., 2015; Yang et al., 2018). The root exudate signals identified thus far present several limitations regarding their broader implications for general plant-plant signalling. For instance, allelopathic signals are unlikely to be relevant for plant species like *A. thaliana*, which lacks this capacity. Additionally, signals primarily involved in plant-symbiont communication, such as strigolactones, are unlikely to be released or relevant in species like *A. thaliana* that do not participate in these interactions. Since *A. thaliana* is a major focus of this thesis, it is frequently referenced. However, it is important to note that many species lack allelopathic capacity, and notably, the entire *Brassicaceae* family does not form mycorrhizal symbioses, but still neighbour responsiveness and tolerance are widely observed phenomena.

5.1.2 Volatile organic compounds as mediators of interaction

Root volatile organic compounds (VOCs) are gaseous chemicals released by plant roots (Delory et al., 2016; van Doan et al., 2020). Ethylene is likely the best characterised root-released VOC due to its endogenous role as a phytohormone and multiple functions it plays exogenously in the rhizosphere (Binder & Schaller, 2017; Borch et al., 1999; Chen et al., 2020; Pierik et al., 2006). Ethylene plays tremendous roles in aboveground plant-plant interactions where it is involved in shade avoidance response and canopy sensing (Pierik et al., 2003, 2004, 2007; Smalle et al., 1997). Belowground, ethylene is involved almost in all aspects of root system development

playing crucial roles in regulating root growth and architecture. It has inhibitory effect on primary root elongation, especially under stress conditions like waterlogging or soil compaction, while promoting lateral root and root hair formation to enhance nutrient and water uptake (Dreyer & Edelmann, 2018; Lei et al., 2011; Park et al., 2018; Pandey et al., 2021; Shukla et al., 2017; Tian et al., 2009; Visser & Pierik, 2007).

Ethylene biosynthesis and release primarily occur within the root epidermis, strategically located closest to the rhizosphere to facilitate prompt passive release and sensing (Vaseva et al., 2018). The possible role of ethylene in plant-plant signalling was proposed before (Cahill et al., 2005; Falik et al., 2005; Masclaux et al., 2012). However, ethylene biosynthesis and perception are relatively complex with numerous genes involvement therefore it is unlikely that studies assessing single genes were able to undoubtedly confirm or deny its role (Figure 5.2) (Cahill et al., 2005). A comprehensive assessment of ethylene signalling network on plant-plant interactions has not been carried out till date.

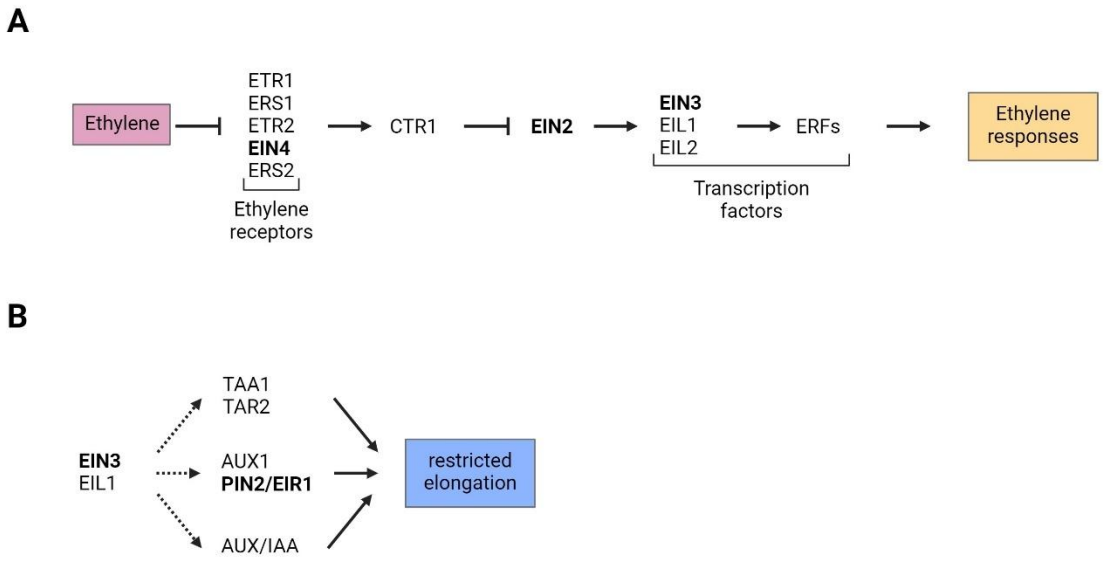


Figure 5.2. An overview of ethylene signalling.

(A) A model for ethylene signalling, derived from studies in *Arabidopsis*. Key components are the ethylene receptors (ETR1, ERS1, ETR2, EIN4, and ERS2), followed by the kinase CTR1, and its substrate EIN2, which relays signals to

transcription factors like EIN3, EIL1, and EIL2. These transcription factors then activate further transcription factors (ERFs), culminating in ethylene responses. In this framework, CTR1 acts as a negative regulator of the signalling pathway. Ethylene operates as an inverse agonist by inhibiting these receptors, thus reducing CTR1 activity and freeing downstream elements from CTR1 inhibitory effects. Figure adapted from Binder, 2020. (B) An integrative model highlights the interplay between ethylene and auxin homeostasis at the root tip. Within this model, ethylene reception at the root tip reduces the inhibitory action of CTR1 on EIN2 and the EIN3/EIL1 complex. This relief of suppression allows EIN3/EIL1 to indirectly promote the activity of AUX, PIN2, AUX/IAA, and TAA1 in the lateral root cap and epidermis, thereby enhancing their roles in auxin regulation. The gene components that were assessed in this thesis are indicated in bold. Figure was adapted from Vaseva et. al., 2018.

5.1.3 Summary

Root exudates are widely proposed as key mediators of belowground plant-plant interactions, facilitating neighbour detection and even recognition (Mathieu et al., 2024; Wang et al., 2021). While their role in communication has been confirmed across many species, and their mode of secretion shown to be important for effective signalling, the underlying mechanisms remain unclear. It is also uncertain whether rhizodeposits-driven signalling has lasting effects on plant fitness. Additionally, volatile organic compounds, particularly ethylene, play a crucial role in regulating root growth and architecture, but their role in plant-plant interactions remains ambiguous due to the complexity of ethylene biosynthesis and perception. This chapter provides the comprehensive validation of rhizodeposits role in *A. thaliana* which is needed to clarify these signalling networks and their impact on plant-plant interactions.

5.2. Chapter aims

The gaps in our current understanding raise several questions about the mechanisms of plant-plant interactions which this chapter aims to address:

1. Do rhizodeposits participate in plant-plant interactions?
2. Do rhizodeposits act as proxy of neighbour presence?
3. What constituents are required for belowground communication? Is it root contact, rhizodeposits, root exudates or all?
4. Do rhizodeposits allow for neighbour differentiation?
5. Do rhizodeposits have long-term fitness consequences?

This chapter aimed to evaluate the phenotypic responses and outcomes of rhizodeposits mediated interactions between *A. thaliana* and other members of *Brassicae* and understand their relevance for the fitness of interacting plants.

5.3. Methods

5.3.1 Germplasm

Plant signalling through chemical compounds is highly effective belowground, with rhizodeposits comprising root exudates and volatile organic compounds playing multiple roles in the rhizosphere (Delory et al., 2016; Vives-Peris et al., 2020). Root exudates have been shown to participate in plant-plant signalling, however the nature of signalling molecules presented in them has not been fully comprehended yet (Rahman et al., 2019; Wang et al., 2021). Ethylene is a volatile organic compound (VOC) that is released by roots and influences various processes in the rhizosphere, including root growth, development, and responses to environmental stresses (Binder & Schaller, 2017; Borch et al., 1999; Ju & Chang, 2015; Pandey et al., 2021; Qin & Huang, 2018; Vaseva et al., 2018). In this chapter the general capacity of root exudates to participate in plant-plant signalling belowground and the role of candidate inter-plant signal ethylene was tested.

In all experiments in this chapter *A. thaliana* Col-0 ecotype was used. All *A. thaliana* ethylene mutants were impaired in ethylene perception or biosynthesis were also in Col-0 background. The ethylene biosynthesis and signalling mutants were obtained as a courtesy from Prof. Malcolm Bennett (University of Nottingham), Prof. Sheng Zhong and Prof. Li-Jia Qu (Peking University) and from NASC (Table 5.1). The further details regarding particular mutants can be found in the following publications: *ET-free-2* (Li et al., 2022) and other ethylene mutants (Binder & Schaller, 2017; Pandey et al., 2021).

Table 5.1. The details of *A. thaliana* ethylene mutants used in this study.

Mutant	Gene	Locus	Comments	Source
aco1	ACO1	AT2G19590	Impaired in ethylene biosynthesis	NASC (N682904)
aco2	ACO2	AT1G62380	Impaired in ethylene biosynthesis	Bennett M
aco4	ACO4	AT1G05010	Impaired in ethylene biosynthesis	Bennett M

ET-free-2	ACO1/ ACO2/ ACO3/ ACO4/ ACO5	AT2G19590/ AT1G62380/ AT1G12010/ AT1G05010/ AT1G77330	A quintuple mutant of all five ACO genes, free of ethylene production	Zhong S & Qu L-J
ein2-1	EIN2	AT5G03280	Ethylene insensitive	NASC (N3071)
ein3	EIN3	AT3G20770	Ethylene insensitive	Bennett M
ein4	EIN4	AT3G04580	Ethylene insensitive	Bennett M
eir1	EIR1	AT5G57090	Ethylene insensitive, root-specific role in auxin transport (other name PIN2)	Bennett M

5.3.2 Experimental design

This chapter assessed the impact of belowground interactions using multiple experimental approaches allowing for differentiation between the effects of: physical root contact combined with rhizodeposits, the *exchange* of rhizodeposits only, *application* of root exudates and the overall impact of root secretion. The same approach used in previous chapters was applied, where the impact of rhizodeposits was deemed significant only if it produced a notable effect on plant fitness, measured through reproductive output such as seed biomass or total silique number. It has to be acknowledged that, although changes in fitness are not strictly necessary to confirm or deny a role in signalling, establishing a link between fitness alterations, signalling and plant-plant interactions is essential to identify meaningful effects. The specific experimental designs are presented in Figure 5.3 and outlined in subsections below.

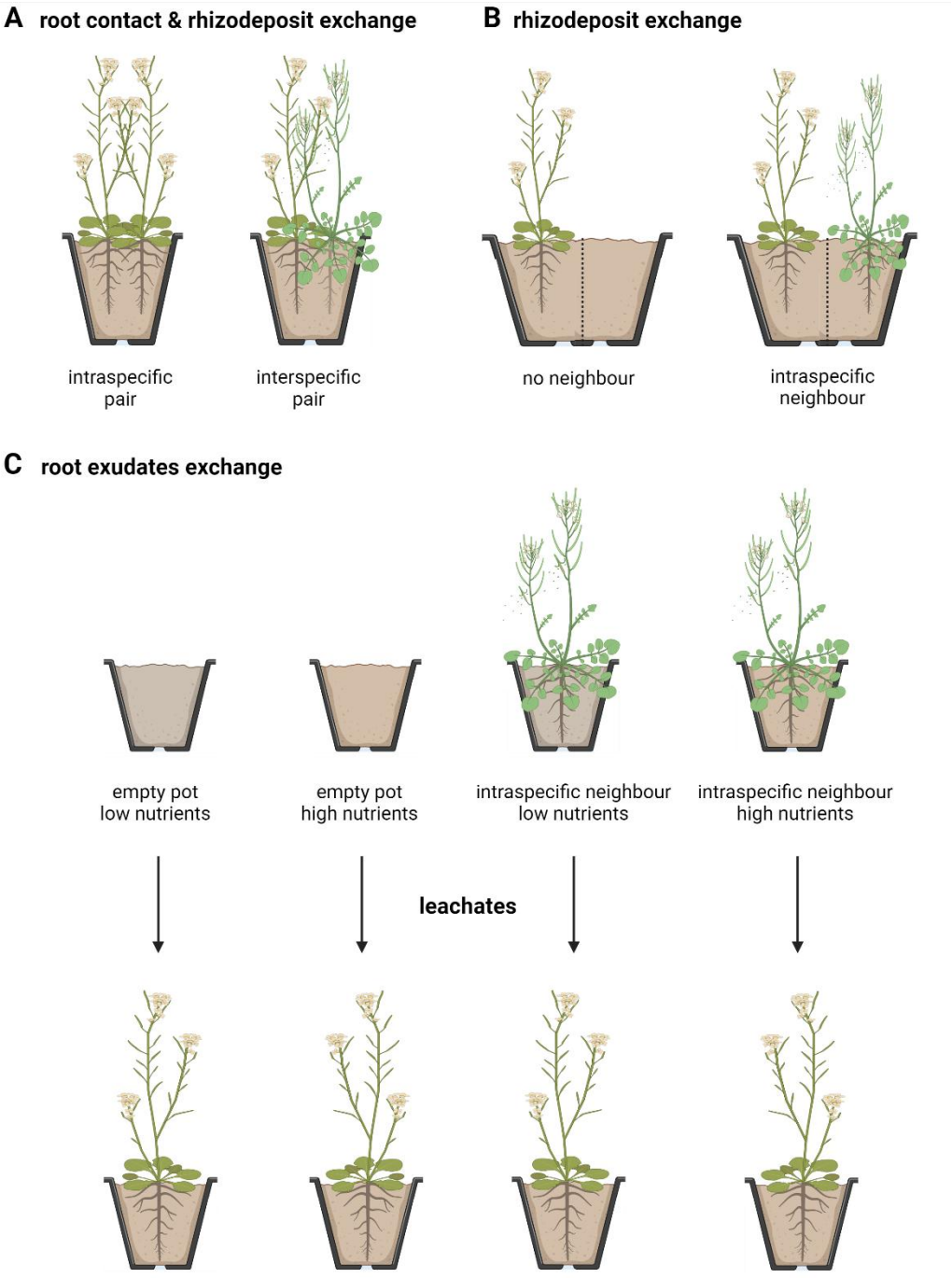


Figure 5.3. The overview of experimental designs used for assessing the role of rhizodeposits in plant-plant interaction.

The focal *A. thaliana* Col-0 and a neighbouring *C. hirsuta* are demonstrated as examples. (A) Plants were grown in pairs together in one pot with root contact and rhizodeposits exchange allowed. (B) Plants were grown either as single or in pairs in one pot but separated with permeable mesh, root contact was prevented but rhizodeposits exchange was allowed. (C) All plants were grown as single, and donor plants were grown in high and low nutrients. Leachates (containing root exudates)

were collected from watering donor plants and used to irrigate focal *A. thaliana* Col-0 recipient plants. Leachates from empty pots served as controls.

5.3.2.1 Assessment of the effect of root physical contact and rhizodeposits exchange on interaction

The combined effect of physical root contact and root rhizodeposits was investigated by growing plants together in pairs in the same pot without any separation. Various plant neighbours, including different ecotypes and species, were evaluated to increase the likelihood of detecting an effect. As mentioned in the previous chapter, species and ecotypes vary in their responsiveness and tolerance to neighbours (Chapter 4). Therefore, *A. thaliana* ecotype Col-0 served as a focal plant and was paired either with another *A. thaliana* ecotype Col-0, *A. thaliana* ecotype Ler, *C. rubella* or *C. hirsuta*. The pot volume used was 100 ml. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The total number of siliques was measured as an indicator of the reproductive output. The total number of pots in this experiment was 31; that comprised 8 replicates of *A. thaliana* Col-0 paired with another Col-0, 7 replicates of Col-0 paired with Ler, 9 replicates of Col-0 paired with *C. rubella* and 10 replicates of Col-0 paired with *C. hirsuta*.

5.3.2.2 Assessment of the effect of rhizodeposits exchange on interaction

The isolated effect of rhizodeposits exchange between plant roots was examined by growing plants individually or in pairs using mesh-separated pots (following method used by Semchenko et al. 2007 with adjustments). A permeable mesh was positioned in the center of the pots, allowing chemical diffusion between plants while preventing direct root contact. Various plant neighbours, including different ecotypes and species, were evaluated to increase the likelihood of detecting an effect. As mentioned in the previous chapter, species and ecotypes vary in their responsiveness and tolerance to neighbours (Chapter 4). Therefore, *A. thaliana* ecotype Col-0 served as a control and was grown as single with no neighbour present in the other half of

the mesh-separated pot, or in pairs either with another *A. thaliana* Col-0, Ler, *O. pumila*, *C. rubella* and *C. hirsuta*. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The seed biomass was measured as an indicator of the reproductive output. The total number of pots in this experiment was 26; that comprised 7 replicates of *A. thaliana* Col-0 grown without neighbour, 7 replicates of *A. thaliana* Col-0 paired with another Col-0, 3 replicates of Col-0 paired with Ler, 4 replicates of Col-0 paired with *O. pumila*, 3 replicates of Col-0 paired with *C. rubella* and 4 replicates of Col-0 paired with *C. hirsuta*.

5.3.2.3 Assessment of the effect of root exudates application on interaction

The primary effect of root exudate application was tested by growing plants individually and irrigating them with leachates containing root exudates, collected from other individually grown plants. This method was applied from Semchenko et al., 2014, with adjustments. Given that the leachate collection procedure used here likely encouraged the release and loss of volatile organic compounds from rhizodeposits, it is assumed that the leachates primarily consisted of root exudates — liquid-dissolved primary and secondary metabolites. Various root exudate donors, including different ecotypes and species, were evaluated to increase the likelihood of detecting an effect. As mentioned in the previous chapter, species and ecotypes vary in their responsiveness and tolerance to neighbours (Chapter 4). What is more, species and ecotypes differ in exudates content and exudates content was shown to change depending on nutrients condition (Dietz et al., 2019; Monchgesang et al., 2016; Semchenko et al., 2014; Strehmel et al., 2014; Sun & He, 2019; Vives-Peris et al., 2020). To better simulate the exudates that would be released by paired plants, two nutrient conditions were applied: a high-nutrient condition consisting of 100% soil and a low-nutrient condition consisting of 10% soil mixed with sand. Briefly, *A. thaliana* ecotype Col-0 focal plants (acceptor) was grown singly in high nutrients conditions (100% soil) and irrigated with leachate collected from watering donor plants. Donor plants comprised of singly grown *A. thaliana* Col-0, *A. thaliana* Ler or

C. hirsuta plants grown singly in high (100% soil) or low (10% soil and 90% sand) nutrients conditions. Acceptor and donor plants were grown in parallel therefore, matching each other developmental stages. The experiment comprised mock treatment of empty pots filled with high and low media. Each day each donor plant was watered with 200 ml of water ensuring media was rinsed thoroughly. All of the flow-through solution was collected, filtered through Whatman filter paper and 100 ml of this solution was used per acceptor focal plant for watering. The application was maintained throughout the entire growth period of the focal *A. thaliana* Col-0 plants. Plants were grown until arrest, after which were treated and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The total siliques number was measured as an indicator of the reproductive output. The total number of pots in this experiment was 65. That comprised 34 Col-0 focal plants treated with leachates from donors grown in high nutrients media: 9 replicates of mock treatment (no plant), 9 replicates of from Col-0, 8 replicates of from Ler and 8 replicates from *C. hirsuta*. 31 Col-0 focal plants were treated with leachates from donors grown in low nutrients media: 7 replicates of mock treatment (no plant), 9 replicates of from Col-0, 7 replicates of from Ler and 8 replicates from *C. hirsuta*.

5.3.2.4 Assessment of the effect of root secretion on interaction

Rhizodeposits are complex and dynamic mixtures of chemical compounds (Canarini et al., 2019; York et al., 2016). It is likely that there are at least as many signals with unknown functions as there are described and characterised compounds. Although numerous studies highlight the roles of root exudates in plant-plant interactions, so far neither a universal signal, receptor nor transporter involved in neighbour detection was identified (Wang et al., 2021). The potential involvement of an unidentified transporter from the ATP-binding cassette (ABC) family in belowground plant-plant interactions was suggested in several studies, which demonstrated disruption of interactions when ABC transporters were inhibited (Biedrzycki et al., 2011; Yang et al., 2018). Sodium orthovanadate is a commonly used inhibitor of the membrane ATPases, inhibits ABC transporters in a non-specific way and was shown to inhibit

root secretion (Loyola-Vargas et al., 2007). In order to verify the role of root secretions transported by ABC-transporters on root-root interactions, *A. thaliana* Col-0 plants were grown singly or in pairs in standard medium or medium supplemented with 1.2 mg/L of sodium orthovanadate (Na_3VO_4) (New England BioLabs Inc.). Plants were grown and processed according to the procedures outlined in Chapter 2.2 Plants and growth conditions. The root morphology was assessed as an indicator of neighbour responsiveness. The total number of plates in this experiment was 38; that comprised 14 replicates of *A. thaliana* Col-0 grown single, 12 replicates of *A. thaliana* Col-0 grown paired and 12 replicates of *A. thaliana* Col-0 grown paired in media with sodium orthovanadate.

5.3.2.5 Assessment of the effect of ethylene on interaction

Ethylene has well established role in plant-plant interactions aboveground where it is involved in canopy sensing (Pierik et al., 2003, 2004, 2006, 2007). What is more, ethylene also plays multiple roles in the rhizosphere by modulating root growth and development, particularly under stressful conditions (Binder & Schaller, 2017; Borch et al., 1999; Pandey et al., 2021; Qin & Huang, 2018). The potential role of ethylene in plant-plant interactions belowground has been previously suggested (Cahill et al., 2005; Falik et al., 2005; Mutic & Wolf, 2007), but it has yet to be thoroughly investigated. In order to verify ethylene's involvement in interactions, *A. thaliana* Col-0 wild type and ethylene biosynthesis and perception impaired mutants (Table 5.1) were grown either singly or in pairs of all possible wild type and mutant combinations as in the Neighbour swap method as described in Chapter 2.3 Experimental design. Plants were co-cultivated until growth arrest, after which plants were treated and processed according to the procedures outlined in Chapter 2.3 Plants and growth conditions. The seed biomass was measured as an indicator of the reproductive output. The ethylene mutants did not differ from the wild type in terms of shoot biomass, apart from *aco4* which was bigger (Appendix C, Figure C.1). The total number of pots in this experiment was 146; that comprised 15 replicates of *A. thaliana* wild type grown single, 16 replicates in intragenotypic pair; 8 replicates of

ein4 grown single, 16 replicates in intragenotypic pair; 8 replicates of *ein2-1* grown single, 18 replicates in intragenotypic pair; 9 replicates of *ein3* grown single, 15 replicates in intragenotypic pair; 10 replicates of *aco1* grown single, 20 replicates in intragenotypic pair; 8 replicates of *eir1* grown single, 19 replicates in intragenotypic pair; 8 replicates of *aco2* grown single, 20 replicates in intragenotypic pair; 6 replicates of *aco4* grown single, 20 replicates in intragenotypic pair; 6 replicates of *ET-free* grown single, 8 replicates in intragenotypic pair.

5.4. Results

5.4.1. Assessment of the role of rhizodeposits in interactions

The role of rhizodeposits in plant-plant interactions of *A. thaliana* remains ambiguous due to the lack of the evidence for this signalling consequences on plant's fitness in previous reports (Biedrzycki et al., 2010; Biedrzycki et al., 2011; Biedrzycki et al., 2011; Caffaro et al., 2011, 2013). In this study, the role of rhizodeposits on plant-plant interactions was evaluated through various approaches, enabling the distinction between the effects of physical root contact, root exudate exchange and secretion.

The combined effect of physical root contact and rhizodeposits was examined by growing plants together in the same pot (Figure 5.4A). This enabled the close proximity and overlap of roots and their zones of influence, was that required for functionality. No significant differences in the total number of siliques produced by the focal *A. thaliana* Col-0 plants were observed across any of the plant pairings studied. Next, the effect of rhizodeposits exchange was tested under conditions where plants were grown together in the same pot but separated by a permeable mesh that allowed diffusion of chemical compounds (Figure 5.4B). Notably, no significant differences were found in seed biomass either between *A. thaliana* Col-0 grown alone and in intragenotypic pair nor with Ler ecotype or *C. rubella* and *C. hirsuta* species. However, the rhizodeposits derived from *O. pumila* significantly influenced the focal *A. thaliana* as it produced higher seed biomass than with another *A. thaliana* Col-0. Lastly, the impact of root exudates was assessed by irrigating singly grown *A. thaliana* Col-0 plants with leachates collected from other plants grown in either high or low nutrient conditions. Only exudates derived from *C. hirsuta* grown in high nutrient conditions had significant effect on the fitness of focal *A. thaliana* Col-0 such as it produced higher total number of siliques (Figure 5.4CD).

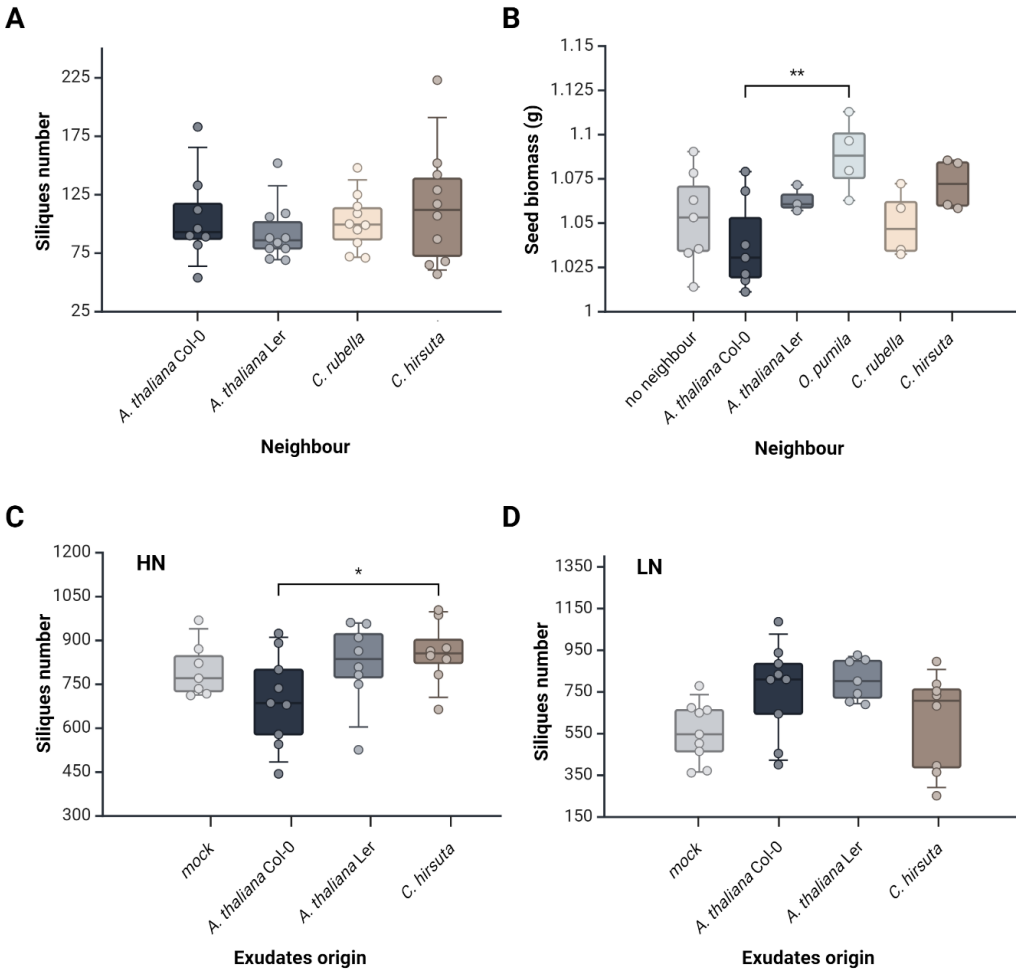


Figure 5.4. Rhizodeposits function in belowground interaction but it is complex and context dependent.

The species in the graph are arranged in phylogenetic order, from the closest to the most distantly related to *A. thaliana* Col-0 ecotype. (A) *A. thaliana* Col-0 ecotype grown with intra- and interspecific neighbours with root contact and exudates exchange allowed. 1-way ANOVA with Dunnett multiple comparisons test, n = 5-10. (B) *A. thaliana* Col-0 ecotype grown with intra- and interspecific neighbours with exudates exchange allowed but root contact permitted by mesh separator. 1-way ANOVA with Dunnett multiple comparisons test, $p < 0.05$, n = 4-8. (C) *A. thaliana* Col-0 ecotype grown single and irrigated with leachates from intra- and interspecific neighbours grown in high nutrients media. 1-way ANOVA with Dunnett multiple comparisons test, $p < 0.05$, n = 8-9. (D) *A. thaliana* Col-0 ecotype grown single and irrigated with exudates from intra- and interspecific neighbours grown in low nutrients media. 1-way ANOVA with Dunnett multiple comparisons test, $p < 0.05$, n = 7-9.

The possible involvement of root secretion through the ATP-binding cassette (ABC) transporters family in belowground plant-plant interactions has been suggested by several studies (Biedrzycki et al., 2011; Yang et al., 2018). To explore the role of ABC transporters in root-root interactions, plants were grown either singly or in pairs in standard medium or in medium supplemented with ABC-transporters sodium orthovanadate (Na_3VO_4) (Figure 5.5ABCD). No difference was observed between paired *A. thaliana* Col-0 plants upon inhibition of root secretion.

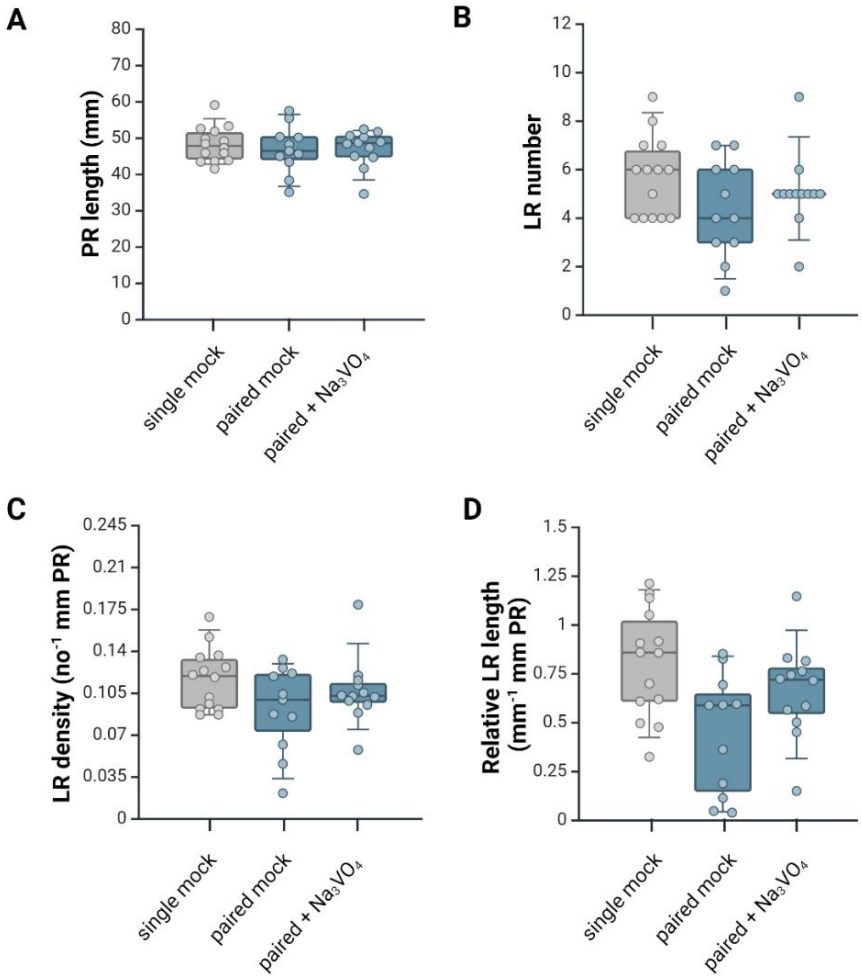


Figure 5.5. The disruption of root secretion through inhibition of ABC-transport does not affect belowground intraspecific interactions.

The comparison of *A. thaliana* Col-0 ecotype root traits between single, paired and paired conditions where ABC-transporters inhibitor was applied. (A) primary root length, (B) lateral root number, (C) lateral root density, (D) relative lateral roots length. Student's t-test, $p > 0.05$, $n = 11\text{-}14$.

5.4.2. Assessment of the role of ethylene in interactions

Ethylene, a volatile organic compound released by roots, plays various roles in the rhizosphere by influencing root growth and development, especially in response to stressful conditions (Binder & Schaller, 2017; Borch et al., 1999; Pandey et al., 2021; Qin & Huang, 2018). While its involvement in belowground plant-plant interactions has been proposed (Cahill et al., 2005; Falik et al., 2005; Muñoz-Parra et al., 2017; Mutic & Wolf, 2007), this area remains largely unexplored. Here, the role of ethylene biosynthesis and perception in interactions was assessed. *A. thaliana* wild type plants and ethylene mutants that were insensitive to ethylene or impaired in its production were grown singly or paired in all possible combinations and the impact on fitness measured as seed biomass was assessed.

In general, the consequences of the intragenotypic neighbour presence were similar between *A. thaliana* wild type plants and the majority of ethylene mutants as they also experienced decrease in the seed biomass (Figure 5.6A). However, in the case of one of the tested ethylene biosynthesis mutants — *aco1* this decrease was smaller and non-significant ($p = 0.0838$). The Relative Interaction Index (RII) revealed further differences in competition intensities between genotypes. The intragenotypic competition was less intense between *ein2-1* and *aco1* mutants and higher between *aco2* and *aco4* mutants than between wild type plants (Figure 5.6B). Further, it was tested whether having a neighbour that is impaired either in ethylene perception or biosynthesis had an effect on the interaction outcome of the wild type plant. The wild type plant benefitted significantly from interaction with one of the perception mutants – *ein2-1*. The seed biomass output of wild type paired with *ein2-1* was significantly higher than with another wild type and not different from single grown wild type plant (Figure 5.7A). This was further corroborated by the lowest competition intensity in interaction between wild type and *ein2-1* that was also ~5 times lower than between two wild type plants (Figure 5.7B).

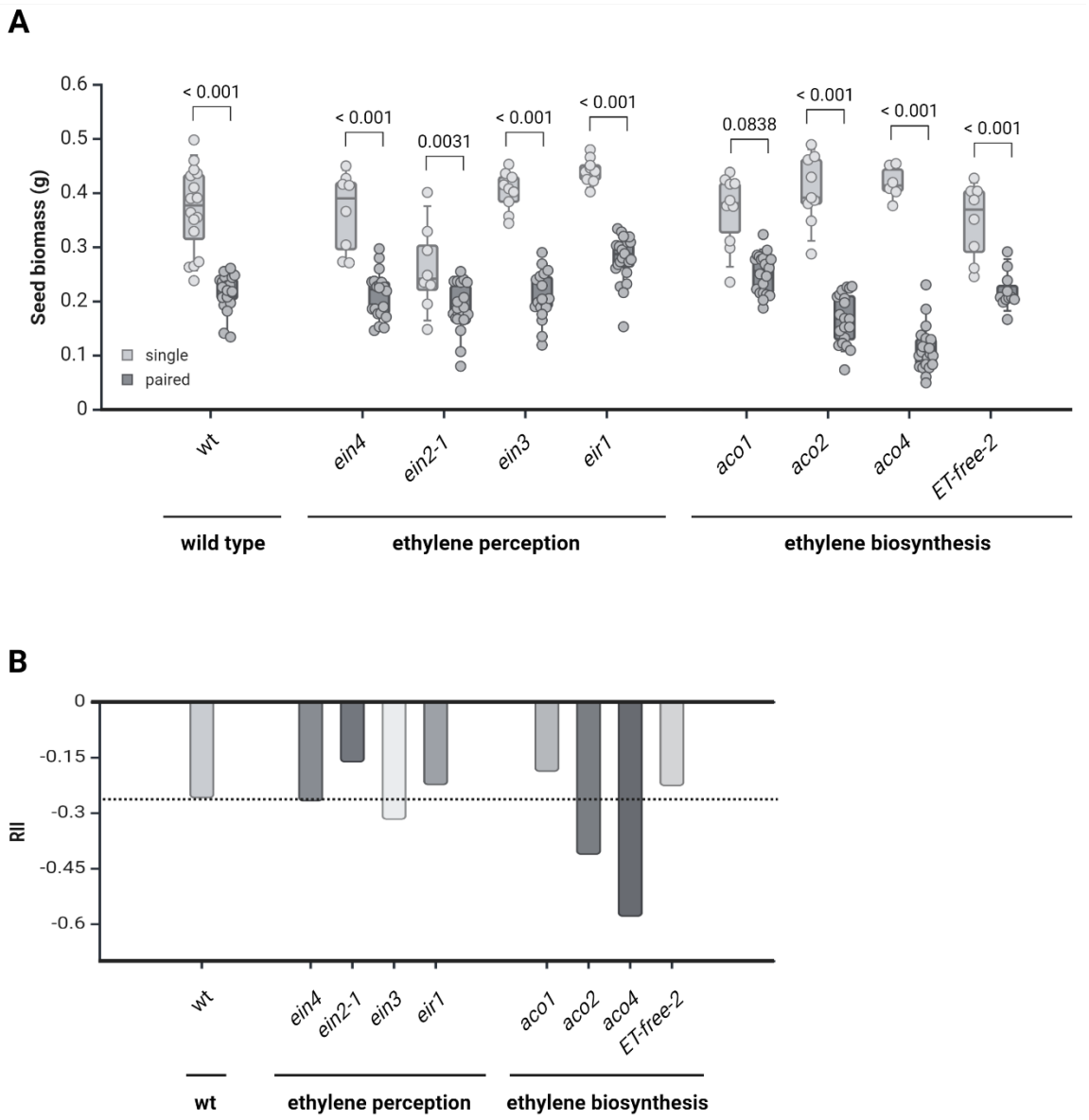


Figure 5.6. Ethylene biosynthesis and perception are not essential for neighbour detection but have impact on competition intensity.

The wild type and mutants impaired in ethylene perception or biosynthesis responses to being grown with a intragenotypic neighbour. Ethylene receptor mutants appear in order of the signalling cascade (Figure 5.2). (A) The seed biomass of plants grown singly and in pairs. Student's t-test, n= 8-20. (B) The Relative Interaction Index (RII) as a function of seed biomass. The level of RII experienced by wild type indicated with dashed line. n = 8-20.

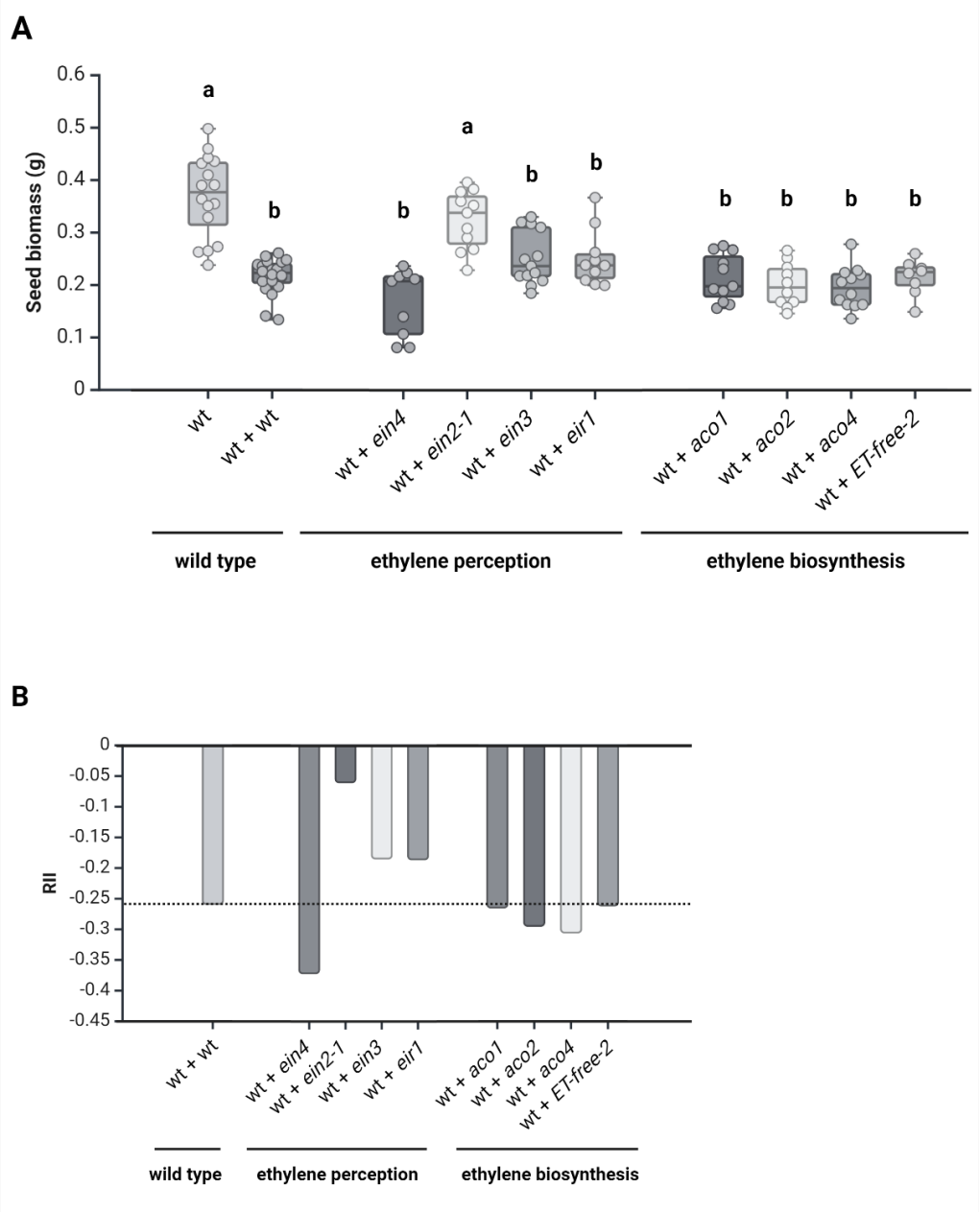


Figure 5.7. The perception of ethylene *via* EIN-2 is particularly essential for detecting and responding to neighbouring plants.

The wild type response to being paired with another wild type, mutant impaired in ethylene perception or biosynthesis. Ethylene receptor mutants appear in order of the signalling cascade (Figure 5.2). (A) The seed biomass of wild type plants grown singly and in pairs with another wild type or different mutant neighbours. Kruskal-Wallis test with Dunn's multiple comparisons correction, n = 8-20. (B) The Relative Interaction Index (RII) as a function of seed biomass. The level of RII experienced by wild type indicated with dashed line. n = 8-20.

5.5 Discussion

Root exudates have been heavily implicated in plants belowground communication and multiple species or context specific signals have been characterised (reviewed in Wang et al., 2021). However, no common neighbour detection signal neither its transporter nor receptor have been reported so far. This is surprising because the ability to detect the neighbour appears to be a common phenomenon observed among plethora of species (Callaway, 2002; Chen et al., 2012). It certainly have been observed in *A. thaliana* on multiple occasions (Biedrzycki, 2010; Caffaro et al., 2013; Cahill et al., 2005; Willis et al., 2010). Among others, ethylene was suggested as a promising common belowground plant detection signal however this was not comprehensively tested yet (Cahill et al., 2005; Falik et al., 2005; Mutic & Wolf, 2007). The role of root exudates, or more broadly rhizodeposits, in the context of plant-plant interactions remains unclear due to the lack of robust methodological assessments in the literature, as well as the absence of reference fitness outcomes related to this form of communication. Here it was hypothesised that rhizodeposits participate in plant-plant interactions with consequences for plants fitness, and that neighbour-derived ethylene may be used by plants as a proxy for neighbour detection.

5.5.1 Rhizodeposits have complex roles in interactions

The chemical signalling between plants is particularly well described in the realm of aboveground interactions (Ballaré & Pierik, 2017; Bilas et al., 2021; Huber et al., 2021; Kegge & Pierik, 2010; Pierik et al., 2003, 2013). Nevertheless, it is thought to be remarkably efficient belowground as well with multiple examples of root released signals and their functions in plant-plant interactions (Kong et al., 2018; Li et al., 2020; Semchenko et al., 2014; Yang et al., 2018). ABC transporters have been implicated in secretion of plant-plant signals in *A. thaliana* and *O. sativa* (Biedrzycki et al., 2011; Yang, 2018). The role of rhizodeposits in neighbour detection in *A. thaliana* remains unclear, as few studies have explored this topic. Moreover, the available literature does not fully address the potential impact of rhizodeposits on

plant fitness (Biedrzycki, 2010; Biedrzycki et al., 2011; Caffaro et al., 2011, 2013; Masclaux et al., 2010). In this study, several methodological approaches, previously successful in root exudate research in other species (Figure 5.3) (reviewed in McNickle, 2020), were utilised to investigate the role of rhizodeposits in plant-plant interactions in *A. thaliana*. Also, the possible role of ABC transporters in the secretion of relevant signals was assessed.

There were only two instances where belowground chemical interactions influenced *A. thaliana's* fitness. In the first case, *A. thaliana* exchanging rhizodeposits with *O. pumila* showed increased seed biomass production. Interestingly, in the same experiment, *A. thaliana's* fitness did not differ whether it was grown alone or in intraspecific pair, suggesting that the effect of *O. pumila* was likely due solely to its rhizodeposits, not those of *A. thaliana* or nutrient diffusion. A similar approach using mesh-separated pots has been employed in previous studies, yielding comparable results. In intraspecific pairs of Avena sativa, plants grown without partition, with a mesh partition, and with an impermeable plastic partition showed no significant differences in shoot, root, or total biomass (Semchenko et al., 2007). However, reproductive output was not assessed in that study. In contrast, Zhu and Zhang (2013) used a similar design and reported differences in interspecific combinations, attributing them to exudate sensitivity between two *T. aestivum* cultivars. The old landrace Monkhead benefited from being grown alongside the modern cultivar, although this benefit was not significantly proportional to the number of modern plants. Conversely, the modern cultivar 92-46 experienced a reduction in seed biomass, which appeared to be somewhat proportional to the number of old landrace plants (Zhu & Zhang, 2013). However, the study did not address potential allelopathic interference, nor is there information available on allelopathy for these specific cultivars. In addition, the modern cultivar was much smaller than the old landrace which introduces asymmetric competition for nutrients as a possible confounding factor. More recently, a study using similar methods found that *G. max* exhibited no response, while *H. annuus* showed a reduction in seed and shoot biomass, although

root biomass remained unaffected (Chen et al., 2021). Therefore, it may be concluded that rhizodeposits-driven interactions are not common among plant species.

In this work, the second instance where *A. thaliana* seemed to respond to root exudates included *C. hirsuta* grown in high but not low nutrient conditions, which resulted in significantly higher silique production. In a study using similar method Semchenko and colleagues (2014) reported *D. caespitosa* to be able to differentiate genetic relatedness, population origin and species identity of neighbours based on root exudates and adjust its root system architecture accordingly. A handful of studies that reported *A. thaliana* response to root exudates also mainly focused on root system modification and reported increased lateral root number in response to exudates from other accessions (Biedrzycki et al.,, 2010) and root system segregation with another ecotype (Caffaro et al., 2013). This is the first study that reports relationship between rhizodeposits and fitness in *A. thaliana*.

The inhibition of ABC transporters did not have an effect on root morphology in *A. thaliana* grown in pairs. Biedrzycki and colleagues (2011) reported that in hydroponics system ABC transporters were upregulated as a response to neighbouring plant and that their inhibition with the use of sodium orthovanadate generated lack of neighbour response in *A. thaliana*. However, a subsequent transcriptomics study reported contradictory results with ABC transporters being downregulated (Biedrzycki et al., 2011). Results presented here suggest that results from hydroponics studies are not observable in plants grown on plates with solid medium and that ABC transporters are most likely not involved in root exudates driven neighbour detection.

5.5.2 Plant detection of neighbours is mediated by ethylene and involves EIN2 and ACO1

Ethylene is released by roots and its build-up in soil space inhibits root growth (Pandey et al., 2021). Here it was hypothesised that neighbour-derived ethylene

contribution to its concentration in the rhizosphere may be used as a proxy for neighbour detection. Indeed, *aco1* — a mutant impaired in ethylene biosynthesis and *ein2-1* mutant which is insensitive to ethylene showed different impact on interaction than wild type plants. In the case of *aco1*, the ethylene biosynthesis impairment in intragenotypic pairs resulted in a smaller reduction in seed biomass and less intense competition compared to wild type plants. However, this characteristic of *aco1* was not significant when interacting with wild type plants. In the intergenotypic pairs wild type competed with *aco1* in similar way as with another wild type. In *A. thaliana* ACO genes showed tissue specific expression, ACO1 is expressed specifically at the root tip, ACO2 in the vasculature whereas also tested here ACO4 does not show notable expression in roots (Park et al., 2018). The root tip is especially sensitive to environmental cues as it is at the frontier of soil exploration (Falik et al., 2005; Okada & Shimura, 1990; Schenk, 2006). Therefore, it is likely that ethylene release from the root tip is particularly important for proper neighbour detection. Interestingly, Cahill and colleagues (2005) reported *eto1-1* – an ethylene overproducer, as a weaker competitor that allowed wild type *A. thaliana* gain in siliques number (Cahill et al. 2005). More research would be needed to explain the phenomenon of ethylene biosynthesis role and mechanism by which its synthesis affects plant-plant interactions. However, this is the first study reporting the role of ACO1 in neighbour detection in plants.

The *ein2-1* mutants which are insensitive to ethylene also experienced lower, albeit still significant decrease in seed biomass in intragenotypic pairing and the competition intensity between them was as well lower than between wild type plants. Their competition intensity was of similar level to the one between *aco1* mutants. However unlike ethylene biosynthesis and *aco1*, the less competitive approach of *ein2-1* had consequences also in the intergenotypic interaction with wild type. The wild type benefitted from the interaction with *ein2-1* as it experienced less intense competition and lost significantly less seed biomass than with another wild type. Only one other study assessed the role of EIN2 in plant neighbour perception and

reported no effect of *ein2* mutation on density sensing in *A. thaliana* (Muñoz-Parra et al., 2017). It should be acknowledged however, that density and crowding studies cannot be directly referred to plant-plant interactions studies as they differ fundamentally in their principles and resolution. Briefly, density studies focus on the *overall* effects of population size on growth and resource use, while plant-plant interaction studies explore the *specific* relationships and dynamics between neighbouring plants. Therefore it is likely that in density studies subtle effects may be overlooked. This is the first study reporting the role of EIN2 in neighbour detection in plants.

5.5.3 Concluding remarks

It is widely recognised that plant rhizodeposits are involved in interactions within the rhizosphere, engaging with both other plants and microbes (Callaway, 2002; Chen et al., 2012; Sun et al., 2021; Vives-Peris et al., 2020; Wang et al., 2021). This study thoroughly examined the role of rhizodeposition in plant-plant interactions and its effect on plant fitness, while also assessing the widely suggested neighbour detection signal, ethylene. It was shown that while rhizodeposits play a role in *A. thaliana* interactions with other plants, *A. thaliana* does not appear to respond to its own rhizodeposits in a way that alters its fitness. When rhizodeposits had significant effect on *A. thaliana* it was specific to species that released them and dependent on the nutrient environment that the donor plant was grown in. The role of ABC transporters in the secretion of interaction relevant signals was concluded unlikely in intragenotypic interaction that was tested. Here, for the first time ethylene was reported to mediate neighbour detection with involvement of EIN2 receptor and ACO1-driven biosynthesis. Further research would be required to identify specifically the compound(s) that evoked differential response in *A. thaliana* as a reaction to interspecific rhizodeposits and exudates. Alike, it would be interesting to test the involvement of ethylene in neighbour detection in other species to verify the commonality of this signal.

Chapter 6
Description of root exuded proteome profile

Abstract

Root exudates play a crucial role in plant-plant and plant-microbe interactions, yet their protein content remains poorly characterised. This study investigates the *Arabidopsis thaliana* root exuded proteome, focusing on proteins and small peptides under varying nutrient conditions. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), 2,425 proteins were identified under high nutrient conditions and 1,388 under low nutrient conditions, with 1,108 proteins shared across both environments. Approximately 25% of the common proteins contained secretion signals, with overrepresented biological processes linked to plant defence and rhizosphere interactions. High nutrient conditions led to a more diverse exudate proteome, while low nutrient conditions favoured selective secretion. Notably, receptor-like kinases, lectins, subtilisin-like proteases, and chitinases were prevalent, suggesting roles in biotic interactions. Small peptides were not directly detected in root exudates, despite prior evidence of their signalling roles. This study provides a foundational dataset for understanding root exudate composition and its potential influence on rhizosphere dynamics. The findings highlight the variability of root exuded proteins in response to nutrient availability and suggest that exudate proteins play a functional role in belowground signalling. Further research is needed to elucidate their specific roles in plant-plant interactions and microbial recruitment.

6.1 Introduction

Plant neighbour detection and response play a crucial role in determining the outcomes of plant-plant interactions (Callaway, 2002; Kong et al., 2018; Pierik et al., 2013). One hypothesis proposes that root exudates contain signals that plants use to detect the presence of nearby neighbours. However, root exudates are not well characterised, making it difficult to identify and test potential signalling compounds (Vives-Peris et al., 2020; Wang et al., 2021). In particular, the protein content of root exudates remains largely unexplored. Proteins are highly variable molecules in terms of structure, sequence, and function, making the proteome a promising source of compounds that may influence rhizosphere dynamics and plant-plant interactions.

The root exuded proteome refers to the diverse array of proteins exuded by plant roots into the surrounding soil environment that plays a crucial role in mediating plant-soil interactions, influencing nutrient acquisition, soil structure, microbial community composition, and overall plant health (Kapale et al., 2020; Sun et al., 2020; Tanveer et al., 2014). The root protein secretion profile was shown to change depending on the identity of the interacting microorganism (De-la-Peña et al., 2008) and plant neighbour identity (Badri et al., 2012). The literature concerning nutrients' impact on root exudates composition is scarce. Carvalhais and colleagues (2011) studying Zea mays reported that phosphorus deficiency promoted exudation of c-aminobutyric acid and carbohydrates, and nitrogen starvation led to decrease in secretion of amino acids (Carvalhais et al., 2011). Another example comes from analysis of Helianthus annuus exudation in half nutrient strength of media and concluded fumaric acid, quinic acid, and glucose to become significantly more abundant (Bowsher et al., 2016). Some of those organic acids were proven to have role in phosphorus mobilization (Pantigoso et al., 2020). However, the basic constitutive set of exuded protein is unknown.

To date, only a limited number of studies have focused on the qualitative assessment of the root exuded proteome. The first report by Basu and colleagues

(2006) identified 16 proteins in oilseed rape (*Brassica napus*) and 52 proteins in *A. thaliana* exudates (Basu et al., 2006). Later, ~100 proteins were identified in pea (*Pisum sativum*) root exudates (Wen et al., 2007). More than 100 proteins were reported to be exuded by *A. thaliana* and *Medicago truncatula* interacting with microbes (De-la-Peña et al., 2008). The proteins detection efficiency improved over time and in 2010 Ma and colleagues reported 2848 proteins in exudates of maize (*Zea mays*) (Ma et al., 2010) whereas in 2012 Badri and colleagues reported 426 exuded proteins in *A. thaliana* Col-0 ecotype, 343 in Ler ecotype and 123 in *C. rubella* root exudates (Badri et al., 2012). Exudates released by roots of three legume species contained 150 proteins in white lupin (*Lupinus albus*), 121 in soybean (*Glycine max*), and 79 in cowpea (*Vigna sinensis*) (Liao et al., 2012). More recently, 2861 exuded proteins were reported in grasspea (*Lathyrus sativus*) (Rathi et al., 2022). There has been a significant progress in root exudates proteomics, yet due to high variability in reported results this field remained far outside the realm of general conclusions and functional assessment of particular proteins in the rhizosphere.

The potential of small peptides (> 50 aa) to function as exogenous signals is even less explored than the one of proteins. Unlike larger proteins, small peptides often act as hormones or signalling molecules that mediate short and long distance signalling, growth, development, stress responses, and defence mechanisms. Small peptide signalling has been shown to regulate root meristem development and primary and lateral root emergence and growth, lateral root angle regulation and root hair and Casparian strip development (De Coninck & De Smet, 2016; Ohyama et al., 2008; Takahashi et al., 2019). The high evolutionary conservation of plant small peptides together with the enzyme families required for their processing implies a hidden but potentially significant benefit for their conservation (Ogilvie et al., 2014) and numerous small peptides families emerged after plants gained the root. The *A. thaliana* genome encodes approximately 6000 genes of predicted small signalling peptides out of which more than 300 are expressed in roots (Hanada et al., 2013; Wang et al., 2020). The field of peptidomics emerged recently and is rapidly

progressing. So far, the scarce evidence for small peptides' presence in roots originates from expression analysis or overexpressed peptide detection, but the external application of synthetic forms is widely applied and sufficient to invoke response. Whether small peptides have exogenous roles in the rhizosphere remains unknown.

6.1.1. Summary

This chapter focuses on the characterization of root exudates constituents, particularly proteins and small peptides. While root exudates are hypothesised to play a key role in neighbour detection, their composition, especially the protein content, remains poorly characterised. Progress has been made in identifying root exuded proteins, with studies reporting hundreds to thousands of proteins in different plant species, yet high variability in results has limited general conclusions. The potential role of small peptides as signalling molecules in the rhizosphere is even less explored, though they are known to regulate critical root functions. Root exudates content has been shown to differ depending on the nutrient environment, however it is not known whether this applies to proteins. This chapter provides root exuded dataset to understand the exogenous roles of these proteins and peptides in the rhizosphere and has general implications for broadening our knowledge also beyond plant-plant interactions.

6.2 Chapter aims

Given the existing knowledge gaps, this chapter seeks to generally characterise the root exuded proteome in *A. thaliana*. Following the approaches of studies reporting root exudates metabolic composition and other reports on root exuded proteome this study aims to address the following objectives:

1. Define proteins that are constitutively present in root exudates.
2. Provide a summary of the functions of root exuded proteins.
3. Define the impact of nutrients conditions on root exuded proteome.
4. Attempt to determine small peptides presence in root exuded proteome.
5. Propose root exuded proteins with potential roles in plant-plant interactions.

6.3 Methods

6.3.1 Plant growth conditions

For mass spectrometry analysis of root exuded proteome, *A. thaliana* Col-0 seeds were surface sterilised in 70% ethanol and 7% bleach, resuspended in 0.1% sterile agar, and stratified in 4°C for 48 hours. Afterwards seeds were placed on plates and germinated in controlled environment room (ATS (Wilson et al., 1990) with 1% sucrose and 1% phytagel (Duchefa); 16hrs light, 8 hrs dark; 20°C). After 14 days 50 seedlings per sample were transferred to Erlenmeyer flasks which had been filled with 100 ml of 100% or 10% ATS media with 1% sucrose. Plants were grown in sterile conditions for two further weeks prior to hydroponates collection. Each treatment, high and low nutrients conditions was comprised of 3 replicates.

6.3.2 Exuded proteins extraction and LC-MS/MS analysis

Hydroponates were collected, passed through 0.45 µm filter (Nalgene, Thermo Fisher Scientific), and then high molecular weight (HMW) proteins were separated from low molecular weight (LMW) by tangential flow filtration with 10 000 Da cut-off membrane (Centramate Ultrafiltration system; PALL Europe, Portsmouth, Hampshire, UK) and freeze-dried. Peptides were extracted using o-chlorophenol method (Ohyama et al., 2008). Briefly, 20 ml of o-chlorophenol with 1% n-ethylmorpholine (NEM) was added to each sample and shaken for 1 min at RT (25°C). Samples were centrifuged at 10 000 g for 10 min. The phenolic phase was collected, and 20 volumes of acetone were added. Samples were left for precipitation at −20°C overnight. Samples were centrifugated at 10 000 g for 10 min. Precipitated samples were dissolved in 500 µl of 1.0% trifluoroacetic acid (TFA). The small peptide CEP5 of sequence DFR{HYP}TT{HYP}GHS{HYP}GIGH (Q058G9, At5g66815) was synthesised by GenScript USA Inc. with at least 90% purity and dissolved in ddH2O as per manufacturer's recommendations. The standard samples were spiked to 10, 1 and 0.1 µM CEP5 concentration and analyzed.

HMW samples were subjected to trypsin (Promega) digestion prior to further analysis using the S-TRAP Micro column (PROTIFI, NY, USA) following the manufacturer's instructions. 3 µL sample (approx. 0.6 ug of protein) were injected onto an in house-packed 20cm capillary column (inner diameter 75µm, 3.5µm Kromasil C18 media). An Ultimate 3000 nano liquid chromatography system was used to apply a gradient of 2–30% ACN in 0.1% formic acid over 30 min at a flow rate of 300 nl/min. Total acquisition time was 60 min including column wash and re-equilibration. Peptides were eluted from the column and into an Orbitrap Exploris 240 Mass Spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK) via a nanospray flex ion source using a capillary voltage of 2.7 kV. Precursor ion scans were acquired in the Orbitrap with resolution of 60000. EASY-IC internal calibration was used for precursor ion scans. Up to 20 ions per precursor scan (charge state 2+ and higher) were selected for HCD fragmentation using a normalised collision energy of 30%. Fragments were measured in the Orbitrap at a resolution of 15000. Dynamic exclusion of 30 s was used. Peptides were analyzed through DDA (Data-Dependent Acquisition) in Ion Trap with a 50 millisecond injection time (run length 35 min, column length 50 cm).

6.3.3 Bioinformatic analysis

Peptide MS/MS data were processed with PEAKS Studio XPro (Bioinformatic Solutions Inc, Waterloo, Ontario, Canada) and searched against the Uniprot Arabidopsis thaliana database. MS mass tolerance was 20 ppm, and fragment ion mass tolerance was 0.05 Da. The peptide false discovery rate was set to 1%. Proteins that were detected in at least 2 out of 3 replicates were considered valid.

6.3.3.1 Identification of secreted proteins

To predict the subcellular localization of proteins identified in the root exudates of *A. thaliana* TargetP v2.0 was utilised (Armenteros et al., 2019). TargetP predicted the presence of N-terminal signal peptides that direct proteins to specific cellular compartments, including the secretory pathway. It distinguished between proteins

targeted to the secretory pathway (*via* a N-terminal signal peptide, SP), the mitochondrion (mTP), thylakoid lumen (lTP), the chloroplast (cTP), and proteins without clear targeting signals (other). Proteins predicted to contain an SP signal were considered candidates for secretion into the extracellular space, including cell membrane and root exudates. These results were used to identify proteins that might perform extracellular functions, such as involvement in rhizosphere interactions.

6.3.3.2 Proteins overrepresentation

The Venn diagram was constructed using Venny 2.1 (Oliveros, 2007). The proteins were subjected to PANTHER Overrepresentation Test based on GO biological process term against *A. thaliana* whole genome as a reference with Fisher's Exact test type and FDR correction (Released 20240226, PANTHER) (Mi et al., 2019).

6.3.3.3 Peptides detection

The identified peptide sequences generated by *de novo* sequencing were searched against CEP5 aa sequence (DFRPTTPGHSPGIGH) with in R Bioconductor package (Gatto & Christoforou, 2014; Pagès et al., 2019) with 5 aa fragment length match requirement. The *contains_fragment* function was defined to check if a fragment of the peptide sequence exists as a substring within another peptide sequence. This function utilised the *grepl* function for substring matching from base R (R Core Team 2023). The *check_fragments* function was implemented to iterate over all possible fragments of a given length within the target peptide sequence. For each fragment, it checked if the fragment was present in any of the cleaned peptide sequences from the dataset. The function constructed the fragments by iterating over the target sequence with a sliding window approach.

6.4 Results

In order to characterise the protein content of *A. thaliana* root exudates, plants were grown in high and low nutrients liquid media which was collected and analyzed. To address the objective regarding small peptides detection, a filtration was performed in order to separate and enrich small peptides fraction hence, the high molecular weight (HMW, > 10 kDa) and low molecular weight (LMW, < 10 kDa) fractions were obtained. In plants, small peptides are typically 5 to 20 aa in length, though this can vary. To estimate the average mass of a small peptide with 15 aa, the average molecular weight of an amino acid, which is about 110 Da can be applied. Therefore, the average mass of a 15 aa peptide would be approximately 1.65 kDa. This is a rough estimate, as different amino acids have slightly varying masses. While there is no universal classification system for protein sizes, grouping proteins by small (< 20 kDa), medium (20-60 kDa), large (60-100 kDa) and very large sizes (> 100 kDa) is a common practice in many fields, especially in mass spectrometry. Analysis of fraction samples from high and low nutrient conditions revealed a distinct separation in protein abundance across different size categories (Figure 6.1A).

A greater number of proteins was detected under high nutrient conditions, particularly in the HMW fraction. Both HMW and LMW fractions exhibited similar protein size distributions in both nutrient conditions. However, further analysis indicated that the filtration process was not effective in enriching small proteins. Actually, the HMW fractions spanned a wider range of protein sizes and also captured smaller proteins better: in high nutrient conditions, the range of detected proteins sizes was 2–405 kDa, while in low nutrient conditions, it ranged from 2–328 kDa. In contrast, the smallest proteins detected in the LMW fractions were approximately 9 kDa and 13 kDa, respectively. Consequently, HMW and LMW samples were combined based on nutrient conditions, with duplicated proteins removed. This resulted in the detection of 2,425 unique proteins under high nutrient conditions and 1,388 unique proteins under low nutrient conditions. The Venn diagram illustrates the

distribution of unique proteins detected under high and low nutrient conditions (Figure 6.1B).

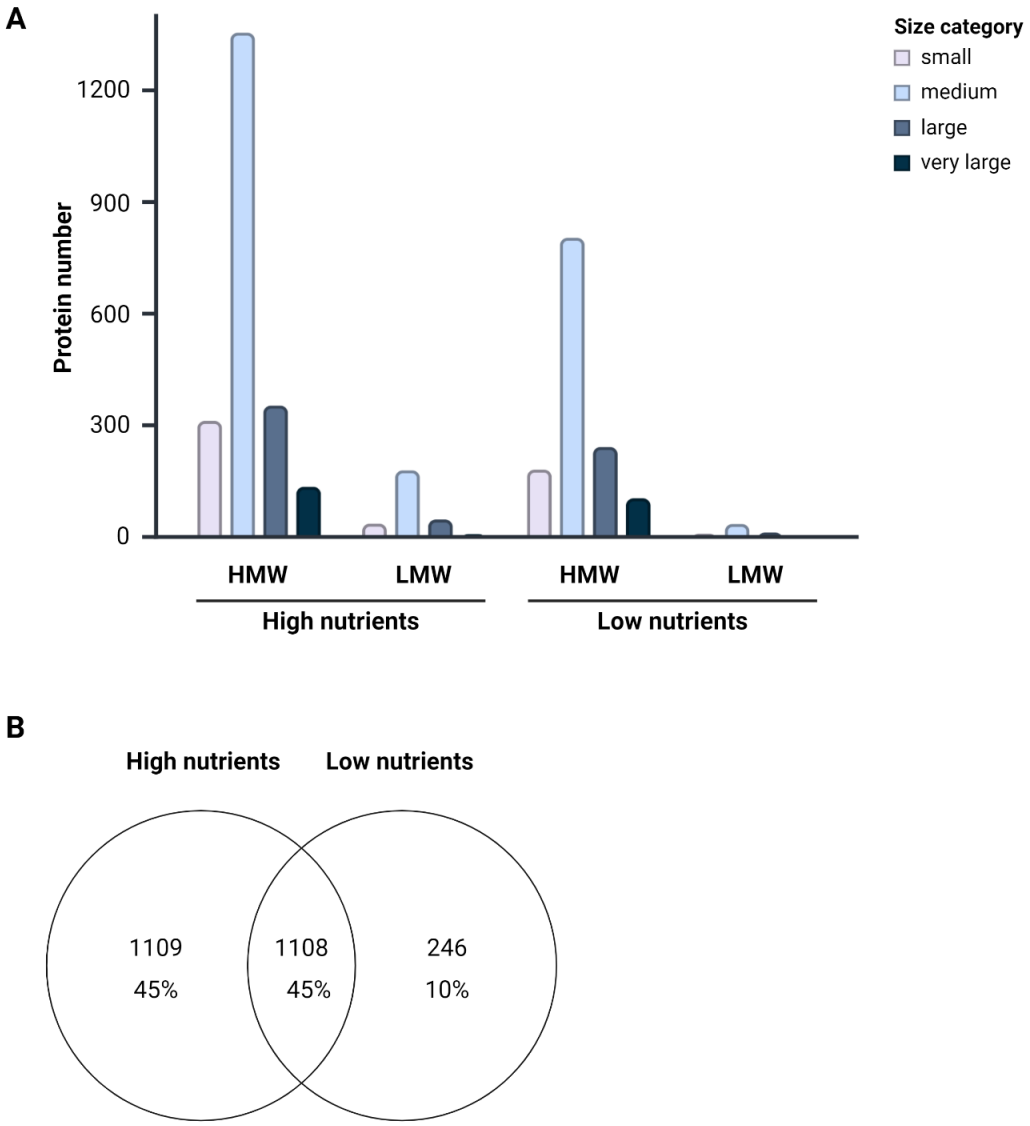


Figure 6.1. Distinct and overlapping protein profiles in *A. thaliana* root exudates.

(A) The distribution of high molecular weight (HMW) and low molecular weight (LMW) proteins, categorised by size, in the root exudates of A. thaliana grown under high and low nutrient conditions. Bar charts display the number of proteins detected within each mass fraction. Detected proteins were classified by size as follows: small < 20 kDa, medium 20-60 kDa, large 60-100 kDa, very large > 100 kDa. (B) The Venn diagram with the overlap of proteins detected under different nutrient conditions. The numbers within each section indicate the number of proteins unique to or shared. The percentages represent the proportion of the total proteins detected.

While there was a substantial overlap between the proteins detected commonly in both conditions (45%), there were distinct sets of proteins found exclusively in either high or low nutrient environments, with high nutrient conditions showing a larger proportion of unique proteins compared to low nutrient conditions (45% and 10% respectively).

In none of the experimental sample's CEP or other annotated small peptides were identified. The smallest protein detected in high and low nutrients condition was actually the same protein CWP04 (P80828) with mass of 2.3 kDa which is a small uncharacterised cell wall protein. The next small proteins detected in both nutrient treatments were of size > 5 kDa and comprised of ribosomal subunits. Standard CEP5 peptide was detected only in control samples spiked with 0.1uM concentration but was not detected with confidence in samples spiked with 1 and 10µM concentration of CEP5.

6.4.1 The commonly present root exudates proteins

Approximately 45% of the proteins detected in exudates, comprising 1,108 unique proteins, were common to both high and low nutrient conditions (Figure 6.1B).

To verify how many of these proteins were truly intended for secretion and to eliminate background proteins potentially resulting from root cell damage, an analysis was conducted using TargetP (Armenteros et al., 2019). TargetP determines whether a protein is secreted by identifying the presence of an N-terminal signal peptide (SP), which directs the protein into the classical secretory pathway. In this pathway, the protein is transported to the endoplasmic reticulum, where the signal peptide is cleaved, allowing the protein to either be secreted into the extracellular space or integrated into the plasma membrane. More than half of the common proteins lacked targeting signal (656 proteins, 59% of total common proteins). Approximately 25% of common exudate proteins had a predicted signal peptide (SP), which suggested that these protein are destined for the secretory pathway (Figure 6.2). The protein overrepresentation analysis was conducted to identify enriched

biological processes associated with 282 secreted proteins common in root exudates. In general, 85 biological processes were significantly overrepresented among common secreted proteins (Appendix D, Table D.1).

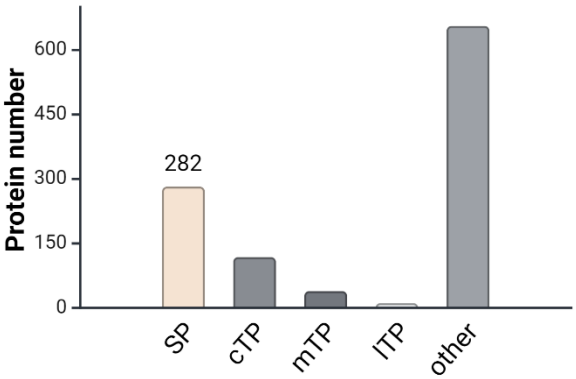


Figure 6.2. The abundance of proteins predicted for secretion represent a significant proportion of common root exudates.

SP signal peptide, directed to the ER and possibly secreted or membrane-bound; mTP mitochondrial transfer peptide, directed to the mitochondria; cTP chloroplast transfer peptide, directed to the chloroplast; lTP thylakoid luminal transfer peptide, specifically targeted to the thylakoid lumen in the chloroplast; other, likely cytoplasmic or without a detectable targeting signal.

The carbohydrate and cell wall processes were highly overrepresented. For example, processes of cell wall catabolic process and polysaccharide catabolic process showed large fold enrichments. Several metabolic processes, including L-arabinose metabolic process, mannose metabolic process, and tryptophan catabolic process, showed significant fold enrichment. Among the overrepresented processes, 19 were related to plants interactions with other organisms (Table 6.1). A strong enrichment in defence-related processes was identified, particularly in systemic acquired resistance, defence responses to fungus, and symbiont interactions. These processes showed high fold enrichment values and were supported by highly significant p values. Several enriched biological processes were related to response to biotic stimuli, response to other organisms, and interspecies interaction. Broader processes like response to external stimulus and response to

chemical showed lower fold enrichment (around 2-3), but still indicated that exudate proteins are responsive to a wide range of environmental signals and stressors.

Table 6.1. Overrepresentation of common root exudate proteins in biological processes related to interactions with other organisms.

GO biological process complete	Reference proteins #	This study proteins #	Expected proteins #	Fold Enrichment	p value	FDR
systemic acquired resistance	67	12	0.68	17.64	3.46E-12	1.20E-09
response to chitin	23	4	0.23	17.13	7.91E-05	4.93E-03
response to insect	43	4	0.44	9.16	9.41E-04	4.78E-02
defense response to fungus	279	19	2.83	6.71	8.80E-11	2.03E-08
response to fungus	383	25	3.89	6.43	2.17E-13	8.60E-11
defense response to symbiont	269	16	2.73	5.86	1.91E-08	2.58E-06
defense response to other organism	882	41	8.96	4.58	4.23E-16	2.35E-13
immune system process	220	10	2.23	4.48	9.11E-05	5.55E-03
response to ethylene	179	8	1.82	4.40	5.12E-04	2.70E-02
response to external biotic stimulus	1209	50	12.28	4.07	1.98E-17	1.83E-14
response to other organism	1209	50	12.28	4.07	1.98E-17	1.57E-14
response to biotic stimulus	1212	50	12.31	4.06	2.19E-17	1.52E-14
involved in interspecies interaction between organisms	1223	50	12.42	4.03	3.17E-17	1.95E-14
defense response	1108	43	11.25	3.82	4.20E-14	1.79E-11
response to bacterium	510	19	5.18	3.67	1.37E-06	1.27E-04
response to external stimulus	1420	51	14.42	3.54	2.80E-15	1.41E-12
defense response to bacterium	390	14	3.96	3.54	5.12E-05	3.42E-03
response to stimulus	5862	127	59.53	2.13	1.40E-19	1.94E-16
response to chemical	2711	58	27.53	2.11	4.39E-08	5.53E-06

The detailed overview of the secreted 282 common proteins in the root exudates of *A. thaliana* revealed a diverse array of proteins covering a broad spectrum of functions, including potential roles in rhizosphere interactions (Appendix D, Table D.2). Notably, several receptor families involved in biotic interactions were detected, such as lectins, jacalin-related proteins, Lysin motif receptor-like kinases (LysM), Leucine-rich repeat receptor-like kinases (LRR-RLKs), Cysteine-rich receptor-like protein kinases (CRKs), and a group of uncharacterised GPI-anchored proteins

(Buendia et al., 2018; Furumizu et al., 2021; Tsaneva & Van Damme, 2018; Zeiner et al., 2023). An overrepresentation of other proteins associated with both abiotic and biotic stress responses was also observed. These included general abiotic stress proteins like heat shock proteins and peroxidases, as well as proteins involved in alkaloid biosynthesis (Figueiredo et al., 2018; Grover, 2012). In relation to biotic stress, proteins involved in plant defence and pathogen response were enriched, with five subtilisin-like proteases and six chitinases detected. Subtilisins play a role in processing peptides that act as signalling molecules in plant defence, while chitinases release chitin fragments that are recognised by receptors such as LysM receptors, triggering immune signalling pathways (Figueiredo et al., 2018; Grover, 2012). Additionally, other biotic stress-related proteins were identified, including pathogenesis-related proteins and defensins (Parisi et al., 2019). Of particular note was the presence of several purple acid phosphatases (PAPs), which are commonly involved in phosphate mobilization in the rhizosphere (Bhadouria & Giri, 2022).

6.4.2 Root exudates proteins specific to high nutrients

Around 45% of the proteins found in the exudates, totaling 1,109 unique proteins, were exclusively associated with the high nutrient condition. With the use of TargetP (Armenteros et al., 2019) it was determined that approximately 65% of high nutrients specific proteins lacked targeting signal (718 proteins) whereas only ~12% had a predicted signal peptide (SP) (137 proteins) (Figure 6.3).

The protein overrepresentation analysis was conducted to identify enriched biological processes associated with 137 secreted proteins in high nutrient specific root exudates. In general, 30 biological processes were significantly overrepresented among common secreted proteins (Appendix D, Table D.3). Several metabolic processes involved in cell wall structure maintenance were overrepresented. That included lignin, xyloglucan and pectin related processes.

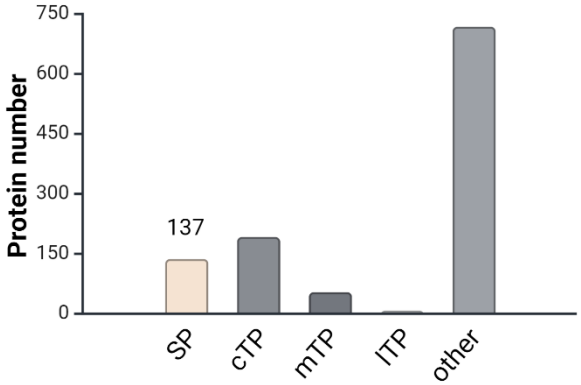


Figure 6.3. The abundance of proteins predicted for secretion represent a small fraction of high nutrient specific root exudates.

SP signal peptide, directed to the ER and possibly secreted or membrane-bound; mTP mitochondrial transfer peptide, directed to the mitochondria; cTP chloroplast transfer peptide, directed to the chloroplast; lTP thylakoid luminal transfer peptide, specifically targeted to the thylakoid lumen in the chloroplast; other, likely cytoplasmic or without a detectable targeting signal.

Among the overrepresented processes, 5 were related to plants' interactions with other organisms (Table 6.2). Both general responses such as response to biotic stimulus, and response to other organism as well as more specific terms such as defence response to symbiont could be noted.

Table 6.2. Overrepresentation of high nutrient specific root exudate proteins in biological processes related to interactions with other organisms.

GO biological process complete	Reference proteins #	This study proteins #	Expected proteins #	Fold Enrichment	p value	FDR
defense response to symbiont	269	7	1.29	5.42	3.32E-04	4.28E-02
response to external biotic stimulus	1209	16	5.81	2.75	2.32E-04	3.48E-02
response to other organism	1209	16	5.81	2.75	2.32E-04	3.39E-02
response to biotic stimulus	1212	16	5.82	2.75	2.39E-04	3.39E-02
involved in interspecies interaction between organisms	1223	16	5.88	2.72	2.64E-04	3.66E-02

The detailed analysis of the 137 proteins specifically secreted under high nutrient conditions in the root exudates of *A. thaliana* revealed a diverse array of proteins with a broad range of functions, including potential roles in rhizosphere interactions (Appendix D, Table D.4). Most of these belonged to the protein groups already identified in common set, as described in subsection 6.4.1, though additional genes were also discovered within these groups. Notably, several additional receptor families involved in biotic interactions were identified, such as lectins, Lysin motif receptor-like kinases (LysM), Leucine-rich repeat receptor-like kinases (LRR-RLKs), Cysteine-rich receptor-like protein kinases (CRKs), and an uncharacterised GPI-anchored protein (Buendia et al., 2018; Man et al., 2020; Tsaneva & Van Damme, 2020; Zeiner et al., 2023). An overrepresentation of proteins related to stress responses was observed, with a prevalence of biotic stress-associated proteins. Additional plant defence and pathogen response proteins were enriched, including one subtilisin-like protease and two chitinases. Seven additional defensins were detected, alongside pathogenesis-related proteins (dos Santos & Franco, 2023; Parisi et al., 2019). What is more, eight additional purple acid phosphatases (PAPs), commonly involved in phosphate mobilization (Bhadouria & Giri, 2022).

6.4.3 Root exudates proteins specific to low nutrients

About 10% of the proteins detected in the exudates, totaling 246 unique proteins, were exclusively associated with low nutrients. Those proteins were analyzed to predict the presence of secretion signals (Figure 6.4). Approx. half of proteins lacked signal peptides, indicating they are likely cytoplasmic (119 proteins, ~48% of low nutrient-specific proteins). Interestingly, around 48% of the low nutrient-specific proteins were predicted to have signal peptides, suggesting they are directed for secretion.

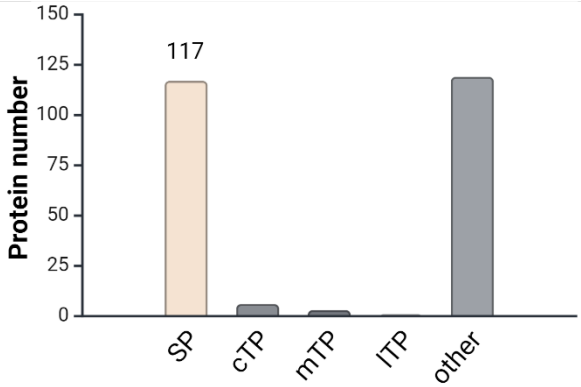


Figure 6.4. The abundance of proteins predicted for secretion represent the majority of low nutrient specific root exudates.

SP signal peptide, directed to the ER and possibly secreted or membrane-bound; mTP mitochondrial transfer peptide, directed to the mitochondria; cTP chloroplast transfer peptide, directed to the chloroplast; lTP thylakoid luminal transfer peptide, specifically targeted to the thylakoid lumen in the chloroplast; other, likely cytoplasmic or without a detectable targeting signal.

A total of 29 biological processes were found to be overrepresented in the proteins specific to low nutrient exudates (Appendix D, Table D.4). Nearly all of the overrepresented processes were related to cell wall maintenance, particularly catabolic activities such as cell wall disassembly, cell wall modification during abscission, and the pectin and hemicellulose catabolic processes. Notably, no processes associated with biotic interactions were identified to be overrepresented. However, a detailed analysis of proteins detected specifically in low nutrient root exudates revealed several proteins groups with potential roles in rhizosphere (Appendix D, Table D.6). A high number of receptors from LRR-RLK and CRK families were found including well-characterised receptors for brassinosteroids (BRI1) (Hothorn et al., 2011) and root growth factors (RGI5) (Ou et al., 2016a). One additional subtilisin-like protease was found (SBT3.6) (Figueiredo et al., 2018) as well as two additional PAPs (PPA6 and PPA13) (Bhadouria & Giri, 2022). 11 additional biotic stress response proteins were found mainly from the germin-like proteins, but pathogenesis-related protein and defensin were noted as well (Govindan et al., 2024).

6.5 Discussion

The *Arabidopsis thaliana* genome was sequenced 20 years ago, and hundreds of ecotypes have since been analyzed at the genome and transcriptome level (The Arabidopsis Genome Initiative, 2000). By contrast, the *A. thaliana* proteome as the main executer of most biological processes is far less comprehensively characterised and its mass-spectrometry-based draft was released in 2020 (Mergner et al., 2020). Due to this it is unsurprising that challenging samples such as protein content of root exudates remains vastly uncharacterised with few reports on their content (Basu et al., 2006; Charmont et al., 2005; Liao et al., 2012; Ma et al., 2010; Staudinger et al., 2022; Tian et al., 2009). Unlike root organs, tissues or cells that often take advantage of transcriptomics, in the case of root exudates, only the direct identification of particular compound can verify its presence (Vives-Peris et al., 2020). In this study, the updated basal proteome of *A. thaliana* Col-0 root exudates is characterised, with additional insights into protein alterations in response to different nutrient conditions.

6.5.1 Root exudates contain a common set of proteins present regardless of nutrients conditions

1,108 proteins were commonly present in root exudates regardless of the nutrient condition, out of which ~25% (282 proteins) had predicted secretion signals. Numerous processes related to biotic stress were overrepresented. This corroborates the hypothesis that root exudates indeed contain proteins aimed at functioning in rhizosphere interactions.

Among the set of common proteins a number of receptor families were found including lectins (9 proteins), LysM (4 proteins), LRR-RLK (11 proteins), CRK (6 proteins) and uncharacterised GPI-anchored proteins (4 proteins). The presence of receptors typically bound to the cell membrane is likely due to the release of border-like cells during root growth or ectodomain shedding, which renders the receptors non-functional but still carries important roles. These include regulating signalling by

controlling receptor activation or deactivation, acting as decoys in pathogen defence, releasing soluble signalling molecules into the extracellular environment, and aiding in stress adaptation and protein turnover management (Antolín-Llovera et al., 2014; Burkart & Stahl, 2017; Tör et al., 2009).

Lectins are an important class of proteins involved in the recognition of microbes and are considered non-specific defence proteins (Bian et al., 2024; Peumans & Van Damme, 1995; Tsaneva & Van Damme, 2018). Lectin proteins bind to oligosaccharides and can be associated with kinases forming lectin receptor-like kinases (LecRLKs). However, soluble lectins are also known i.e. amaranthins, calreticulins/calnexins, Euonymus lectin- (EUL) related lectins, and nictaba lectins, ricin-B lectins and, detected here, jacalin-related lectins (JRL) (Lannoo & Van Damme, 2014; Nagano et al., 2008). The jacalin-related lectins were prevalent with 3 receptors detected JAL8, JAL9 and JAL10. Strikingly, jacalin-related lectins were also found in root exudates in other studies, JAL10 was found in 3 other independent studies on *A. thaliana* exudates (Badri et al., 2012; Basu et al., 2006; Charmont et al., 2005) whereas JAL9 was reported in *A. thaliana* and *C. rubella* exudates (Badri et al., 2012). JRLs bind to microbial surfaces and inhibit their growth or facilitate their recognition by the plants immune system (Esch & Schaffrath, 2017) and were shown in rhizobia-host selectivity (Downie, 2010; Wolpert & Albersheim, 1976). A similar process could be helping plants to differentiate and identify their neighbour.

Another example of proteins detected here and found in other exudates proteomics studies as well are subtilisin-like proteases. Those were found in exudates of *A. thaliana* and *C. rubella* (Badri et al., 2012; Basu et al., 2006), tobacco (Wendlandt et al., 2015), wheat (Staudinger et al., 2022) and in outer root tissue of asparagus (Döll et al., 2021). Subtilisin-like proteases were shown to facilitate lateral root emergence (Neuteboom et al., 1999). Here, common proteins in root exudates include 5 subtilisin-like proteins: SBT1.4, SBT1.7, SBT3.3, SBT 3.5 and CRSP (CO_2-response secreted protease). Subtilisins were shown to be involved in several small peptides processing. For example, SBT1.7 found here, was recently shown to be vital

in flagellin processing that allows efficient initiation of plant defence (Matsui et al., 2024). Interestingly, another subtilisin SBT1.1 was shown to be involved in roots in processing a plant growth promoting peptide Phytosulfokine 4 (PSK4) to its active form (Srivastava et al., 2008) whereas S1P relieves the growth-retarding effect of a RALF 23 peptide growth factor (Srivastava et al., 2009). Another class of processing enzymes reported here are chitinases with 6 representatives. Chitinases break down chitin, a key component of fungal cell walls. These proteins play a crucial role in plant defence by degrading fungal pathogens, triggering defence signalling, and restricting fungal growth (Grover, 2012).

A few other groups of proteins related to biotic stress were also identified. The two pathogenesis-related proteins PR1 and PR5 are molecular markers for SAR response to a variety of pathogens (Han & Schneiter, 2024). In addition, two defensins PDF1.5 and ATTI1and five different germin-like proteins were detected. Interestingly, PDF1 genes together with other defensin genes were shown to be upregulated in response to intergenotypic root exudates in *A. thaliana* (Biedrzycki et al., 2011).

To summarise, the set of commonly found proteins that were identified contains interesting candidates with proven functions in signalling, including both perception and signal processing, making them promising candidates in the realms of belowground plant signalling. Whether these proteins have specific roles in plant-plant signalling remains to be verified.

6.5.2 Root exudates contain sets of proteins that are specific to high and low nutrients conditions

The analysis of protein sets specific to particular conditions provided further insight into the general patterns of differences in exudate composition under varying nutrient conditions. In general, the high nutrients condition promoted a higher diversity of detected proteins (1,109 specific proteins detected), whereas low nutrients reduced the overall number of detected proteins (246 specific proteins detected). In high nutrients only ~12% (137 proteins) whereas in low nutrients ~48% (117 proteins) were

intended for secretion. The findings suggest that nutrient availability significantly influences the composition and function of root exudates in *A. thaliana*. The high nutrient conditions lead to a broader diversity of proteins, but a smaller proportion is specifically secreted. In contrast, low nutrient conditions not only reduce the overall number of proteins but promote a much higher percentage of proteins intended for secretion. This indicates that in nutrient limited environments, plants may prioritise the targeted release of proteins and minimise the loss of essential molecules through unintended leakage. This is to be expected since so far, numerous other root exuded compounds such as sugars, amino acids, organic acids and other primary metabolites have been repeatedly shown to be heavily dependent on nutrient conditions (Canarini et al., 2019; Carvalhais et al., 2011).

The main differences between the samples are characterised by the unique biological processes and pathways enriched in each condition. The high nutrient conditions favoured metabolic and biosynthetic processes, stress responses, and cellular regulation, with a notable emphasis on glutathione biosynthesis, amino acid metabolism, and oxidative stress response. Low nutrient conditions highlighted stress response mechanisms, pH regulation, cell wall modification, and energy metabolism, with significant enrichment in processes related to cell wall dynamics and hormonal regulation. The high nutrients condition exhibited a broader range of stress response processes (i.e., response to salt stress, cold, cadmium ion, fungus) compared to the low nutrients condition which showed a more generalised response to stress. The ample nutrient environments might allow plants to maintain redundant and overlapping stress response pathways, making their overall stress response more robust and versatile (Khan et al., 2023). The high nutrients condition showed a more diverse set of metabolic processes, including various amino acid biosynthesis and catabolic processes, indicating a higher metabolic activity compared to the low nutrients condition. The high nutrients conditions show enrichment in processes related to cell cycle regulation, protein phosphorylation, and transcription regulation, highlighting active cell division and growth processes. Interestingly, the

mitochondrial transport processes were significantly enriched in low nutrients conditions, reflecting possible shifts in energy management under nutrient-limited scenarios. Indeed, the connection between mitochondrial activity as a mean to enhance nutrients uptake have been investigated (Foyer et al., 2011; Gupta et al., 2005; Haozi-Taskovh et al., 1998; Rychter et al., 1992).

Interestingly, the only proteins directly related to nutrients detected in this study were purple acid phosphatases (PAPs) (Bhadouria & Giri, 2022). The common exudate protein set included 4 phosphatases, while the high nutrient specific set contained an additional 8 phosphatases, and the low nutrient specific set had only 2 additional phosphatases. This finding, though initially surprising given that phosphatases are typically associated with low phosphorus conditions, is reasonable when considering that PAPs are part of a highly diverse family, with members exhibiting differential regulation and a wide range of functional roles (Li et al., 2002).

6.5.3 Small peptides are not directly detectable in root exudates

No annotated small peptides were directly detected in the exudate samples, and the standard small peptide CEP5 was confidently identified only in the 0.1 µM sample. The efficient sample preparation for proteomic analysis of plants represents a challenge. The obtainment of good quality samples and mass-spectrometry protocols for plant proteins and peptides is not a trivial task as require to deal with low concentration of compounds, high level of degradation and interfering secondary metabolites (Hussein & El-Anssary, 2018). So far, CEP1 when overexpressed was found in whole-plant liquid culture of A. thaliana (Ohyama et al., 2008b) and in root exudates of hairy roots of M. truncatula (Mohd-Radzman et al., 2015). What is more, in general the characterization of peptide families has been hindered by several factors: the small size of peptide coding genes, which often prevents their detection by annotation algorithms and causes the scarcity of mutants, functional redundancy within these large multigene families; and the lack of similarity in precursor sequences, making it challenging to knock down multiple genes with a single

construct (Hsu & Benfey, 2018; Tabata & Sawa, 2014; Wang et al., 2020). While 10 µM concentration of proteins is generally not too high for mass spectrometry, it may require careful handling to avoid issues such as ion suppression, detector saturation, and carryover. Often, diluting the sample to a lower concentration (e.g., 0.1 to 1 µM) is even recommended to achieve optimal results (Han et al., 2008; Mikulášek et al., 2021). However, in this initial analysis a strong signal and robust detection were necessary, therefore appropriate adjustments to instrument settings and sample preparation techniques may be necessary in future experiments to effectively manage the discrepancy between native low abundance and detection of higher concentrations of standards. Alternatively, the use of novel techniques to obtain valid overexpressors and knock-out lines for small peptides or single-cell proteomics should advance their detection.

6.5.4 Concluding remarks

The study of root exudate content is challenging, primarily due to the difficulties in sample collection and the lack of comprehensive analytical methods capable of detecting multiple groups of compounds simultaneously (Oburger & Jones, 2018). What is more, studying the proteome present in root exudates adds another layer of difficulty since plant proteins are highly sensitive to degradation and the analysis of plant proteins is still developing, require extremely high quality of samples as it is often characterised by low reproducibility (Han et al., 2008; Yan et al., 2022). The results presented in this chapter suggest that a high diversity of proteins is present in *A. thaliana* exudates. Moreover, quantitative differences are evident between proteins commonly present and those specific to high and low nutrient conditions. While the main groups of biological processes associated with these proteins are largely similar across the three datasets, nutrient conditions lead to the presence of additional proteins within these groups, as well as the expression of different proteins under varying conditions.

Chapter 7
Assessment of the role of root receptors in neighbour perception and response

Abstract

Leucine-rich repeat receptor-like kinases (LRR-RLKs) play key roles in plant signalling, but their involvement in plant-plant interactions remains largely unexplored. This study investigates the role of root-expressed LRR-RLKs in *Arabidopsis thaliana*, focusing on their potential function in belowground signalling. A screening of 15 LRR-RLK mutants revealed that most receptors did not influence plant-plant interactions, except for an uncharacterised LRR-RLK (At1g68400) and CEPR1 (C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1). CEPR1 was found to mediate asymmetric competition, with wild-type plants competing more strongly against each other than against *cepr1* mutants. CEPR1 also influenced nutrient transporter expression and root architecture, particularly lateral root development. While internal CEPR1 signalling contributes to neighbour response, exogenous CEP1 application did not induce root morphological changes, suggesting that CEP1 is not directly involved in rhizosphere neighbour detection. The *cepr1* mutant exhibited reduced competitive ability and failed to execute typical neighbour responses. These findings suggest that root receptors like CEPR1 contribute to belowground plant interactions by modulating nutrient signalling and competitive responses. This study provides insights into how receptor-mediated signalling influences plant interactions and highlights the complexity of root perception mechanisms in competitive environments.

7.1 Introduction

In order to detect molecules released by other plants in the rhizosphere, root receptors are required to respond to the signals of neighbouring plants presence. The receptor-like kinase (RLK) family is promising for this function, as its members play significant roles in plant signalling pathways, particularly in recognizing external stimuli such as pathogens, symbiotic organisms, and environmental stress (Liu et al., 2017). The RLK family consists of several hundred members and is the largest receptor family in *A. thaliana* whereas the leucine-rich repeat receptor-like kinases (LRR-RLKs) subfamily is the largest subfamily within it (Morris & Walker, 2003). The *Arabidopsis* genome contains over 200 LRR-RLKs categorised into 15 distinct subfamilies based on their structural features and phylogenetic relationships, underscoring their significance and diversity within the plant's signalling networks. Around 40 of these are expressed in roots epidermis (ten Hove et al., 2011).

Despite extensive studies on LRR-RLKs activity and responsiveness to hormonal treatments or environmental factors, the characterization of ligands is troublesome. There is limited knowledge about the specific ligands that interact with many LRR-RLKs. Identifying and characterizing these ligands remains a significant challenge. Only a subset of ~ 50-70 LRR-RLKs have well-characterised functions, the roles of the majority of LRR-RLKs remain poorly understood (Ou et al., 2016b; Shinohara et al., 2019; W. Song et al., 2016; Tabata Ryo et al., 2014). Studying LRR-RLKs is particularly compelling when investigating root epidermis-localised receptors due to their integral role in the detection and transduction of plethora of extracellular signals such as bacterial proteins and fungal cell wall components, facilitating interactions between plants and their environment (Morris & Walker, 2003).

LRR-RLKs are composed of three spatially and structurally different domains. The extracellular domain (ECD) contains 20-25 leucine-rich repeats (LRRs) (consensus sequence LxxLxxLxLxxNxLSGxIPxxLGx), that facilitate ligand binding and protein-protein interactions (P. Liu et al., 2013; Man et al., 2020; Mubassir, 2019). The

number of LRRs can vary significantly as well as the content of the GxIP sequence and contribute to the specificity and diversity of ligand recognition (Afzal et al., 2008; Kobe Bostjan & Kajava Andrey, 2001; P. Liu et al., 2013; P. L. Liu et al., 2017). The transmembrane domain (TMD) is a single α-helix that anchors the protein in the plasma membrane, ensuring proper positioning for signal reception and transduction. The intracellular kinase domain (KD) is responsible for signal transduction through phosphorylation of specific target proteins. This domain initiates downstream signalling cascades upon activation by ligand binding to the ECD.

The mode of action of many LRR-RLKs involves the conformational changes caused by ligand binding, which enables recruitment of a co-receptor and only this complex is fully functional in signal perception and amplification (Mubassir, 2019). Alternatively, homo- or heterodimerisation with either complete receptors or forms that lacks receptor or kinase domains may occur (De Coninck & De Smet, 2016; Johnson & Ingram, 2005; Nimchuk et al., 2011; Shinohara & Matsubayashi, 2015). The processes and conditions under which LRR-RLKs form functional receptor complexes, and what are the co-receptors, are not well understood. Such complexity of these receptors extends even further their potential to distinguish minor differences between signals. However, the mechanisms by which LRR-RLKs distinguish between subtle differences in extracellular signals are not fully elucidated. Even in the case of well described receptors such as CEPRs. there is a lack of comprehensive knowledge about the downstream signalling pathways activated due to receptor and ligand redundancy.

7.1.1 CEP-CEPR1 signalling pathway

CEP-CEPR1 signalling is a crucial pathway in plants that link nutrient acquisition and root development. CEPs (C-TERMINALLY ENCODED PEPTIDEs) are small peptide signals primarily involved in response to nitrogen deficiency and lateral roots development (Taleski et al., 2018). The genome of *A. thaliana* encodes 15 CEP genes which are not characterised in detail but appear to play redundant roles in primary

and lateral roots growth inhibition (Delay et al., 2013; Ogilvie et al., 2014; Roberts et al., 2013). When nitrogen levels are low, CEP1 peptides are produced in the roots and transported to the shoot, where they interact with the CEPR1 (CEP RECEPTOR 1), a leucine-rich repeat receptor-like kinase. This drives the expression of CEPDs, a CC-type glutaredoxin polypeptides (ROXYs) which travel from the shoot to the root as systemic N-demand signals (Ohkubo et al., 2017; Ota et al., 2020; Tabata et al., 2014; Taleski et al., 2023). In parallel to this, CEPR1 perception of CEP3 and CEP5 peptides can also trigger downstream signalling pathways that adjust root architecture (Delay et al., 2019; Roberts et al., 2016). This signalling pathway plays a vital role in modulating root growth, promoting lateral root development, and maintaining nutrient homeostasis under nutrient stress. CEPR1 also regulates the expression of nitrate transporters like NRT2.1, ensuring that plants efficiently adapt to fluctuating environmental nutrient conditions. The CEP-CEPR1 signalling mechanism exemplifies how plants use peptide-receptor interactions to coordinate growth and nutrient responses in the rhizosphere.

7.1.2 Summary

The leucine-rich repeat receptor-like kinase (LRR-RLK) family is the largest receptor family in *Arabidopsis thaliana*, playing key roles in growth, development, and responses to abiotic and biotic stress. However, many LRR-RLKs remain uncharacterised, and even well-studied members continue to reveal new functions, highlighting their multifunctionality. Despite their significance, LRR-RLKs have not yet been recognised as potential receptors involved in plant-plant interactions. The CEP-CEPR1 signalling pathway, for instance, regulates nutrient acquisition and root development. Yet, like many LRR-RLK-mediated pathways, CEP-CEPR1 remains only partially understood, with considerable gaps in our understanding of its relevance to other processes.

7.2 Chapter aims

Considering the knowledge gaps, this chapter aims to explore a deeper understanding of the root exudate-driven signalling in *A. thaliana*. Focusing on novel receptor candidates – LRR-RLKs prompts several questions about the mechanisms of plant-plant interactions, which are addressed in this chapter:

1. Do LRR-RLKs have roles in plant-plant interactions?
2. Do LRR-RLKs ligands have roles in plant-plant interactions? What are their functions?

This chapter aimed to evaluate the possible roles of LRR-RLKs in plant-plant interactions and assess their impact on belowground signalling.

7.3 Methods

7.3.1. Germplasm

In order to test the possible LRR-RLK capacity to participate in plant-plant interactions signalling belowground, candidate LRR-RLK genes were chosen based on literature and database searches. The wild type *A. thaliana plants* used in this study were of Col-0 ecotype. All *A. thaliana* mutants used in this study were impaired in receptor function and were in Col-0 background. The candidate LRR-RLK mutants seeds were obtained as a courtesy from Prof. Renze Heidstra (Wageningen University & Research) and were T-DNA insertion mutants, *cepr1-3* mutant seeds were obtained as a courtesy from Prof. Martin Stegmann (Technical University Munich) (Table 7.1).

Table 7.1. The details of *A. thaliana* LRR-RLK mutants used in this study.

Locus	Gene	Description	Line ID	Source
AT1G09970	*RLK7*	LRR XI-23; RECEPTOR-LIKE KINASE 7	SALK_120595c	Prof. Heidstra
AT1G68400		Leucine-rich repeat protein kinase family protein	N872562/ SAIL_256_E01	Prof. Heidstra
AT2G31880	*SOBIR1*	SUPPRESSOR OF BIR1 1; EVERSHED; EVR	SALK_009453C	Prof. Heidstra
AT3G02880	*KIN7*	KINASE 7; QSK1; QIAN SHOU KINASE1	N519840	Prof. Heidstra
AT3G56370	*IRK*	INFLORESCENCE AND ROOT APICES RECEPTOR KINASE	N538787	Prof. Heidstra
AT4G23740		Leucine-rich repeat protein kinase family protein	N505132	Prof. Heidstra
AT4G28650	*MIK1*	MDIS1-INTERACTING RECEPTOR LIKE KINASE1; PXL2; PXY-LIKE2	N536232/N800045	Prof. Heidstra
AT4G33430	*BAK1*	BRI1-ASSOCIATED RECEPTOR KINASE; SERK3; SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3	N616202	Prof. Heidstra
AT4G36180	*MUL*	MUSTACHES-LIKE	AT4G36180	Prof. Heidstra
AT5G01890	*PXC2*	PXY/TDR-CORRELATED 2	N518730/ N800005	Prof. Heidstra
AT5G16590	*LRR1*	LRR1; LEUCINE RICH REPEAT PROTEIN 1; QSK2; QIAN SHOU KINASE 2	N553366	Prof. Heidstra
AT5G43020		Leucine-rich repeat protein kinase family protein	N535437	Prof. Heidstra
AT5G49660	*CEPR1*	C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1; XIP1; XYLEM INTERMIXED WITH PHLOEM 1	*cepr1-3*; GK-467C01	Prof. Stegmann
AT5G56040	*RGI4*	RGF1 INSENSITIVE 4;RGI4; SKM2;STERILITY-REGULATING KINASE MEMBER 2	N537932/ N800047	Prof. Heidstra
AT5G63930		Leucine-rich repeat protein kinase family protein	N874087/ SAIL_429_B07	Prof. Heidstra
AT5G65700	*BAM1*	BARELY ANY MERISTEM 1	*bam1-1*	Prof. Heidstra

7.2.2 Experimental design

7.2.2.1 Assessment of LRR-RLKs effect on interaction

LRR-RLKs represent a significant family of receptor kinases involved in detecting external signals and triggering cellular responses (Man et al., 2020; Mubassir, 2019). In order to test whether LRR-RLK receptors are involved in plant-plant interactions experiments were performed where *A. thaliana* Col-0 wild type plants were paired either with another wild type (intragenotypic interaction) or LRR-RLK mutant with non-functioning receptor (intergenotypic interaction). Plants were grown either singly or in pairs of all possible wild type and mutant combinations as in the Neighbour swap method described in Chapter 2.3 Experimental design. Both above- and belowground growth was assessed according to the procedures outlined in Chapter 2.3 Plants and growth conditions. In this experiment the total number of produced siliques was measured as an indicator of the reproductive output. The total number of pots in this experiment was 121; that comprised 5 replicates paired with *bak1*; 7 replicates paired with *At5g43020*, 9 replicates paired with *llr1*; 8 replicates paired with *At4g23740*; 8 replicates paired with *kin7*; 10 replicates paired with *At1g68400*; 10 replicates paired with *pxc2*; 9 replicates paired with *irk*; 10 replicates paired with *sobir1*; 10 replicates paired with *mik1*; 9 replicates paired with *rgi4*; 9 replicates paired with *At5g63930*; 10 replicates paired with *bam1* and 3 replicates paired with *mul*.

In experiments focused in detail on *cepr1* the total number of pots in this experiment was 161; in high nutrients (100% soil) that comprised 12 replicates of wild type grown single; 24 replicates of wild type grown in intragenotypic pair; 11 replicates of wild type grown *cepr1*; 10 replicates of *cepr1* grown single; 14 replicates of *cepr1* grown in intragenotypic pair and 9 replicates of *cepr1* paired with wild type. In low nutrients (10% soil + 90% sand) that comprised 12 replicates of wild type grown single; 24 replicates of wild type grown in intragenotypic pair; 12 replicates of wild type grown with *cepr1*; 17 replicates of *cepr1* grown single; 17 replicates of *cepr1* grown in intragenotypic pair and 12 replicates of *cepr1* paired with wild type. The total number of plates in this experiment was 144; in high nutrients (100% ATS) that comprised 19

replicates of wild type grown single; 12 replicates of wild type grown in intragenotypic pair; 10 replicates of wild type grown *cepr1*; 16 replicates of *cepr1* grown single; 11 replicates of *cepr1* grown in intragenotypic pair and 10 replicates of *cepr1* paired with wild type. In low nutrients (10% ATS) that comprised 18 replicates of wild type grown single; 8 replicates of wild type grown in intragenotypic pair; 7 replicates of wild type grown with *cepr1*; 19 replicates of *cepr1* grown single; 7 replicates of *cepr1* grown in intragenotypic pair and 7 replicates of *cepr1* paired with wild type.

7.2.2.2 Assessment of CEP1 effect on belowground growth

CEPR1 (C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1) is a Leucine-Rich Repeat Receptor-Like Kinase (LRR-RLK) that plays a crucial role in systemic nitrogen signalling, regulating root growth and nutrient uptake in response to environmental cues. CEPR1 functions as a receptor for C-terminally Encoded Peptides (CEPs), which serve as long-distance signalling molecules. Among the 15 known CEP genes in *A. thaliana*, CEP1 is the best-characterised ligand for CEPR1 (Chapman et al., 2019; Ohkubo et al., 2017; Tabata Ryo et al., 2014; Taleski, 2020). The potential role of CEPR1 in plant-plant interactions was described here, in subsections 7.3.1 LRR-RLK screen and 7.3.2 CEPR1. To investigate whether the CEP1 signalling peptide is involved in plant-plant interactions through CEPR1, experiments were conducted using *A. thaliana* Col-0 wild type plants and *cepr1* mutant, which has impaired receptor function and is unable to perceive CEP ligands. Both were exposed to externally applied CEP1 to assess its effects. Next, the belowground growth was assessed according to the procedures outlined in Chapter 2.3 Plants and growth conditions. The CEP1 synthetic peptide of sequence DFR{HYP}TNPGNS{HYP}GVGH (Q8L8Y3, At1g47485) was synthesised by GenScript USA Inc. with at least 90% purity. CEP1 was supplied to media in 1µM concentration. The total number of plates in this experiment was 60 and each treatment consisted of 15 replicates.

7.2.3 Nutrient transporter and CEP gene expression in response to neighbouring plants

Nutrient transporters

Changes in macronutrient transporters gene expression such as nitrate and phosphate transporters typically signify plant's attempt to optimise nutrient uptake and maintain growth in response to internal needs or external factors, such as nutrient scarcity (Linn et al., 2017; Misson et al., 2004; Okamoto et al., 2006; Orsel et al., 2004; Poirier & Bucher, 2002; Shin et al., 2004; Vidal et al., 2020). Here it was tested whether competition with a neighbour evokes the high-affinity nitrate (NRT1.1, NRT2.1 and NRT3.1) and phosphate (PHT1;1 and PHT1;4) transporters response. Primers are outlined in Table 7.2.

Table 7.2. The list of nutrient transporter genes which expression was assessed in this study and respective primers' sequences. *ACT2*, housekeeping reference. gene**.**

Gene		Locus		Sequence	Reference
ACTIN 2	*ACT2*	At3g18780	F R	AGTGTCTGGATCGGTGGTTC CCCCAGCTTTTTAAGCCTTT	Chamba et al., 2021
NITRATE TRANSPORTER 1.1	*NRT1.1*	At1g12110	F R	GTCGAGCTCAAACGTCTTAGAA CTTCGCCGATACCGACAATAA	
NITRATE TRANSPORTER 2.1	*NRT2.1*	At1g08090	F R	CTTGCGGAGCCACCTTTG TTAAACCCGAGATGATGCCTAGA	Santos et al., 2012
NITRATE TRANSPORTER 3.1	*NRT3.1*	At5g50200	F R	GTCTTGTTGCACACTTCTTCTTT GCATCACCACCGTAGGATTAG	
PHOSPHATE TRANSPORTER 1;1	*PHT1;1*	At5g43350	F R	CCTTTGGGTTCCTATATGCG TAACCTCAGCCTCACCAGAG	Chen et al., 2021
PHOSPHATE TRANSPORTER 1;4	*PHT1;4*	At2g38940	F R	TCAATGGCGTTGCCTTCTG ATCACCAAGCCACCCGAAA	Wang et al., 2020

C-terminally Encoded Peptides

Changes in the expression of C-terminally Encoded Peptide (CEP) genes in plants typically signify the plant's systemic response to nutrient availability, particularly nitrogen, and its adaptive strategies to regulate root architecture and nutrient acquisition. Here it was tested whether competition with a neighbour evokes the CEPs expression response. The CEP primers used were previously published (Rzemieniewski et al., 2022) or designed using PrimerQuest™ Tool (Integrated DNA Technologies IDT) as indicated in the Table 7.3.

Table 7.3. The list of CEP genes which expression was assessed in this study and their respective primers' sequences.

Gene	Locus		Sequence	Reference
CEP1	At1g47485	F	AATGCTAAAGGGGTGTTTGG	Rzemieniewski et al., 2022
		R	ACAAACCCACGACAAAGAC	
CEP2	At1g59835	F	TGGTGACCATTTTGACCATC	Rzemieniewski et al., 2022
		R	CCGACCATCTTTTTCGACTT	
CEP3	At2g23440	F	GACCTACGGAACCTGGTCAT	Rzemieniewski et al., 2022
		R	AAAAAGTCACCAGGCCAATC	
CEP4	At2g35612	F	AGAATACAAAAGCAGCTCGAC	Rzemieniewski et al., 2022
		R	TCCAATACCTTGACTAGGACC	
CEP6	At5g66816	F	GTCGGGTAATCTAGACCTTCCTC	Rzemieniewski et al., 2022
		R	CGGTTTTTCGCAACTGTCTCG	
CEP8	At5g09745	F	AGACACTTGAGGACCCATAGA	Own design
		R	TGCTAATGTCAGTCTTCACAGAG	

7.4 Results

7.4.1. The screen of root-expressed LRR-RLKs candidates

Leucine-Rich Repeat Receptor-Like Kinases (LRR-RLKs) form a large family of receptor kinases that are essential for sensing extracellular signals and initiating responses within the cell. Their primary function is signal detection and transduction (Man et al., 2020; Mubassir, 2019). This chapter examines their potential role in mediating belowground signalling in plant-plant interactions. In order to select candidate LRR-RLK receptors that are relatively highly expressed in *A. thaliana* roots and root epidermis and therefore may potentially be involved in plant-plant belowground interactions were identified through literature and database search (Arabidopsis Thaliana Expression Atlas ATHENA; Brady et al., 2007; ten Hove et al., 2011). This resulted in the selection of 15 LRR-RLK candidates (Figure 7.1AB; Table 7.1). The selected LRR-RLKs represented distinct expression patterns across various organs and root regions. Several candidate LRR-RLKs, including BAK1, LLR1, KIN7, BAM1 and RLK7 exhibited relatively high protein expression levels across multiple organs, with particularly strong expression in the roots, specifically in the root tip. The rest of the candidates showed broader expression across organs and moderate albeit notable expression within roots. LRR-RLKs are further categorised into subfamilies based on their receptor and kinase domains sequences (Man et al., 2020; Mubassir, 2019). Out of 15 LRR-RLK candidates, the majority (7 genes: SOBIR1, MIK1, RGI4, At5g63930, BAM1, MUL and RLK7) belonged to subfamily XI. The next highly represented subfamily was subfamily III (5 genes: At5g43020, LLR1, At4g23740, KIN7 and At1g68400). Both subfamily XI and III are well characterised for their involvement in small peptide signalling (Furumizu et al., 2021).

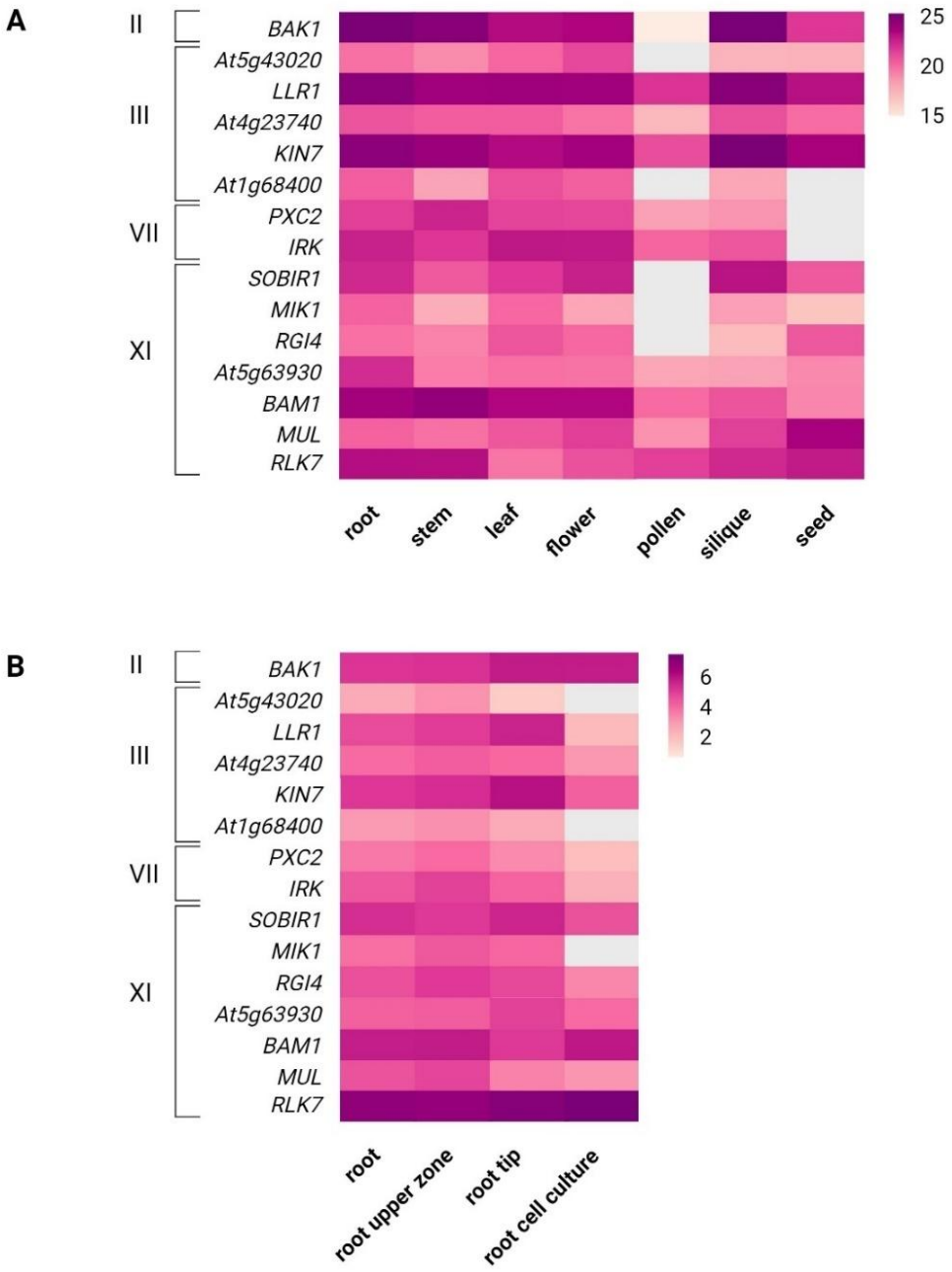


Figure 7.1. The transcript and protein profiles of candidate root-expressed LRR-RLKs in different *A. thaliana* organs.

(A) The candidate LRR-RLKs protein levels in different *A. thaliana* organs. (B) The candidate LRR-RLKs transcript levels in different parts of *A. thaliana* root. Shown values were retrieved from Arabidopsis Thaliana Expression Atlas (ATHENA).

Further, the identified 15 root-expressed LRR-RLK candidates were tested for their involvement in plant-plant interactions. Briefly, mutants for these 15 LRR-RLKs, each with specific gene impairment that rendered the specific receptor non-functional, were obtained (Table 7.1). Next, wild type plants and mutants were grown singly and in pairs and the outcomes of the interactions were compared in order to verify whether the lack of functional receptor has an impact on the wild type plant in the pairing. This would imply that a given receptor plays a role in the interaction.

Most of the LRR-RLK mutants tested as neighbours did not produce interaction outcomes different from those observed with wild type plants as neighbours, suggesting they are likely not relevant to plant-plant interactions (Figure 7.2AB). The *At1g68400* mutant as a neighbour caused moderate albeit significant increasement in the paired wild type's total silique output ($p = 0.0190$) (Figure 7.2A). This locus encodes an uncharacterised LRR-RLK of yet unknown function (The Arabidopsis Information Resource TAIR).

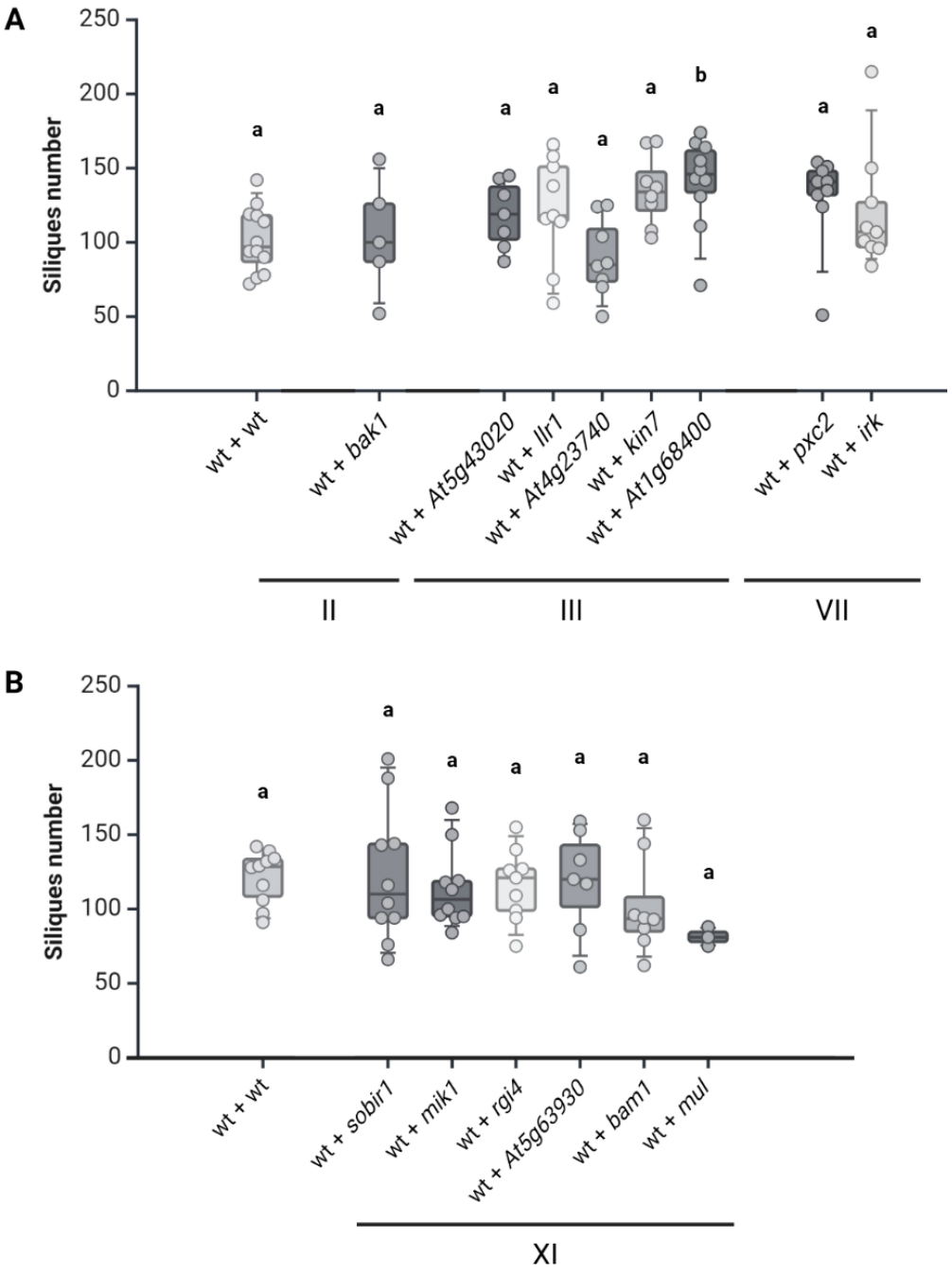


Figure 7.2. Most root-expressed LRR-RLK candidates do not function in interactions.

The wild type *A. thaliana* response to being paired with another wild type or mutant impaired in one of the 15 LRR-RLK candidates. (A) LRR-RLK mutants from subfamilies II, III and VII as neighbours. Kruskal-Wallis with Dunn's multiple comparisons test, $p < 0.01$, n = 5-10. (B) LRR-RLK mutants from subfamily XI as neighbours. One-way ANOVA with Dunnett's multiple comparisons test. $p > 0.05$, n = 3-10.

7.4.2 Assessment of CEPR1 role in interaction

In the preliminary screen presented in the previous section, a member of LRR-RLKs subfamily XI, CEPR1 (C-terminally Encoded Peptide Receptor 1) was also identified to contribute to the plant-plant interaction outcome (Figure 7.3). CEPR1 in *A. thaliana* is known to play distinct roles in nitrogen signalling and root system architecture regulation. In nitrogen signalling, CEPR1 mediates the plant's response to nitrogen availability. It functions by perceiving C-terminally Encoded Peptides (CEPs), which act as long-distance signals from nitrogen-starved roots. This enables the plant to adjust nutrient uptake and allocation, coordinating growth based on nitrogen status (Ohkubo et al., 2017; Ohyama et al., 2008). Regarding root system architecture, CEPR1 influences root growth patterns by regulating lateral root development. In response to nitrogen levels and environmental cues, CEPR1 modulates root proliferation, allowing the plant to optimise its root structure for efficient nutrient acquisition (Chapman et al., 2019, 2020; Delay et al., 2019; Taleski et al., 2023).

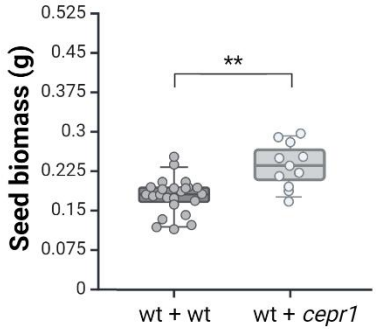


Figure 7.3. CEPR1 contributes to the interaction outcome.

The wild type *A. thaliana* response to being paired with another wild type or mutant impaired in CEPR1 gene. Student's t-test, $p > 0.05$, n = 11-22.

7.4.2.1 High nutrients

First, the interactions between pairs of wild types and pairs comprising a wild type and a *cepr1* mutant in high nutrients conditions were compared to determine if this

gene is important in plant-plant interactions (Figure 7.4). Observing an effect would indicate that this receptor is involved in the interaction process.

The mean percentage change in seed biomass between single grown and paired wt plants indicated a competitive interaction with -36.0% loss whereas competition was weaker with *cepr1* as a neighbour reaching only -15.6% loss (Figure 7.4A). This was further corroborated with RII lower in interaction with wt than with *cepr1* (RII = -0.23 and RII = -0.09 respectively (Figure 7.4B). Indeed, wt paired with *cepr1* produced seed biomass comparable to weight produced by single grown plant and higher than wt paired with another wt (Figure 7.4C). Similar pattern was observed in shoot biomass (Figure 7.4D). Belowground, no PR length changes were observed in neither pairing (Figure 7.4E). Wt plants significantly reduced both the relative LR length and density in response to the neighbouring wt plant but this response was not observed when wt was paired with *cepr1* mutant (Figure 7.4FG). In terms of nutrient transporters expression the nitrate transporters NRT2.1 and NRT3.1 were significantly downregulated whereas the phosphate transporter PHT1;1 was upregulated (albeit less than 2-fold) (Figure 7.5AB). The expression of CEP1 and CEP2 was not detectable (Figure 7.5D), The expression of other CEPs varied significantly however the fold change was low. The CEP3 was significantly upregulated in wt in response to wt neighbour, but not when paired with *cepr1* mutant. Only when paired with *cepr1* the CEP6 and CEP8 were significantly downregulated.

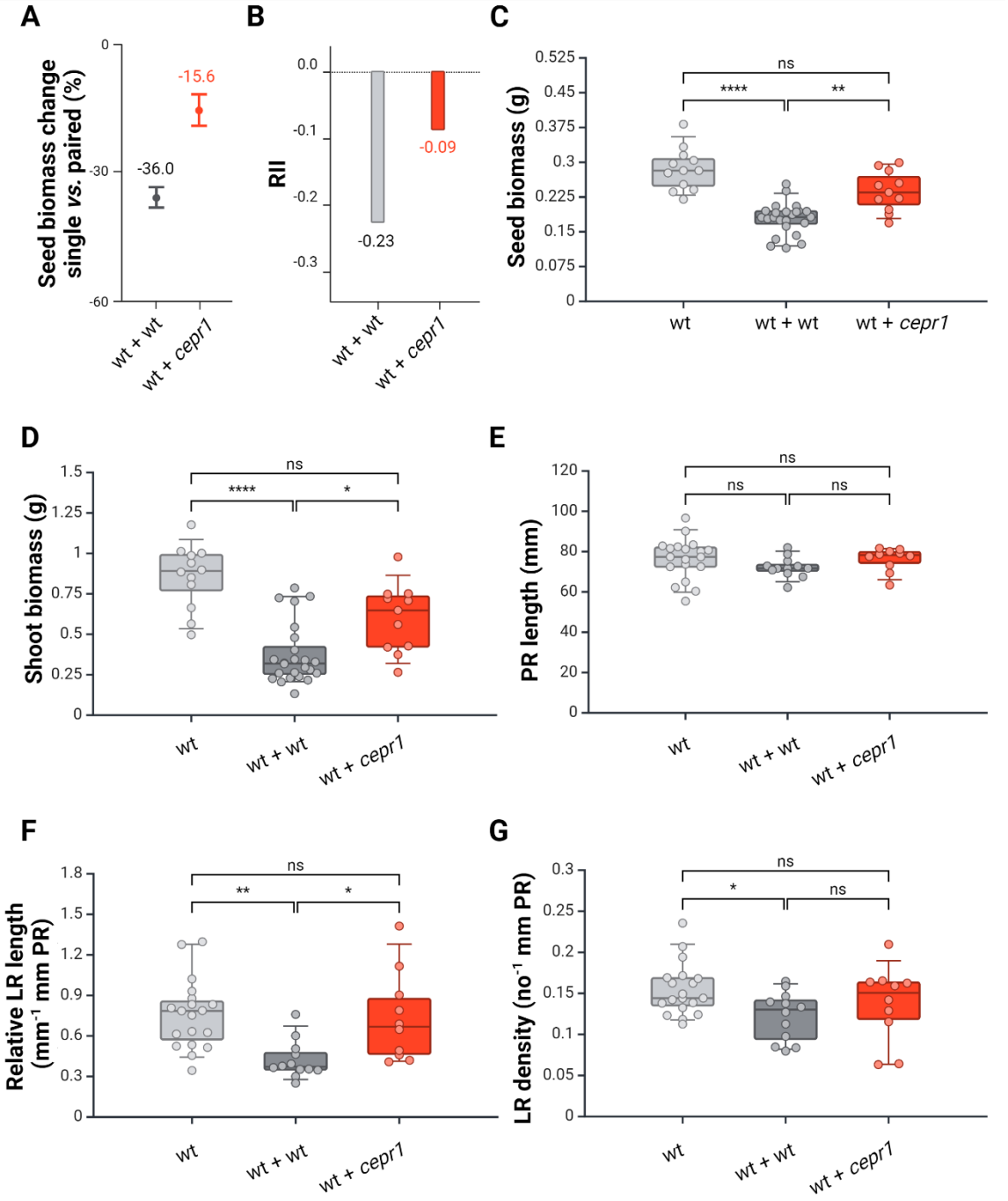


Figure 7.4. Wild type is a stronger competitor than *cepr1* in high nutrient conditions.

The wild type *A. thaliana* interaction with neighbours of different genetic backgrounds: with another wild type and with *cepr1* mutant in high nutrients media. (A) The mean percentage change in seed biomass between single grown and paired plant observed in different neighbour combinations. -100-0% competition, 0% neutral, 0-100% facilitation. (B) The Relative Interaction Index based on the seed

biomass between single grown and paired plant observed in different neighbour combinations. -1-0 competition, 0 neutral, 0-1 facilitation. (C) detailed seed biomass achieved by single grown and paired plant observed in different neighbour combinations. (D) detailed shoot biomass achieved by single grown and paired plant observed in different neighbour combinations. (E) detailed primary root length achieved by single grown and paired plant observed in different neighbour combinations. (F) detailed relative lateral roots length achieved by single grown and paired plant observed in different neighbour combinations. (G) detailed lateral roots density achieved by single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; n_{shoot} = 10-24, n_{seed} = 10-24, n_{root} = 10-19.

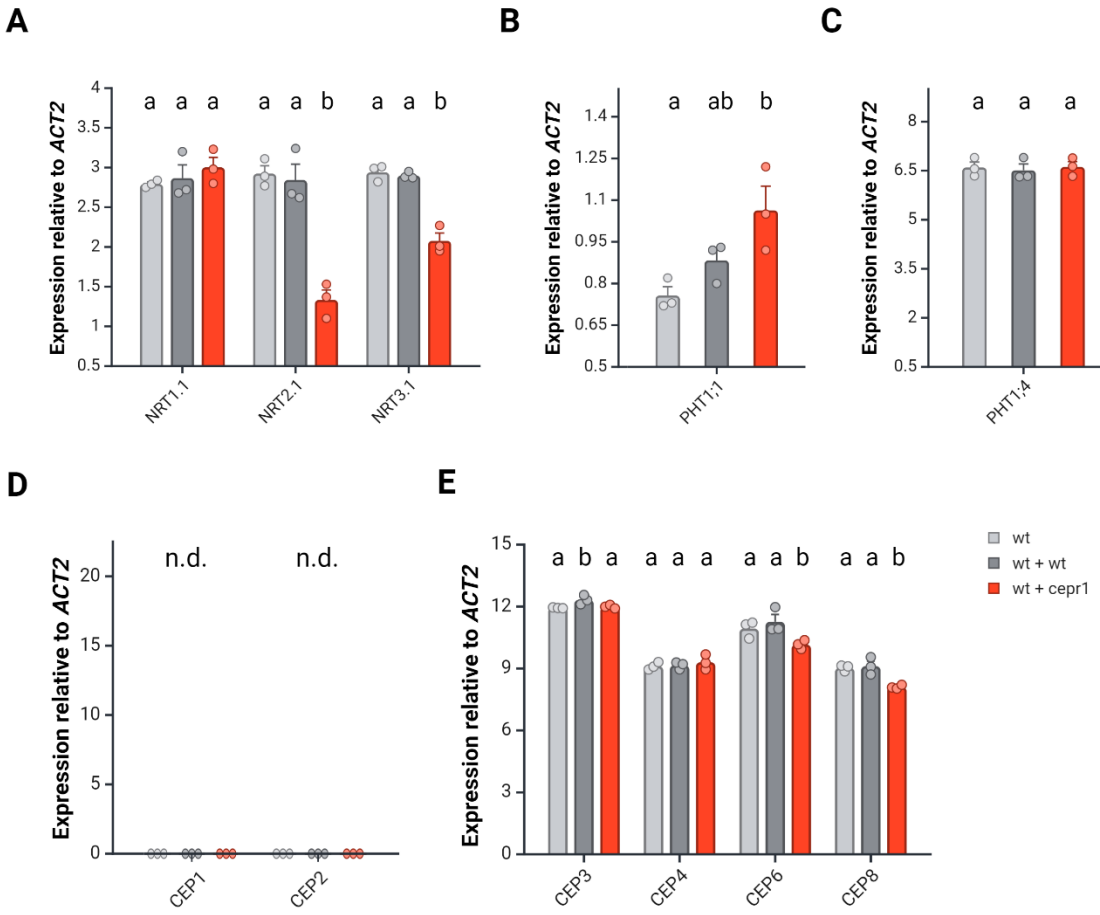


Figure 7.5. Wild type responds to *cepr1* as a neighbour by downregulation of nitrate transport *via* NRT2.1 and NRT3.1 in high nutrient conditions.

The wild type *A. thaliana* interaction with neighbours of different genetic backgrounds: with another wild type and with *cepr1* mutant in high nutrients media. Genes were grouped based on the magnitude of expression for visualization purposes only. (A) the expression of nitrate transporter genes in single grown and

paired plant observed in different neighbour combinations. (B) the expression of phosphate transporter PHT1;1 in single grown and paired plant observed in different neighbour combinations. (C) the expression of phosphate transporter PHT1;4 in single grown and paired plant observed in different neighbour combinations. (D) the expression of CEP genes in single grown and paired plant observed in different neighbour combinations. (E) the expression of CEP genes in single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; $n_{expression} = 3$.

Second, the interactions were assessed from the *cepr1* as a focal plant perspective and single *cepr1*, pairs of *cepr1* and pairs comprising a *cepr1* and wt in high nutrients conditions were compared (Figure 7.6). Observing an effect would further confirm that this receptor is involved in the interaction process.

The mean percentage change in seed biomass between wt pair and *cepr1* pair indicated that competition was stronger within cepr1 pair (-36.0% and -42.4% seed biomass loss respectively) whereas the interaction with wt was the most competitive for cepr1 reaching -66.5% loss (Figure 7.6A). This pattern was further corroborated with RII lower in *cepr1* interaction with wt than with *cepr1* (RII = -0.52 and RII = -0.28 respectively (Figure 7.6B). Indeed, *cepr1* paired with wt produced the lowest seed biomass of all conditions tested (Figure 7.6C). This pattern was not observed in shoot biomass, *cepr1* loss was high but similar in both pair with another *cepr1* and with wt (Figure 7.6D). Belowground, no morphological changes were observed in either pairing in any of traits measured (Figure 7.6EFG).

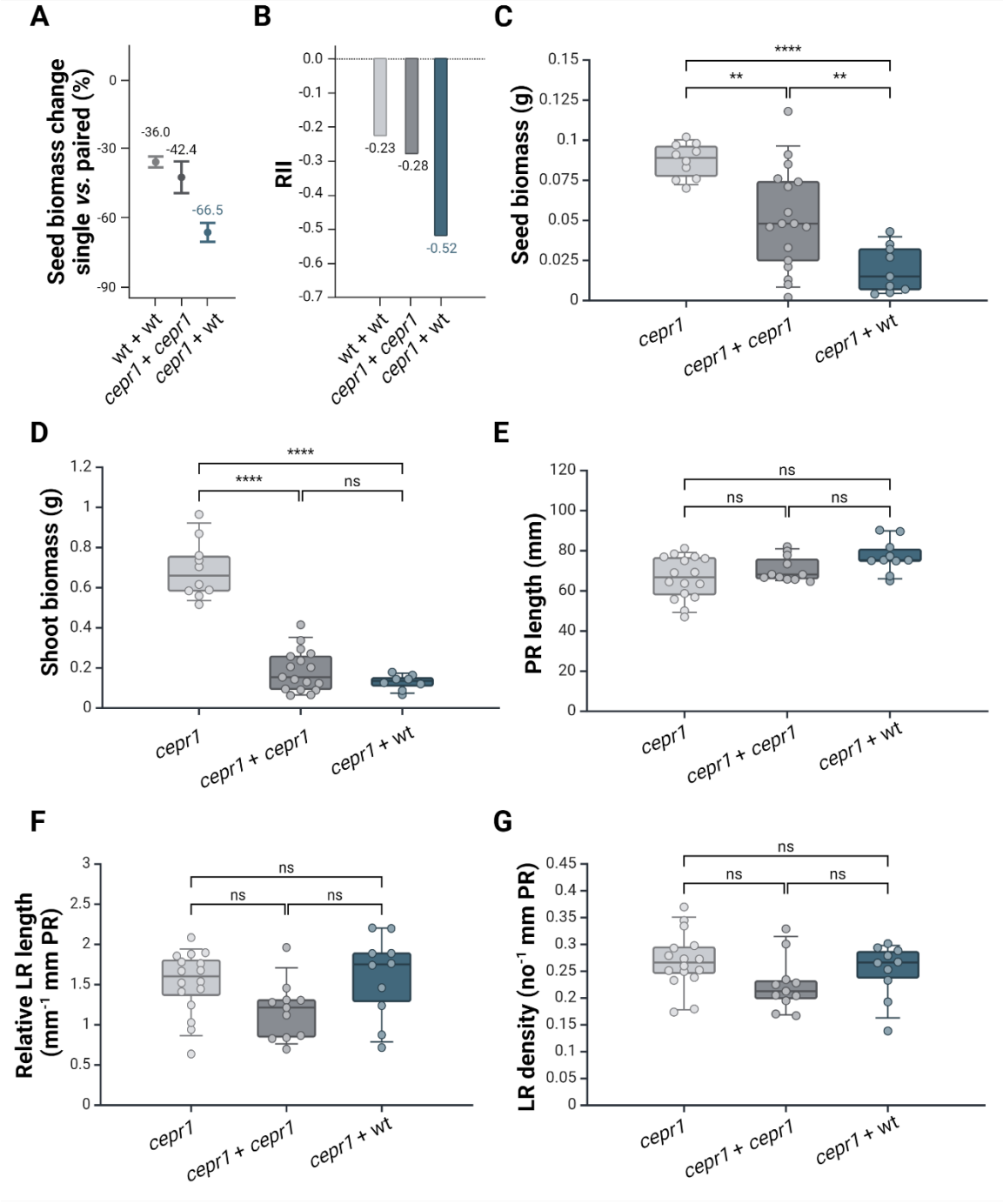


Figure 7.6. *cepr1* is a weaker competitor than wild type and fails to respond to neighbours belowground in high nutrient conditions.

The *cepr1* mutant interaction with neighbours of different genetic backgrounds: with another *cepr1* and with wild type in high nutrients media. (A) The mean percentage change in seed biomass between single grown and paired plant observed in different neighbour combinations. -100-0% competition, 0% neutral, 0-100% facilitation. (B)

The Relative Interaction Index based on the seed biomass between single grown and paired plant observed in different neighbour combinations. -1-0 competition, 0 neutral, 0-1 facilitation. (C) detailed seed biomass achieved by single grown and paired plant observed in different neighbour combinations. (D) detailed shoot biomass achieved by single grown and paired plant observed in different neighbour combinations. (E) detailed primary root length achieved by single grown and paired plant observed in different neighbour combinations. (F) detailed relative lateral roots length achieved by single grown and paired plant observed in different neighbour combinations. (G) detailed lateral roots density achieved by single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; n_{shoot} = 9-17, n_{seed} = 9-17, n_{root} = 10-16.

In summary in high nutrient conditions, the interaction between wild type and *cepr1* mutants showed less competition which was evidenced by higher wt seed biomass. Belowground the root morphology was similar to single grown plant. This suggested that either *cepr1* was not detected or it was detected and these are indicators of wt root overproliferation response, however the nitrate transport was downregulated. In contrast, the interaction between *cepr1* and wt showed higher competition, as evidenced by lowered *cepr1* fitness measured as seed biomass. Interestingly *cepr1* mutant did not show any root morphological neighbour response to another *cepr1* and especially to wt and belowground behaved as it would grow singly. Therefore the competition between wt and *cepr1* was asymmetrical.

7.4.2.2 Low nutrients

Here, the interactions between pairs of wild types and pairs comprising a wild type and a *cepr1* mutant in low nutrients conditions were compared to determine if this gene is important in plant-plant interactions (Figure 7.7). Observing an effect would indicate that this receptor is involved in the interaction process.

The mean seed biomass changes of wt paired with *cepr1* was positive (6.9%) indicating that wt benefits from an interaction with *cepr1* and produces more seeds than in interaction with another wt (Figure 7.7A). The comparison of RIIs between wt paired with another wt and wt paired with *cepr1* proved that the competition was not

only less intense with *cepr1* but in fact the interaction was neutral for wt (wt paired with wt RII = -0.17, wt paired with *cepr1* RII = 0.01) (Figure 7.7B). These results were further corroborated by detailed analysis of seed biomass that revealed that wt paired with *cepr1* achieved seed biomass similar to singly grown wt plants (Figure 7.7C). The shoot biomass followed a similar pattern (Figure 7.7D). Belowground, the primary root length did not differ between inter- and intragenotypic pairs (Figure 7.7E). The relative LR length and LR density were less reduced in wt paired with *cepr1* compared to wt paired with another wt but it was not significant and somewhat showed phenotypes in-between single and paired wt (Figure 7.7FG). The expression of nutrients transporters differed significantly but none of the fold changes indicated major changes. It is perhaps worth to highlight the upregulation of NRT3.1 in wt interaction with *cepr1* (albeit the fold change < 2) (Figure 7.8A). The expression of CEP1 was highly upregulated as a neighbour response between single grown wt and wt paired with another wt and this response was not observed when the neighbour was *cepr1* (Figure 7.8D). CEP3 and CEP8 were significantly upregulated in wt paired with *cepr1* but the fold change was rather low (Figure 7.8E).

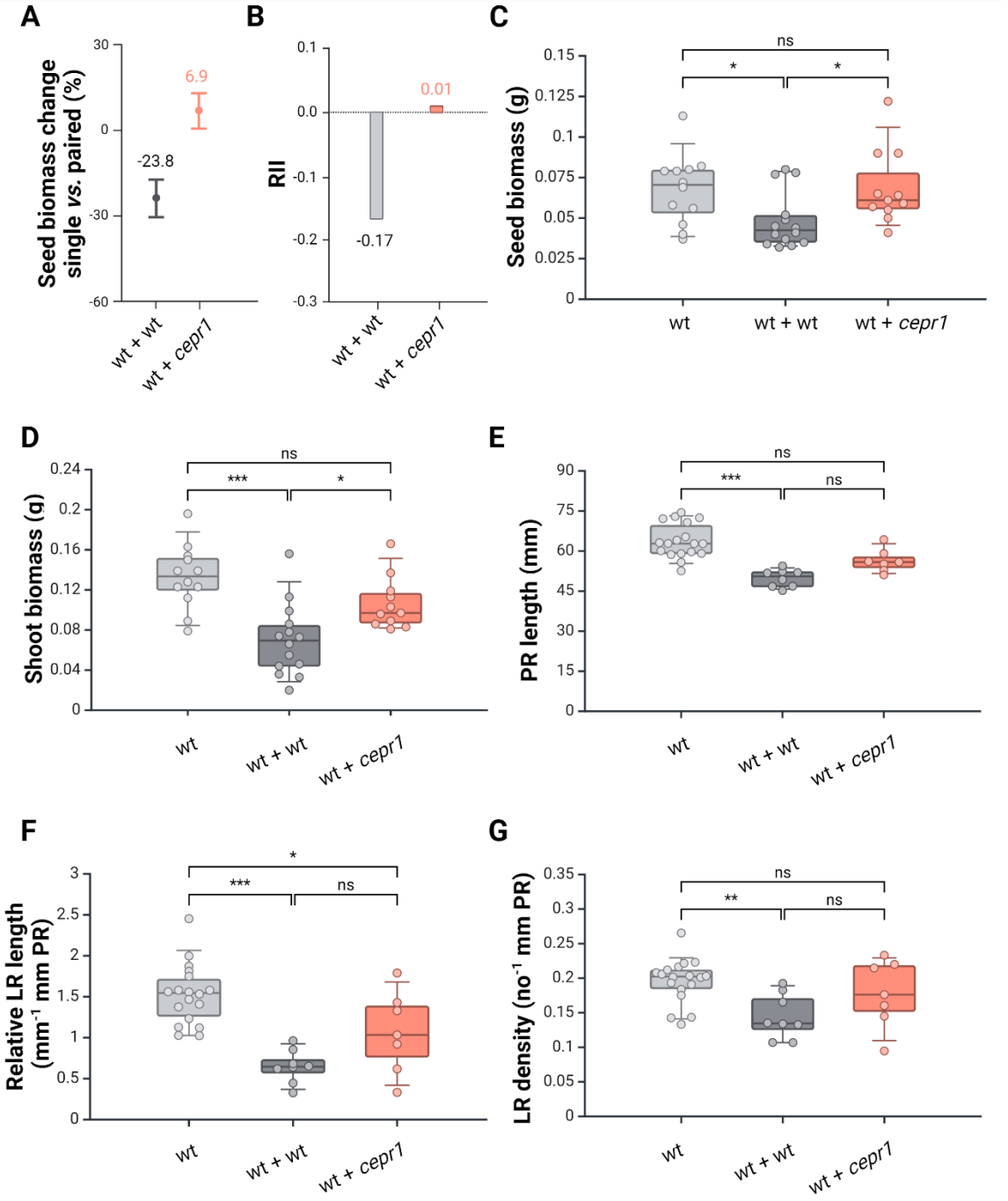


Figure 7.7. Wild type benefits from *cepr1* neighbourhood in low nutrients conditions.

The wild type *A. thaliana* interaction with neighbours of different genetic backgrounds: with another wild type and with *cepr1* mutant in low nutrients media. (A) The mean percentage change in seed biomass between single grown and paired plant observed in different neighbour combinations. -100-0% competition, 0% neutral, 0-100% facilitation. (B) The Relative Interaction Index based on the seed

biomass between single grown and paired plant observed in different neighbour combinations. -1-0 competition, 0 neutral, 0-1 facilitation. (C) detailed seed biomass achieved by single grown and paired plant observed in different neighbour combinations. (D) detailed shoot biomass achieved by single grown and paired plant observed in different neighbour combinations. (E) detailed primary root length achieved by single grown and paired plant observed in different neighbour combinations. (F) detailed relative lateral roots length achieved by single grown and paired plant observed in different neighbour combinations. (G) detailed lateral roots density achieved by single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; n_{shoot} = 11-14, n_{seed} = 11-14, n_{root} = 7-18.

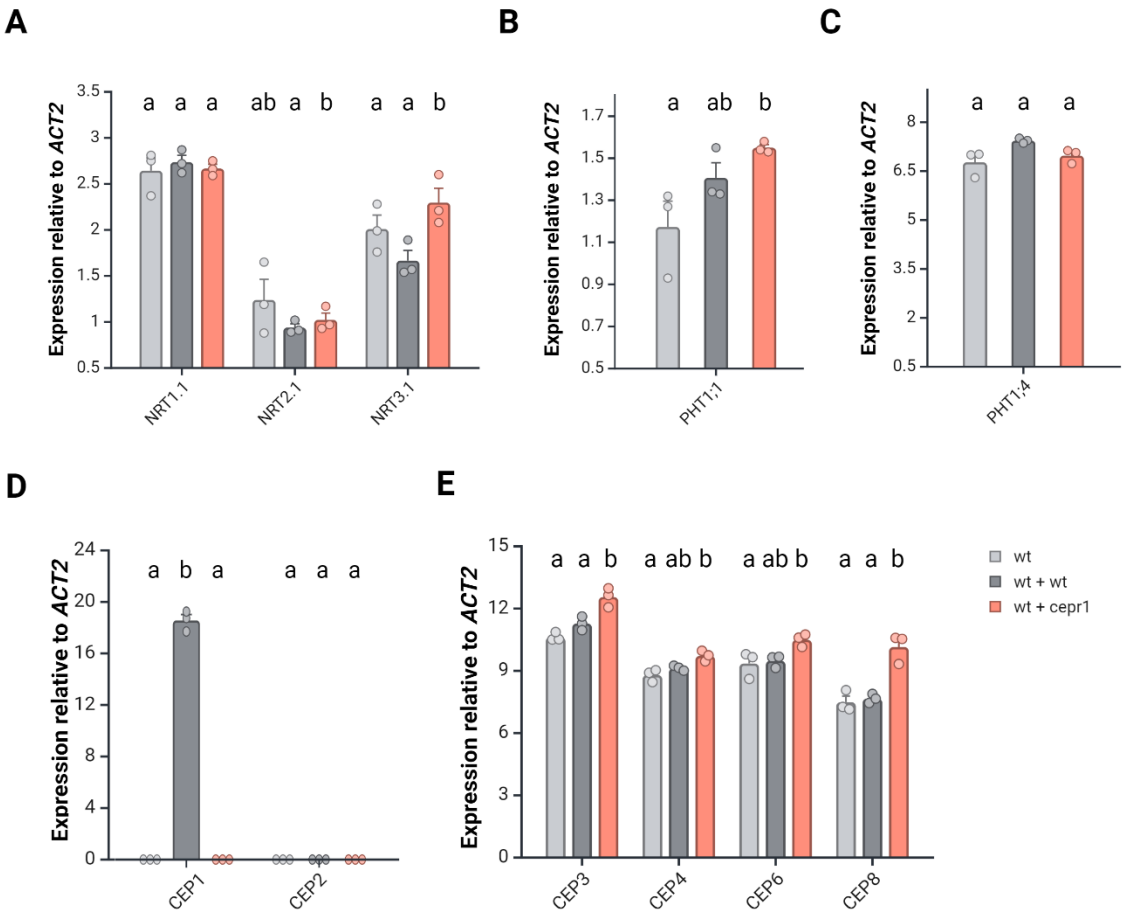


Figure 7.8. Wild type response to *cepr1* lacks the signature CEP1 upregulation in low nutrients conditions.

The wild type *A. thaliana* interaction with neighbours of different genetic backgrounds: with another wild type and with *cepr1* mutant in low nutrients media. Genes were grouped based on the magnitude of expression for visualization purposes only. (A) the expression of nitrate transporter genes in single grown and

paired plant observed in different neighbour combinations. (B) the expression of phosphate transporter PHT1;1 in single grown and paired plant observed in different neighbour combinations. (C) the expression of phosphate transporter PHT1;4 in single grown and paired plant observed in different neighbour combinations. (D) the expression of CEP genes in single grown and paired plant observed in different neighbour combinations. (E) the expression of CEP genes in single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; $n_{expression} = 3$.

Next, the interactions were assessed from the *cepr1* as a focal plant perspective and single *cepr1*, pairs of *cepr1* and pairs comprising a *cepr1* and wt in low nutrients conditions were compared (Figure 7.9). Observing an effect would further confirm that this receptor is involved in the interaction process.

The mean percentage change in seed number between wt pair and *cepr1* pair indicated that competition was markedly stronger within *cepr1* pair (-23.0% and -61.6% seed number loss respectively) and it was stronger than *cepr1* interaction with wt (-57.5%) (Figure 7.9A). This pattern was further corroborated with RII lower in both *cepr1* interactions than wt interaction (wt paired with wt RII = -0.17, *cepr1* paired with *cepr1* RII = -0.45, *cepr1* paired with wt RII = -0.40) (Figure 7.9B). Indeed, *cepr1* paired both with *cepr1* and with wt produced similarly less seed number (Figure 7.9C). This pattern was not observed in shoot biomass as *cepr1* did not reduce shoot biomass in response to neither of neighbours (Figure 7.9D). Belowground, no morphological changes were observed in either pairing in any of traits measured (Figure 7.9EFG).

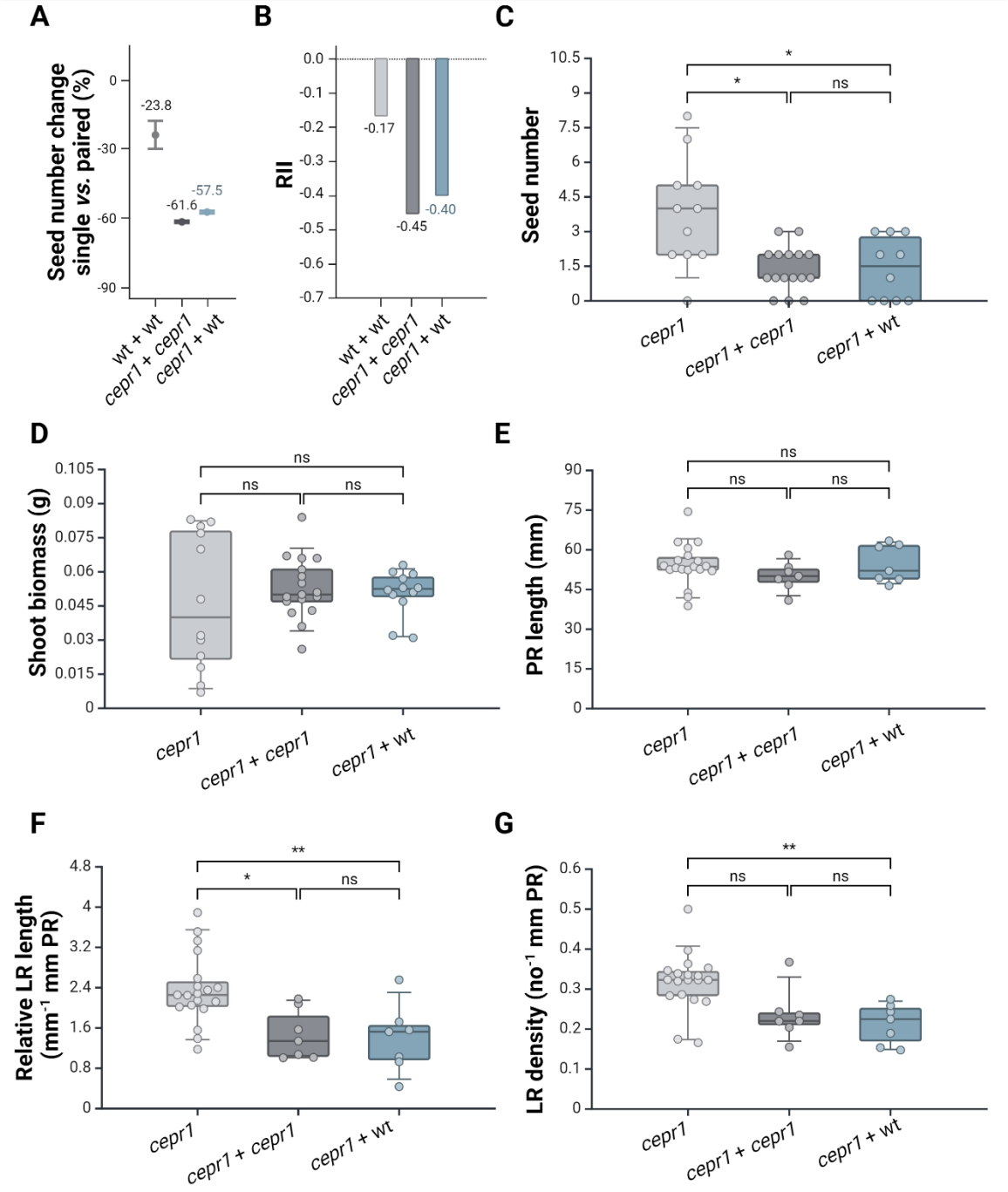


Figure 7.9. *cepr1* response to wild type and *cepr1* neighbours is indistinguishable in low nutrients.

The *cepr1* mutant interaction with neighbours of different genetic backgrounds: with another *cepr1* and with wild type in low nutrients media. (A) The mean percentage change in seed biomass between single grown and paired plant observed in different neighbour combinations. -100-0% competition, 0% neutral, 0-100% facilitation. (B) The Relative Interaction Index based on the seed biomass between single grown and

paired plant observed in different neighbour combinations. -1-0 competition, 0 neutral, 0-1 facilitation. (C) detailed seed number achieved by single grown and paired plant observed in different neighbour combinations. (D) detailed shoot biomass achieved by single grown and paired plant observed in different neighbour combinations. (E) detailed primary root length achieved by single grown and paired plant observed in different neighbour combinations. (F) detailed relative lateral roots length achieved by single grown and paired plant observed in different neighbour combinations. (G) detailed lateral roots density achieved by single grown and paired plant observed in different neighbour combinations. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; n_{shoot} = 12-17, n_{seed} = 12-17, n_{root} = 7-19.

In summary, in low nutrients conditions the interaction between wild type and *cepr1* mutant was neutral for wt as proven by higher wt fitness measured as seed biomass. The belowground lateral root neighbour responses to *cepr1* were weaker than responses to another wt, however not significantly. None of the nutrient transporter was expressed differentially in terms of fold change. CEP1 upregulation appeared as a general wt neighbour response signature that was not employed in response to *cepr1*. Whereas the interaction between *cepr1* with *cepr1* and with wt was highly similar and markedly stronger than interaction between wt plants as evidenced by exceptionally high fitness loses. Interestingly *cepr1* responded to neighbours belowground and those responses also did not differ between wt and *cepr1* as neighbour. Therefore it may be concluded that the competition between wt and *cepr1* was asymmetrical.

7.4.2.3 CEP1 application

Since CEP1 upregulation was identified as a key neighbour response in the previous section, this study involved the external application of CEP1 to assess whether its presence in the rhizosphere could substantially evoke any levels of the neighbour response in single grown wild type plants. Given that CEP1 is a known ligand of CEPR1, single grown *cepr1* mutant plants were also included as an internal control. CEP1 external application was also shown to regulate nitrate transporters expression, therefore NRT1.1, NRT2.1 and NRT3.1 expression was assessed to verify

CEP1 functionality. It has to be acknowledged that 1) it is unlikely that CEP1 would be the sole neighbour detection signal in the rhizosphere, 2) it is unknown whether any actual link exists between the CEP1 upregulation and root traits decrease observed in this study. Therefore, If CEP1 indeed functions as a neighbour detection signal in the rhizosphere *and* CEP1 modulates root response, then single grown wild type plants should exhibit a neighbour response, characterised by a decrease in root traits, while the *cepr1* mutant should remain unaffected.

No differences were detected between *cepr1* mutant plants treated with CEP1 compared to mock which further confirms that functional CEPR1 is required for CEP1 perception. However, no differences were also detected in wild type plants treated with CEP1 (Figure 7.10ABCD). All nitrate transporters were significantly downregulated in CEP1 presence which confirmed that applied CEP1 was biologically functional. It is worth to mention that unlike NRT1.1, NRT2.1 and NRT3.1 were downregulated by more than 2-fold. The downregulation was irrespective of the social setting and expressed in similar manner between single grown plants and plants grown in pairs (Figure 7.10E).

In summary, the exogenously applied CEP1 was not sufficient to mimic any belowground neighbour responses in wild type which indicates that it is not responsible for the root morphology neighbour response phenotype. External CEP1 did evoke nitrate transporters downregulation which indicates its functionality. However, first of all, nitrate transporters expression change is not a neighbour response and second, this phenotype was observed irrespective of social setting. Therefore, it may be concluded that while internal CEP1 signalling is involved in downstream neighbour response, it is not involved in neighbour detection in the rhizosphere.

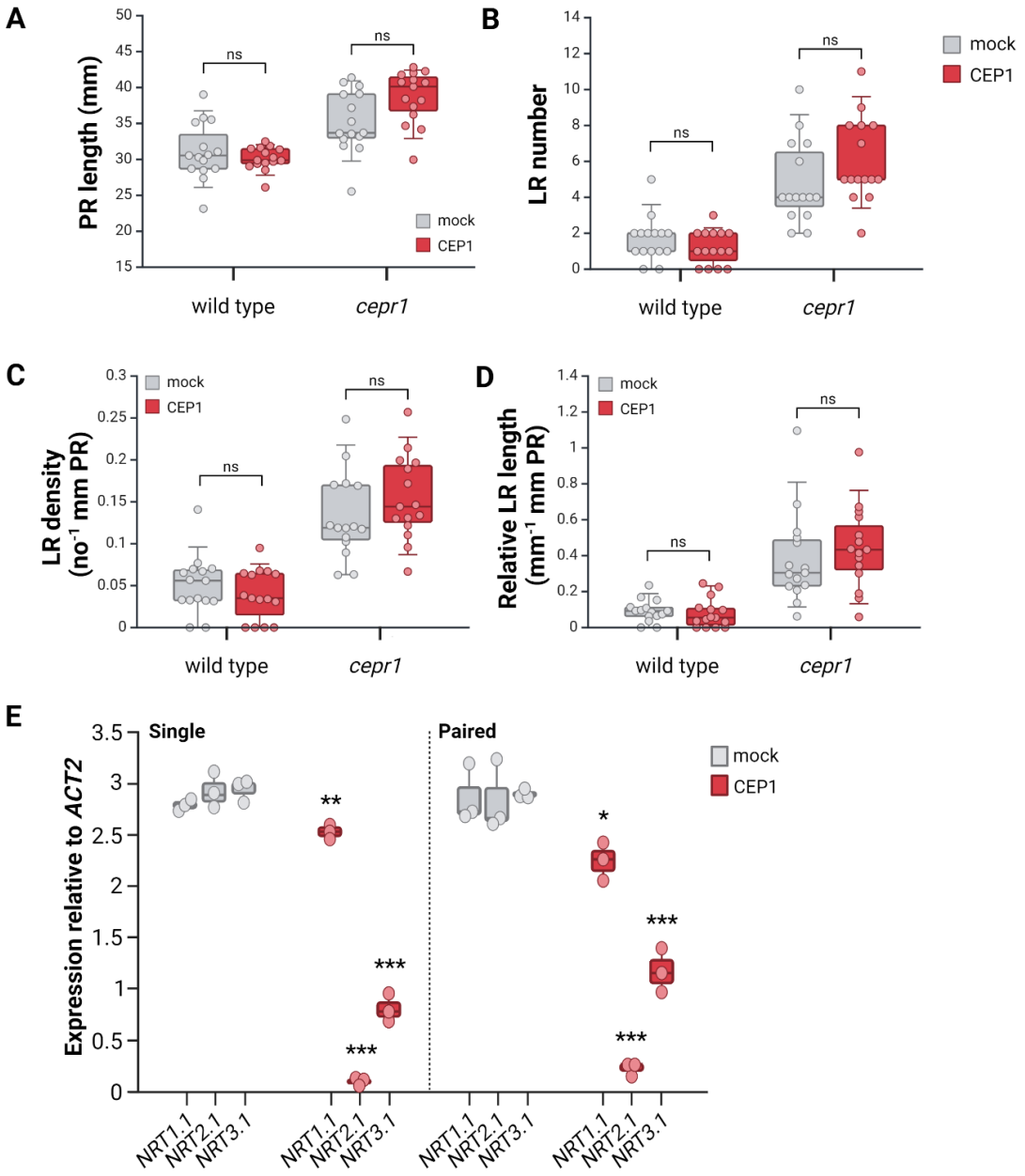


Figure 7.10. Exogenous CEP1 is not responsible for root neighbour response.

The belowground responses of wild type and *cepr1* plants to exogenously applied 1µM of CEP1. (A) primary root length of single grown wild type and *cepr1* plants. (B) lateral root number of single grown wild type and *cepr1* plants. (C) lateral root density of single grown wild type and *cepr1* plants. (D) relative lateral root density of single grown wild type and *cepr1* plants. One-way ANOVA with Tukey multiple comparison test or Kruskal-Wallis with Dunn multiple comparison test; n = 15. (E) nitrate transporters NRT1.1, NRT2.1 and NRT3.1 gene expression in single and paired wild type plants. Student's t-test, n = 3.

7.5. Discussion

The LRR-RLKs screen has found the receptors from subfamily XI prevalently expressed in root epidermis. The subfamily XI LRR-RLKs are particularly notable for their roles in perceiving small peptide ligands that regulate root meristem development and primary and lateral root emergence and growth, lateral root angle regulation and root hair and Casparian strip development (Figure 7.11) (De Coninck & De Smet, 2016; Furumizu et al., 2021; Ohyama et al., 2008; Takahashi et al., 2019). For instance, CLAVATA1 (CLV1) and CLAVATA2 (CLV2) receptors interact with, among others, CLE40 peptide and were shown to regulate nodulation and mycorrhizal symbiosis (Whitewoods, 2021). Another example is RGF1 INSENSITIVE 1 (RGI1) receptor interacting with RGF peptides (also known as GOLVEN (GLV) or CLEL (CLE-like) and playing role in root meristem development (Lu et al., 2020; Ou et al., 2016b). The high abundance of XI subfamily receptors in root epidermis suggests that they may be involved in the perception of external rhizosphere signals.

Here, two LRR-RLK receptors were found to exert significant impact on plant-plant interactions in *A. thaliana*—the unknown LRR-RLK At1g68400 (At1g68400; subfamily III) and CEPR1 (At5g49660; subfamily XI). The At1g68400 appeared as a weaker competitor allowing the wild type to produce more siliques than when paired with another wild type. This is an unknown receptor and its function as well as ligand are yet to be elucidated. Elucidating the function of unknown LRR-RLK receptor is challenging due to the pleiotropic effects they often execute, as well as that the identification of its ligand(s) is difficult due to frequently observed ligands redundancy. Due to the limitations of this study, further work on this gene was not pursued. The second receptor that was found to affect the interaction significantly was CEPR1 and it was investigated further. CEPR1 expression was reported in leaf vasculature, lateral roots initiation sites and in lateral roots, but not in root tips (Tabata et al., 2014). CEPR1 is heavily involved in primary and lateral roots development, nitrate acquisition, nutrient-dependent immunity regulation and

rhizosphere interactions—nodulation and mycorrhizal symbiosis (Rzemieniewski et al., 2022; Taleski et al., 2018, 2024). CEPR1 has known ligands from CEP family which in *Arabidopsis* entails 15 genes CEP1 to CEP15 showing spatiotemporal differences in expression (Roberts et al., 2013) (Figure 7.12AB).

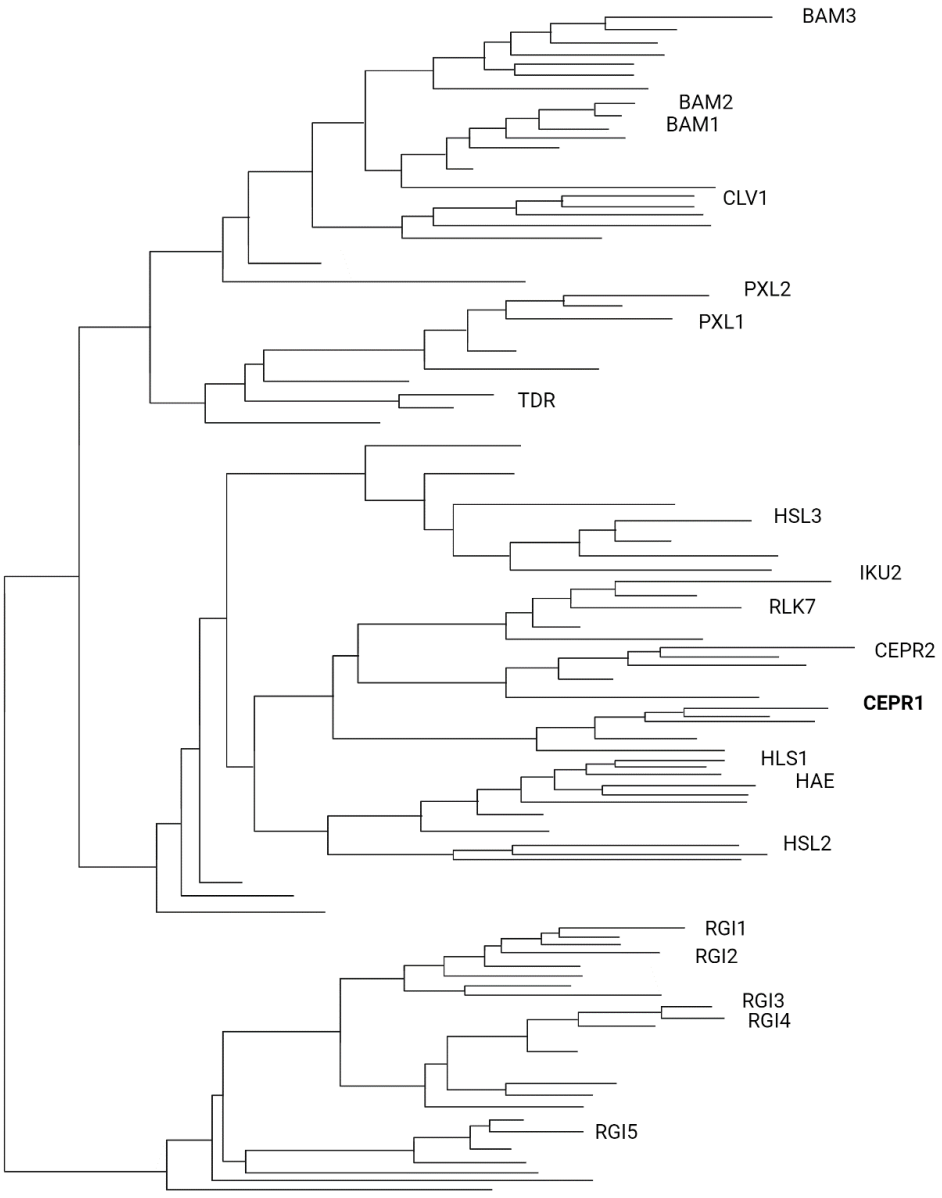


Figure 7.11. The phylogeny of the LRR domains encoded by LRXI genes.

The LRR domain encoded by 249 LRXI genes from 12 species. Branch lengths represent the average number of changes in amino acids per site. CEPR1 highlighted in bold. Figure adapted from Furumizu et al. 2021.

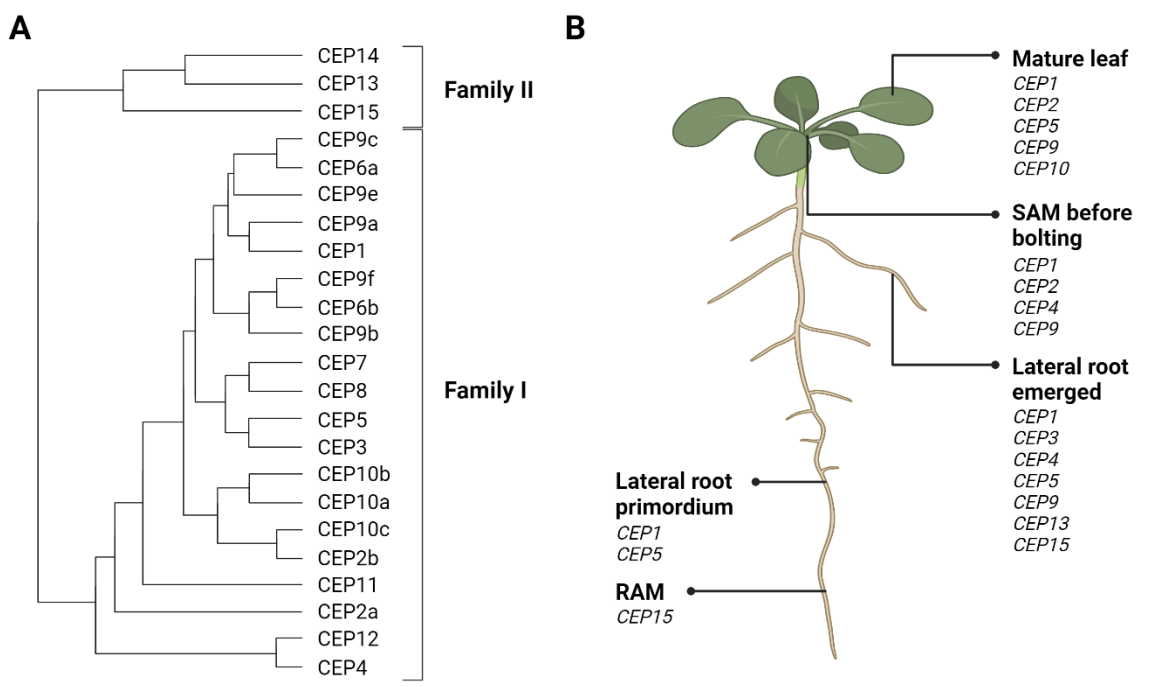


Figure 7.12. An overview of the *Arabidopsis thaliana* CEP gene family and their spatial expression.

(A) The phylogenetic tree of *Arabidopsis* CEP family members based on full length protein sequences. (B) The expression of CEP genes in *Arabidopsis*. Figure adapted from Roberts et al. 2013.

First, the response and outcome of wild type interaction with another wild type was characterised. Due to the fact, that CEPR1-CEPs signalling is involved in nutrients acquisition this study included the analysis of nutrient transporters and CEP genes expression. No differences in expression of nitrate or phosphate transporters in the presence of a wild type neighbour was detected, regardless of whether the nutrients conditions were high or low. This may suggest either that in this conditions the level of nutrients depletion by the neighbour was negligible or that the observed root size decrease was aimed at the prevention of unsustainable growth. In other words, perhaps in both conditions' plants sensed the nutrient depletion by the neighbour but gravitated towards growth reduction rather than the nutrient uptake efficiency increasement. This is in line with results obtained by Masclaux and colleagues (2010) who did not find any differentially regulated nutrient transporter

genes. In a subsequent study comparing single grown plants and a density treatment of 20 plants it was reported, that two high-affinity nitrate transporters NRT2.2 and NRT2.5 were upregulated whereas NRT2.6 and one high-affinity phosphate transporter PHT1;4 were downregulated (Masclaux et al., 2012). Interestingly, here CEP1 peptide was found to be highly upregulated specifically in response neighbour's presence in low nutrients conditions. CEP1 is known to be induced under nitrogen starvation and limitation (Delay et al., 2019; Tabata et al., 2014). All CEP peptides were previously shown to inhibit lateral roots growth (Chapman et al., 2019; Delay et al., 2013; Huang et al., 2023; Ohyama et al., 2008; Roberts et al., 2013). Given that, 1. here the root system growth decrease was observed in both nutrient conditions and 2. CEP1 was upregulated only in low nutrients conditions, and the fact that 3. externally applied CEP1 did not evoke root growth inhibition in high nutrients suggests, and 4. CEP1 triggered downregulation of nitrate transporters regardless of the social context suggests that CEP1 was only partially involved in this phenotype. Thus, although internal CEP1 signalling contributes to downstream neighbour responses, it does not participate in neighbour detection within the rhizosphere.

Given these observations, the comparison of wild type plants response to *cepr1* mutant was also compared. In general, the competitive interaction of wild type with *cepr1* mutant was weaker than with another wild type. In high nutrients it was 2-times weaker and in low nutrients it even became neutral for wild type. In high nutrients, when the neighbour was *cepr1* the focal wild type plant significantly downregulated nitrate transporter NRT2.1, a response not observed in low nutrients. The expression followed a similar pattern NRT3.1 albeit the expression change was lower. NRT3.1 is necessary for stabilizing NRT2.1 and it is essential for high-affinity nitrate uptake induction, hence the similarity of their response is to be expected (Okamoto et al., 2006). *cepr1* itself is characterised by reduced uptake of nitrate (Tabata et al., 2014). Therefore it is likely that since *cepr1* does not exert high pressure on wild type to preempt nitrate resource, hence the observed downregulation of NRTs. Significant differences were observed in CEPs expression during wild type

interaction with *cepr1*. The CEP3, CEP6 and CEP8 were differentially regulated in both nutrients' conditions, however with rather low fold changes. The exact spatial expression of these CEPs is not resolved. However, it may be hypothesised that if these CEPs are particularly expressed i.e. in lateral roots primordia or root tips, the method of assessing the expression with the whole root system applied here could have hindered the ability to measure it and diluted their expression. If that was true, then the fact that significant differences were still detected here is interesting and prompts further research on those peptides' roles. In summary, in general the interaction between wild type and cepr1 can be best described as asymmetric competition. In terms of wild type fitness in both nutrients' conditions, the interaction between it and *cepr1* was less competitive than with another wild type. In both nutrients' conditions, wild type's response to *cepr1* neighbour was the reduction of lateral roots growth albeit lower than when paired with another wild type.

The neighbour responsiveness and tolerance of *cepr1* mutant was also assessed. In general, *cepr1* was a weaker competitor than wild type and experienced higher seed biomass loss both in high and in low nutrients conditions. What is more, in both nutrients' conditions *cepr1* also failed to execute typical neighbour response—reduction of lateral roots growth. This in turn led to higher competition intensity, that was particularly detrimental in low nutrients conditions,

Taken together, it is perhaps reasonable to suggests that either a) *cepr1* was not properly detected b) *cepr1* is properly detected and appears as a stronger competitor than a wild type hence smaller decrease in lateral root growth can be interpreted as competitive root proliferation. If the second is true, then the implication would be as well that neighbour detection occurs mainly *via* the assessment of the physical roots' presence or the amount of exuded compounds and not the assessment of the nutrient depletion.

Chapter 8
General Discussion

8.1 Main findings

In this thesis, I explored the complex and dynamic nature of plant-plant interactions, focusing on how plants influence each other's growth, development, and survival. My research aimed to deepen our understanding of molecular physiology of relationships between plants and mechanisms that drive these interactions particularly in the rhizosphere. Plant-plant interactions are known to shift from competition to cooperation in harsh environmental conditions (Maestre et al., 2009b; Malkinson & Tielbörger, 2010b; Qi et al., 2018). The phenotypic responses and outcomes of plant interactions have been widely studied in model plants as well as in economically important crops (Subrahmaniam et al., 2017). The physiological mechanisms of neighbour detection and response have been proposed and somewhat substantiated in several studies (Callaway, 2002; Chen et al., 2012; Wang et al., 2021). Although the importance of resolving the genetic and molecular mechanisms of plant interactions is widely recognised, there have been relatively sparse initiative to explore them (Becker et al., 2023; Callaway & Li, 2020; Thorpe et al., 2011). For those reasons, my research contributes to the broader effort to build a comprehensive understanding of the mechanisms underlying plant-plant interactions. This thesis advances knowledge of plant behaviour, adaptation, and communication, with wide-reaching implications for the fundamental understanding of biological systems at both individual and community levels. The insights gained here have potential to strengthen the resilience of plant communities against environmental stresses but also emphasise new molecular targets that can improve biodiversity management and promote agroecological practices.

8.1.1 Plant-plant interactions are complex biological systems whose functioning is influenced by a multitude of abiotic and biotic factors

In Chapter 3, I investigated the intricate interplay of abiotic factors, specifically physical space and nutrient availability, in shaping plant-plant interactions. Both nutrient levels and rooting volume, extensively studied in relation to individual plant performance, are essential for growth, development, and reproduction. Resource

scarcity has well-documented negative effects on these processes. Moreover, these resources are subject to exploitation by neighbouring plants, thereby reducing their availability to others. This struggle represents a primary mechanism driving competitive interactions between plants (Harper, 1961; Novoplansky, 2009; West et al., 2002; Wilson, 1988). Although recognised as significant confounding factors in plant-plant interaction research, nutrients and space are seldom the main focus of these studies. Here I conclude that both factors similarly influence neighbour response and *interaction outcomes* — an underreported measure in plant relationships. Notably, rooting volume and nutrient effects were interdependent, with neither substituting for the influence of neighbouring plants. Previous research suggested an approximately linear effect of these factors, without distinguishing between individual and population density levels (Poorter et al., 2012a; Postma et al., 2021). However, here I show that plants grown individually and with neighbours in fact show different sensitivity to these resources and different efficiency in their exploitation. Studies on plant-plant interactions often establish initial assumptions about the system or extend conclusions beyond the reported measures, drawing from individual plant treatments or general knowledge of plant's physiology (Chen et al., 2020; McNickle, 2020; Mobley et al., 2022). However, I was able to demonstrate that the predictive modeling proved unreliable in estimating plant growth during interactions based only on individual plant growth, exactly due to the differences in sensitivity I observed. Moreover, I demonstrate that, in partial alignment with the stress gradient hypothesis, the nature of intraspecific interactions shifts in response to environmental conditions change. However, unlike the classical theory, here strong competition transitioned to a more benign interaction, but did not progress to cooperation or facilitation (Malkinson & Tielbörger, 2010b; Pugnaire & Luque, 2001b; Qi et al., 2018). This outcome may be attributed to the relatively narrow and coarse range of stress applied in this study. Future research with higher resolution could help determine whether cooperation or facilitation are achievable in this context. Nevertheless, this is the first study to propose and report the role of nutrients and

space stress within the framework of the stress gradient hypothesis, which traditionally focuses on climate-related environmental factors.

Chapter 4 delved into biotic factors, including the influence of neighbouring plant age and genotype understood as species and ecotype affiliation on plant-plant interactions. A plant's age, and thus its developmental stage, determines its competitive abilities and potential for niche differentiation with neighbouring plants (Dayrell et al., 2018; Paterno et al., 2016; Schiffers & Tielbörger, 2006). It is also well acknowledged that there is a scope for natural variation between species and ecotypes in their neighbour responsiveness and tolerance, as evidenced by the multitude of research concerning various species and ecotypes (Subrahmaniam et al., 2017). Here I described these phenomena in detail and reported new caveats. Firstly, I was able to confirm that the timing of neighbour emergence plays important role in plant-plant interactions, and extended this with observation that the earlier-emerging plants gain significant reproductive advantage over later ones most likely due to the size-asymmetrical competition. My results align with previous studies, which generally highlight early emergence as a strategy to reduce competition exposure particularly in annual species (Miller et al. 1994; Dyer et al.2000; Geber & Griffen 2003; Mercer et al. 2011). However, most studies on ontogenetic shifts in competition focus on the early stages of seedling development, primarily emphasizing emergence and survival (Bassar et al., 2017; Berendse & Möller, 2009; Mason et al., 2013; Miriti, 2006; Paterno et al., 2016; Schiffers & Tielbörger, 2006). This study enhances this understanding by demonstrating that neighbour's age and the timing of exposure has long-term consequences as it significantly impacts the fitness of the focal plant. I have also identified an early window of opportunity during which the presence of a neighbour does not negatively impact the focal plant's performance. This finding contradicts a handful of studies suggesting that neighbour detection and response is most likely to be observed while taking advantage of the early timing of the interaction which rules out nutrient depletion as a factor (Mcnickle et al., 2016; Padilla et al., 2013; Schenk, 2006b). Certainly, more detailed

experimentation would be required to verify which one of the two statements is correct.

The results discussed in this chapter also reveal that notable differences in neighbour responsiveness and tolerance strategies can be found, not only between closely related species within the same family but also between ecotypes within a single species. It is evident that species vary in their strategies for competition (Subrahmaniam et al., 2018b, 2021b), and different *A. thaliana* ecotypes have also demonstrated varying levels of competitive ability (Masclaux et al., 2010), however the latter is far less explored. I have demonstrated that identifying *common* neighbour responses and linking them to interaction outcomes is a complex and likely unrealistic task. However, I was able to determine *specific* multitrait patterns of neighbour response and tolerance and cluster ecotypes based on them. Additionally, I identified links between competitive traits and changes in seed biomass within ecotypes, suggesting that the strength of the relationship between shoot biomass and seed biomass changes can partially explain the differences between clusters. Surprisingly, none of the individual root architecture traits significantly impacted these cluster distinctions, despite the clear role of root efficiency in determining shoot and seed growth. Plants often display redundancy in their traits, meaning multiple traits can compensate for each other. Therefore, even if certain root traits are not significant individually, other unmeasured traits or compensatory mechanisms in the plant may mitigate the impact of root architecture on overall plant performance (Aibara & Miwa, 2014b; De Kroon, 2012; Lynch, 1995; Novoplansky, 2019).

8.1.2 Belowground neighbour detection and response mechanisms involve ethylene and small peptides

In Chapter 5, I explored the role of rhizodeposits — root exudates and organic compounds released by plant roots — in mediating plant communication and competition. It is well known that plant rhizodeposits are crucial in shaping interactions within the rhizosphere, affecting both nearby plants and microorganisms (Callaway, 2002; Chen et al., 2012; Sun et al., 2021; Vives-Peris et al., 2020; Wang et

al., 2021). This research delved into how rhizodeposition influences plant-plant interactions and its impact on plant fitness. The findings indicated that although rhizodeposits do influence *A. thaliana's* interactions with other species, *A. thaliana* does not respond to its own rhizodeposits in a way that alters its fitness. In rare cases where rhizodeposits did affect *A. thaliana*, the effects were specific to certain species and dependent on the nutrient environment of the donor plant. Earlier studies reported differential expression of ABC transporters in plant interactions and implied their role in exudation of relevant signals (Biedrzycki et al., 2011; Broz et al., 2010; Geisler, 2012; Subrahmaniam et al., 2018b). In this study, I sought to evaluate their actual functionality and concluded that they are likely not relevant in intraspecific interactions, as their inhibition had no impact on the root system architecture of competing plants. This does not definitively rule out their involvement in other types of interactions, and further testing of ABC-transporter mutants along with analyzing their exudate content should help draw broader conclusions. Notably, this study was the first to demonstrate that ethylene plays a role in neighbour detection, with the EIN2 receptor and ACO1-mediated ethylene production being critical factors. Plants that were unable to produce or perceive ethylene were shown to be at a disadvantage in competition, giving their neighbours a competitive edge. It would be valuable to examine ethylene's role in neighbour detection across a wider range of species to see how general this signalling mechanism is.

In Chapter 7, my goal was to identify new receptor candidates that may play a role in neighbour detection and response. I concentrated on candidates from the leucine-rich repeat receptor-like kinases (LRR-RLKs) family because of their capacity to detect a variety of extracellular signals and regulate crucial pathways related to growth, defence, and hormone signalling (Man et al., 2020; Mubassir, 2019; ten Hove et al., 2011). Two receptors, At1g68400 and CEPR1, were identified as affecting plant-plant interactions. CEPR1 binds CEP peptides and regulates various processes, including nitrate acquisition and root development (Ohkubo et al., 2017; Tabata et al., 2014), and I report that it is also significantly influencing competition outcomes in *A.*

thaliana. The interaction between wild type plants and the *cepr1* mutant was marked by asymmetric competition, with wild type plants being less competitive, especially in high nutrient conditions. NRT2.1 nitrate transporter was downregulated in WT plants in high but not low nutrients. While CEP1 was upregulated under low-nutrient conditions, it did not influence root architecture changes, indicating its role in nutrient stress signalling rather than neighbour detection or competitive root growth. This study introduces an additional function to CEPR1, linking its two primary roles: nutrient signalling and lateral root development.

8.1.3 Rhizodeposits proteome presents a promising source of novel rhizosphere interactions mediators

Chapter 6 provided a comprehensive analysis of the root-released proteome. While the primary aim was to identify proteins potentially involved in plant-rhizosphere interactions, the resulting datasets have broader applications, offering insights into the current understanding of proteins present in the root exudates and how they vary with the plant's nutrient status. The findings suggest that nutrient availability significantly influences the composition and function of root exudates in *A. thaliana*. The high nutrient conditions lead to a broader diversity of proteins, but a smaller proportion is specifically secreted. In contrast, low nutrient conditions not only reduce the overall number of proteins but promote a much higher percentage of proteins intended for secretion. This indicates that in nutrient limited environments, plants may prioritise the targeted release of proteins, likely to enhance interactions with the rhizosphere, optimise nutrient acquisition, and minimise the loss of essential molecules through unintended leakage. This suggests that nutrient conditions drive a fundamental shift between growth-oriented processes and survival-oriented responses, reflecting the plant's flexibility in managing its resources. Furthermore, no small peptides were directly detected in the root exudate samples, although the standard CEP5 was detectable. This suggests that native small peptides may either be absent or present in such low abundance that they are undetectable.

8.2 Future perspectives

The findings presented in this thesis provide exciting opportunities for further research across various contexts. These insights not only deepen our understanding of fundamental plant biology but also hold potential avenues for applied sciences, offering practical benefits for agriculture and ecosystem management.

The findings of this thesis suggest that future research should explore a wider range of environmental stresses to fully understand their impact on plant interactions. Investigating other stressors, such as water availability or temperature, in combination with nutrient and space stress, could provide more insights into how plants balance competition and cooperation in diverse ecosystems. The variation in neighbour responsiveness across species and ecotypes opens avenues for future research into the genetic and molecular basis of these differences. Detailed studies on the role of early developmental stages and genetic diversity including biochemical and molecular responses in competition dynamics would help refine our understanding of plant adaptation strategies. While this thesis identified ethylene as a key player in plant-plant interactions, more comprehensive studies across different species are needed to determine the generality of this signalling mechanism. Furthermore, research should focus on small peptides, particularly their internal roles in belowground signalling response, as their detection remains challenging, and their influence on plant interactions is not fully understood.

The insights gained from this research have practical implications for agriculture and biodiversity management. Understanding how plants interact with their neighbours can inform strategies to enhance crop resilience and productivity in nature and agroecosystems. Moreover, the identification of molecular targets, such as ethylene pathways and specific receptors, could be leveraged to breed or engineer plants with improved tolerance to competition and environmental stress.

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Appendices

Appendix A

Table A.1. The summary of 2-way ANOVAs utilised to investigate the effects of explanatory variables: social setting (2 levels: single and paired) and rooting volume (3 levels: 30ml, 100ml and 500ml) on response variables: shoot and seed biomass. 2-way ANOVA, n = 7.

		Df	F value	p value	
Shoot biomass	Social setting	1	28.74	**3.48E-06**	***
	Rooting volume	1	522.79	**< 2e-16**	***
	Social setting × Rooting volume	1	18.73	**9.46E-05**	***
	Residuals	41			
Seed biomass	Social setting	1	44.51	**4.80E-08**	***
	Rooting volume	1	556.05	**< 2e-16**	***
	Social setting × Rooting volume	1	36.88	**3.41E-07**	***
	Residuals	41			

Table A.2. The summary of 2-way ANOVAs utilised to investigate the effects of explanatory variables: social setting (2 levels: single and paired) and nutrients (2 levels: 10% and 100%) on response variables: shoot and seed biomass. 2-way ANOVA, n = 5-7.

		Df	F value	p value	
Shoot biomass	Social setting	1	21.9900	**0.0002**	***
	Nutrients	1	398.3500	**3.31E-14**	***
	Social setting × Nutrients	1	18.4100	**0.0004**	***
	Residuals	19			
Seed biomass	Social setting	1	32.0100	**1.87E-05**	***
	Nutrients	1	405.9300	**2.79E-14**	***
	Social setting × Nutrients	1	23.3900	**0.0001**	***
	Residuals	19			

Appendix B

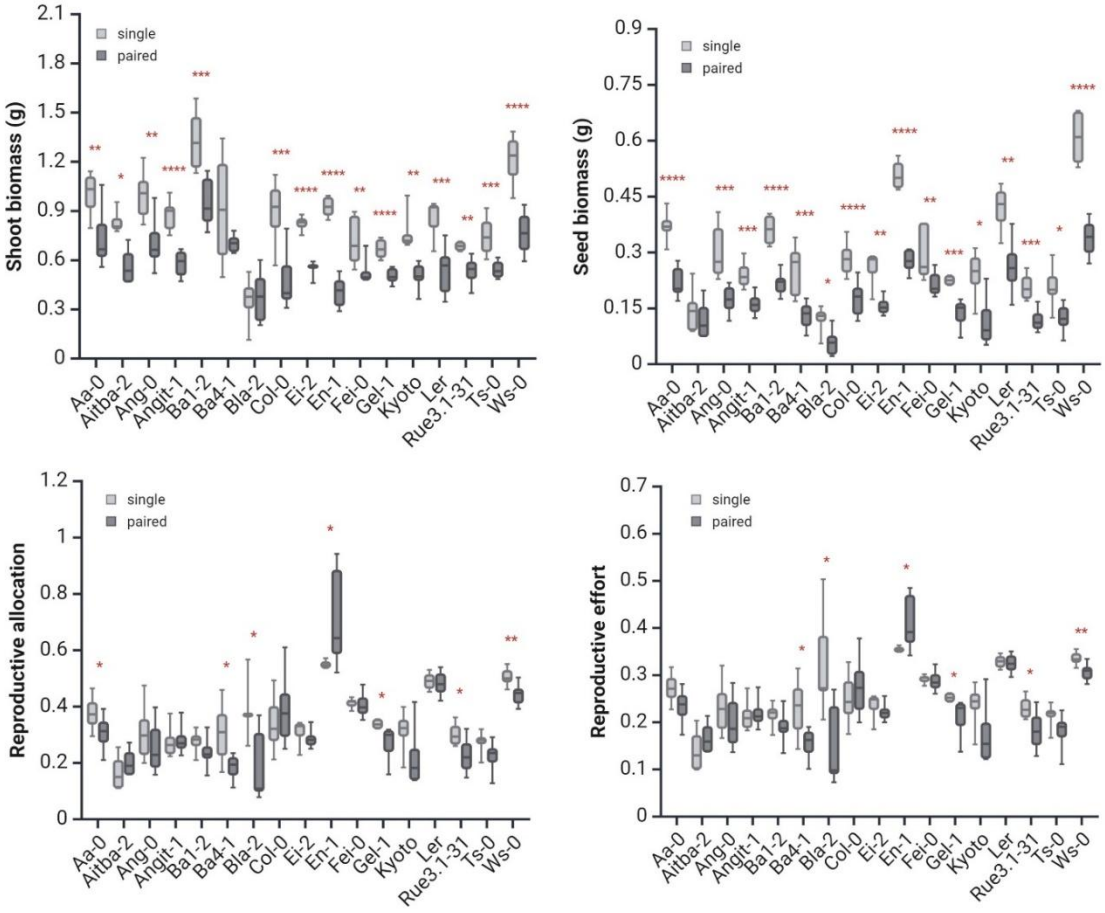

Figure B.1a. The detailed representation of trait expression in *A. thaliana* ecotypes grown individually and in intraspecific pairs. Significant differences indicated with asterisk. Student's t-test, n = 5-15.

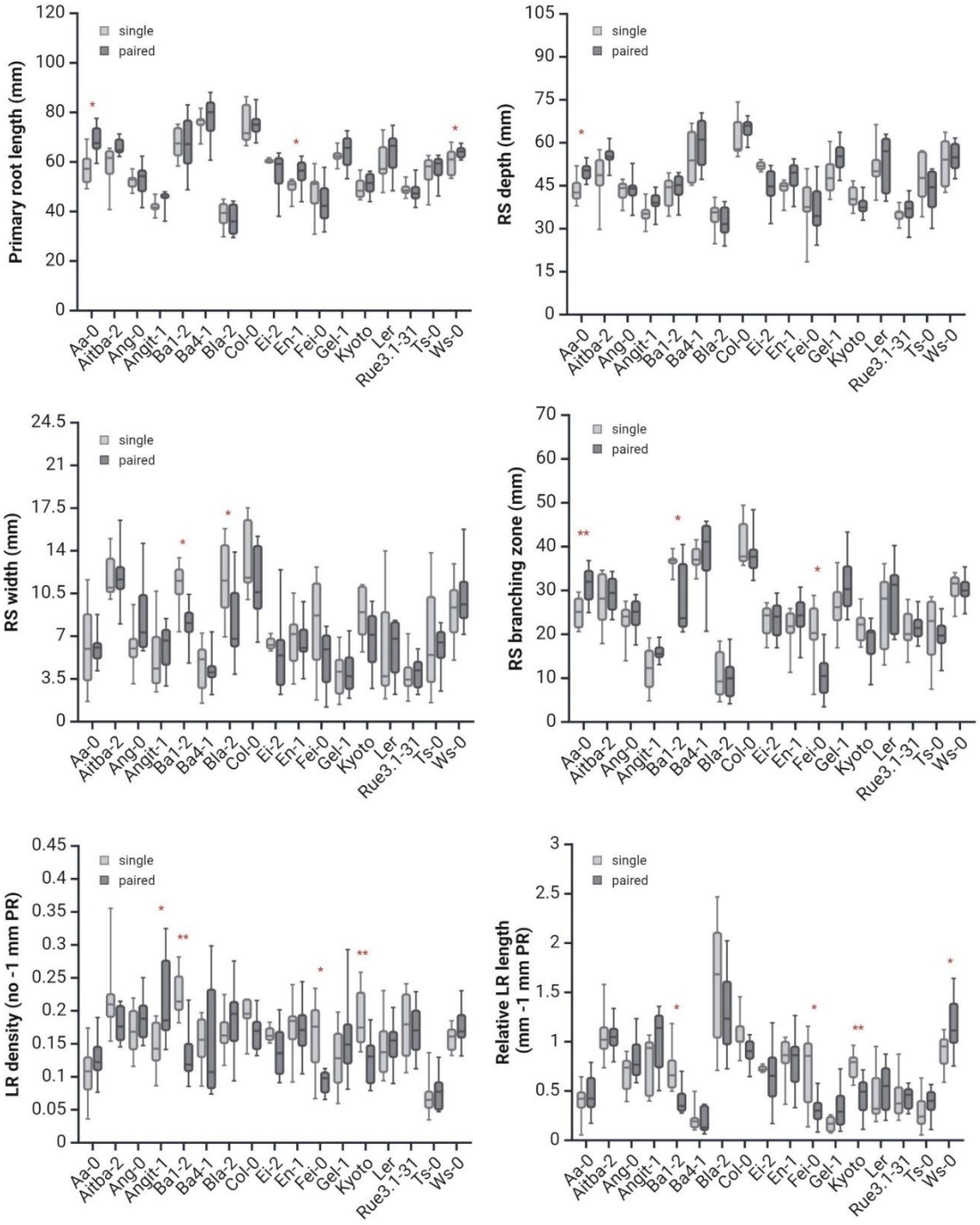

Figure B.1b. The detailed representation of trait expression in *A. thaliana* ecotypes grown individually and in intraspecific pairs. Significant differences indicated with asterisk. Student's t-test, n = 5-15.

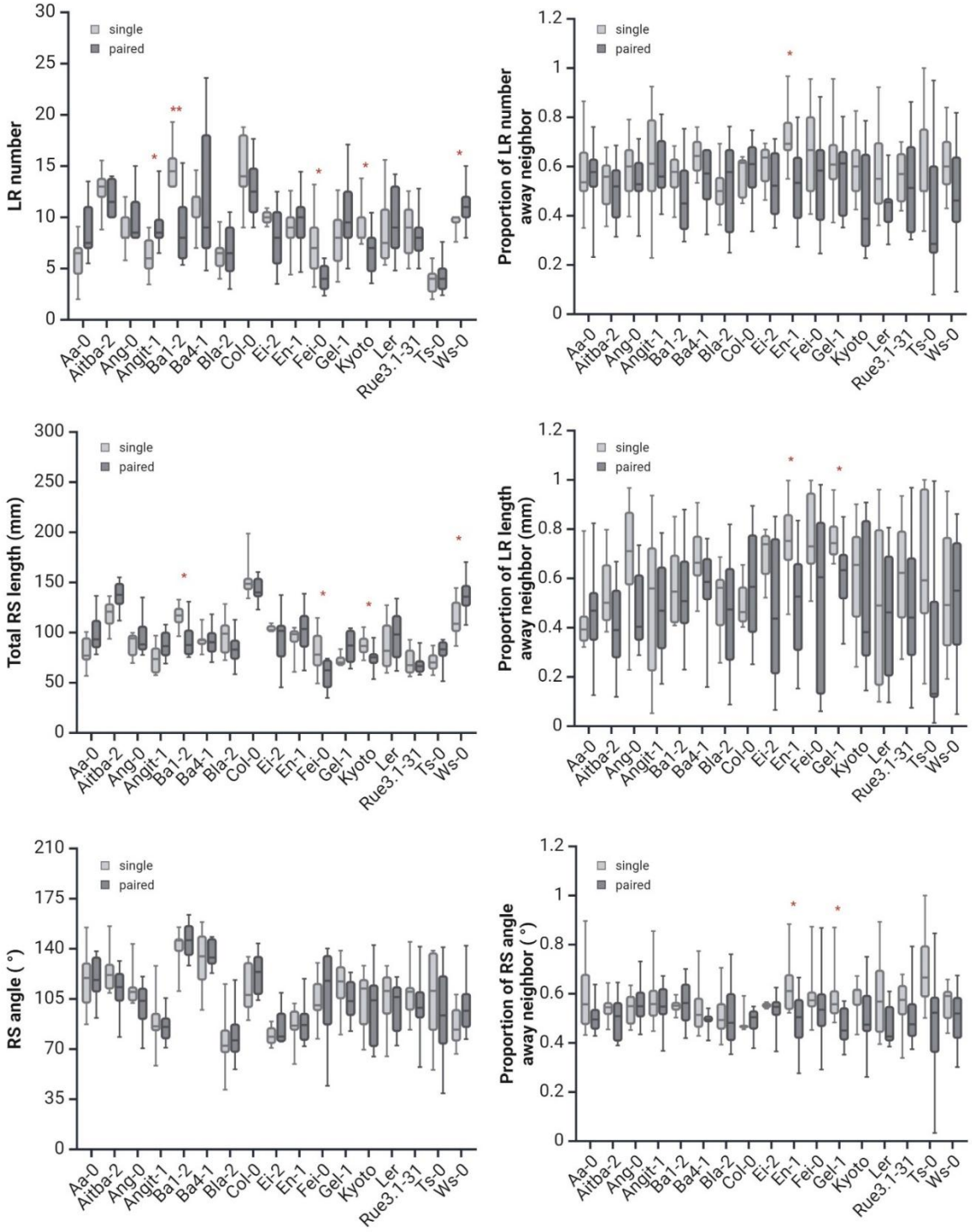

Figure B.1c. The detailed representation of trait expression in *A. thaliana* ecotypes grown individually and in intraspecific pairs. Significant differences indicated with asterisk. Student's t-test, n = 5-15.

Table B.1. The summary of a 2-way ANOVA utilised to investigate whether adding the interaction term between each trait and social setting (single *vs.* paired plants) significantly improves the model fit.

	npar	AIC	BIC	logLik	deviance	Chisq	Df	p value	
baseline model	11	-908.32	-870.36	465.16	-930.32				
interaction model	16	-970.42	-915.21	501.21	-1002.42	72.107	5	**3.73E-14**	***

Table B.2. The summary of Mixed-Effects Model utilised to investigate how different traits affect seed biomass while accounting for variability across clusters. Linear mixed model fitted by REML. t-tests used Satterthwaite's method.

Linear mixed model fit by REML

REML criterion at convergence: 42

Scaled residuals:

Min	1Q	Median	3Q	Max
-1.6298	-0.406	-0.2192	0.41	1.5074

Random effects:

Groups	Name	Variance	Std.Dev.
Cluster	Intercept	0.8091	0.8995
Residual		0.4812	0.6937

Number of observations: 17, groups: Cluster, 4

Fixed effects:

	Estimate	Std. Error	t value	p value
Intercept	-0.3288	0.501	-0.656	0.6741
PR length	-0.4032	0.2401	-1.68	0.1191
Shoot biomass	0.5437	0.2451	2.218	**0.0469**
Repr. allocation	0.3783	0.2343	1.615	0.1378
LR density	-0.128	0.2841	-0.451	0.6604

Correlation of Fixed Effects:

	(Intr)	PR length	Shoot biomass	Repr. allocation
PR length	0			
Shoot biomass	0.098	-0.068		
Repr. allocation	-0.066	-0.095	0.252	
LR density	0.041	0.091	0.23	0.136

Appendix C

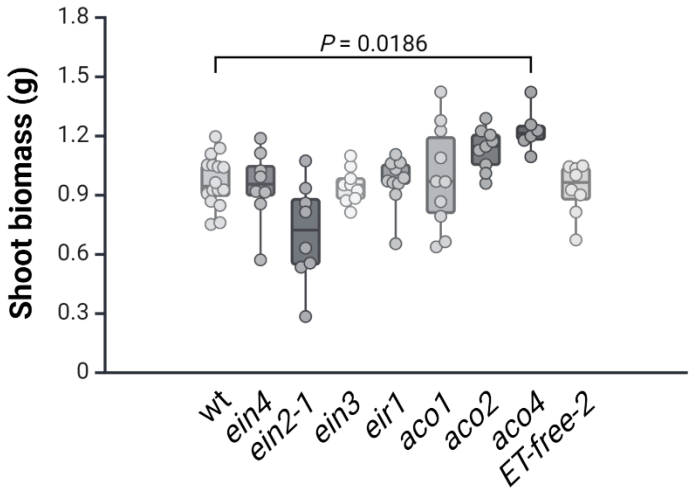

Figure C.1. The majority of ethylene biosynthesis and perception mutants do not differ from wild type plants in terms of shoot biomass.

The wild type and mutants impaired in ethylene perception or biosynthesis shoot biomass. Ethylene receptor mutants appear in order of the signalling cascade (Figure 5.2). *aco4* mutant is the only mutant that differs from wild type and has significantly bigger shoots. Kruskal-Wallis test with Dunn's multiple comparisons test, n = 6-16.

Appendix D

Table D.1. Overrepresentation of biological processes of common root exudate proteins. Processes related to interactions with other organisms are highlighted in blue.

GO biological process complete	Fold Enrichment	p value	FDR
L-arabinose metabolic process	65.65	3.06E-04	1.75E-02
mannose metabolic process	59.09	1.02E-05	7.97E-04
tryptophan catabolic process to kynurenine	59.09	1.02E-05	7.86E-04
kynurenine metabolic process	59.09	1.02E-05	7.75E-04
guard cell morphogenesis	49.24	6.08E-04	3.18E-02
tryptophan catabolic process	42.2	3.52E-05	2.41E-03
regulation of peptidase activity	39.39	1.01E-03	5.03E-02
intracellular phosphate ion homeostasis	39.39	1.01E-03	4.98E-02
guard cell development	39.39	1.01E-03	4.94E-02
arabinan catabolic process	36.93	5.59E-05	3.64E-03
arabinan metabolic process	32.83	8.32E-05	5.12E-03
indole-containing compound catabolic process	32.83	4.83E-06	4.06E-04
xylan catabolic process	30.77	4.15E-07	4.34E-05
hemicellulose catabolic process	30.77	4.15E-07	4.26E-05
cell wall macromolecule catabolic process	29.84	7.48E-13	2.76E-10
cell wall polysaccharide catabolic process	28.96	5.83E-07	5.88E-05
S-glycoside catabolic process	23.17	1.48E-09	2.56E-07
glucosinolate catabolic process	23.17	1.48E-09	2.48E-07
systemic acquired resistance	17.64	3.46E-12	1.20E-09
response to chitin	17.13	7.91E-05	4.93E-03
sulfur compound catabolic process	16.76	2.28E-08	3.01E-06
glycosyl compound catabolic process	16.72	3.03E-09	4.66E-07
cellular response to cold	14.07	2.64E-05	1.85E-03

response to endoplasmic reticulum stress	12.31	4.96E-11	1.31E-08
pectin catabolic process	10.05	6.33E-08	7.63E-06
xyloglucan metabolic process	9.69	3.61E-05	2.44E-03
polysaccharide catabolic process	9.65	5.48E-11	1.38E-08
response to insect	9.16	9.41E-04	4.78E-02
response to organonitrogen compound	9.03	4.24E-08	5.47E-06
cell wall macromolecule metabolic process	8.86	1.12E-11	3.66E-09
hemicellulose metabolic process	8.56	2.90E-07	3.09E-05
ERAD pathway	8.35	3.33E-04	1.87E-02
xylan metabolic process	8.07	3.90E-04	2.12E-02
lipid transport	8.02	1.43E-07	1.59E-05
plant-type secondary cell wall biogenesis	7.69	4.87E-04	2.60E-02
cell wall polysaccharide metabolic process	7.29	1.10E-07	1.28E-05
carbohydrate catabolic process	6.95	5.14E-10	1.02E-07
cell wall organization or biogenesis	6.93	5.80E-21	1.07E-17
S-glycoside metabolic process	6.91	2.22E-05	1.60E-03
glucosinolate metabolic process	6.91	2.22E-05	1.58E-03
carbohydrate derivative catabolic process	6.84	2.31E-06	2.00E-04
cell wall modification	6.71	2.72E-07	2.96E-05
defense response to fungus	6.71	8.80E-11	2.03E-08
lipid localization	6.61	1.01E-06	9.84E-05
carbohydrate metabolic process	6.58	2.04E-33	1.13E-29
response to fungus	6.43	2.17E-13	8.60E-11
protein folding	6.25	5.86E-07	5.80E-05
monosaccharide metabolic process	6.11	5.40E-05	3.56E-03
response to nitrogen compound	6.02	2.52E-06	2.15E-04
defense response to symbiont	5.86	1.91E-08	2.58E-06
pectin metabolic process	5.83	9.60E-06	7.72E-04
glycosyl compound metabolic process	5.79	2.81E-05	1.95E-03

galacturonan metabolic process	5.79	1.01E-05	8.01E-04
cell wall organization	5.64	1.21E-08	1.71E-06
external encapsulating structure organization	5.2	6.19E-09	9.27E-07
response to oxidative stress	5.19	5.89E-11	1.42E-08
polysaccharide metabolic process	5.09	2.24E-10	4.77E-08
cell wall biogenesis	5.05	5.44E-06	4.50E-04
plant-type cell wall organization or biogenesis	4.75	1.83E-06	1.61E-04
defense response to other organism	4.58	4.23E-16	2.35E-13
protein maturation	4.48	8.21E-06	6.69E-04
immune system process	4.48	9.11E-05	5.55E-03
response to ethylene	4.4	5.12E-04	2.70E-02
macromolecule catabolic process	4.37	4.63E-15	2.14E-12
response to external biotic stimulus	4.07	1.98E-17	1.83E-14
response to other organism	4.07	1.98E-17	1.57E-14
response to biotic stimulus	4.06	2.19E-17	1.52E-14
biological process involved in interspecies interaction between organisms	4.03	3.17E-17	1.95E-14
defense response	3.82	4.20E-14	1.79E-11
catabolic process	3.74	3.67E-18	4.07E-15
response to bacterium	3.67	1.37E-06	1.27E-04
response to external stimulus	3.54	2.80E-15	1.41E-12
defense response to bacterium	3.54	5.12E-05	3.42E-03
organonitrogen compound catabolic process	3.3	1.16E-08	1.69E-06
response to salt stress	3.04	1.50E-04	8.92E-03
cellular catabolic process	3.03	4.27E-04	2.30E-02
response to stress	2.87	3.38E-24	9.36E-21
protein catabolic process	2.74	1.86E-04	1.10E-02
proteolysis involved in protein catabolic process	2.68	3.73E-04	2.07E-02
cellular response to stress	2.57	1.61E-06	1.46E-04
response to osmotic stress	2.53	9.96E-04	5.02E-02

proteolysis	2.48	2.88E-04	1.66E-02
response to stimulus	2.13	1.40E-19	1.94E-16
response to chemical	2.11	4.39E-08	5.53E-06
response to oxygen-containing compound	2.06	7.83E-05	4.93E-03

Table D.2. The list of proteins common to *A. thaliana* root exudates.

	Protein	Gene	Description
Lectin	Q9LK72	At3g16530	Lectin-like protein
	Q9LJR2	LEC	Lectin-like protein
	Q9LNN3	At1g53080	Lectin-like protein
	Q9ZVA2	At1g78830	EP1-like glycoprotein 2
	Q9ZVA4	At1g78850	EP1-like glycoprotein 3
	Q9ZVA5	At1g78860	EP1-like glycoprotein 4
	F4IB94	JAL8	Jacalin-related lectin 8
	F4IB95	JAL9	Jacalin-related lectin 9
	Q8GWI7	JAL10	Jacalin-related lectin 10
LysM	O23006	LYM2	LysM domain-containing GPI-anchored protein 2
	A8R7E6	CERK1	Chitin elicitor receptor kinase 1
	Q93ZH0	LYM1	LysM domain-containing GPI-anchored protein 1
	Q6NPN4	LYM3	LysM domain-containing GPI-anchored protein 3
LRR-RLK	Q9ZU46	ZAR1	Receptor protein kinase-like protein
	Q9SCZ4	FER	Receptor-like protein kinase FERONIA
	Q9LK43	TMK4	Receptor-like kinase
	Q9LUL4	SRF7	Leucine-rich repeat receptor kinase-like protein SRF7
	Q9SIT1	TMK3	Leucine-rich repeat receptor-like kinases TMK3
	Q9C8I6	IOS1	LRR receptor-like serine/threonine-protein kinase
	Q8GZ99	HPCA1	Leucine-rich repeat receptor protein kinase
	Q9C8M9	SRF6	Leucine-rich repeat receptor kinase-like protein
	Q9M8T0	At3g02880	Probable inactive receptor kinase
	C0LGG6	At1g51890	Probable LRR receptor-like protein kinase
	Q9FN93	At5g59680	Probable LRR receptor-like serine/threonine-protein kinase
CRK	Q8L7G3	CRK7	Cysteine-rich receptor-like protein kinase 7
	O65468	CRK8	Cysteine-rich receptor-like protein kinase 8
	Q8H199	CRK14	Cysteine-rich receptor-like protein kinase 14
	O65405	CRK28	Cysteine-rich receptor-like protein kinase 28
	Q8S9L6	CRK29	Cysteine-rich receptor-like protein kinase 29
	O65469	CRK9	Putative cysteine-rich receptor-like protein kinase 9

Other receptor	Q84VZ5	At5g19240	Uncharacterized GPI-anchored protein
	Q8GUL8	At5g19230	Uncharacterized GPI-anchored protein
	P59833	At5g19250	Uncharacterized GPI-anchored protein
	Q8GUI4	At1g61900	Uncharacterized GPI-anchored protein
Subtilisin	Q9LVJ1	SBT1.4	Subtilisin-like protease SBT1.4
	O65351	SBT1.7	Subtilisin-like protease SBT1.7
	Q9MAP5	SBT3.3	Subtilisin-like protease SBT3.3
	Q9MAP7	SBT3.5	Subtilisin-like protease SBT3.5
	Q9LNU1	CRSP	CO(2)-response secreted protease
Chitinase	O24658	At2g43590	Endochitinase
	O22841	At2g43620	Endochitinase
	O24603	CHI	Endochitinase
	Q9M2U5	EP3	Endochitinase
	P19171	CHI-B	Basic endochitinase B
	O81862	ChiC	Class V chitinase
Biotic stress	P33154	At2g14610	Pathogenesis-related protein 1
	P28493	At1g75040	Pathogenesis-related protein 5
	Q9FZ31	PDF1.5	Defensin-like protein 18
	Q42328	ATTI1	Defensin-like protein 195
	P92995	GLP1	Germin-like protein subfamily T member 1
	Q9FMA8	At5g38940	Germin-like protein subfamily 1 member 11
	P94014	GLP4	Germin-like protein subfamily 2 member 1
	Q9LMC9	At1g18980	Germin-like protein subfamily T member 2
	P94072	GER3	Germin-like protein subfamily 3 member 3
Abiotic stress	Q9STX5	HSP90-7	Endoplasmin homolog
	Q39043	BIP2	Heat shock 70 kDa protein
	Q9LKR3	BIP1	Heat shock 70 kDa protein
	P50700	OSM34	Osmotin-like protein
Phosphatase	Q9SCX8	PAP17	Purple acid phosphatase 17
	Q9LMX4	PAP1	Probable inactive purple acid phosphatase 1
	Q9LMG7	PAP2	Probable inactive purple acid phosphatase 2
	Q38924	PAP12	Fe(3+)-Zn(2+) purple acid phosphatase 12
Alkaloid	Q9FZC4	FOX1	Berberine bridge enzyme-like 3
	Q9SA85	At1g30700	Berberine bridge enzyme-like 8
	Q9SA87	At1g30720	Berberine bridge enzyme-like 10
	Q9SVG4	At4g20830	Berberine bridge enzyme-like 19
	Q9SVG3	At4g20840	Berberine bridge enzyme-like 21

	Q9SUC6	FAD-OXR	Berberine bridge enzyme-like 22
	P94111	SSL12	Protein STRICTOSIDINE SYNTHASE-LIKE 12
Peroxidase	Q9SY33	PER7	Peroxidase 7
	Q9FX85	PER10	Peroxidase 10
	Q9SI17	PER14	Peroxidase 14
	Q9SI16	PER15	Peroxidase 15
	Q42580	PER21	Peroxidase 21
	P24102	PER22	Peroxidase 22
	O80912	PER23	Peroxidase 23
	Q9SS67	PER28	Peroxidase 28
	Q9LSP0	PER29	Peroxidase 29
	Q9LHB9	PER32	Peroxidase 32
	Q9SMU8	PER34	Peroxidase 34
	Q9LDN9	PER37	Peroxidase 37
	Q9SZE7	PER51	Peroxidase 51
	Q42578	PER53	Peroxidase 53
	Q39034	PER59	Peroxidase 59
	Q9FMR0	PER60	Peroxidase 60
	Q9FMI7	PER70	Peroxidase 70
	Q43387	PER71	Peroxidase 71
	Q9SUT2	PER39	Peroxidase 39
Metabolism	O80497	At2g44570	Endoglucanase 15
	Q93YQ7	At4g39010	Endoglucanase 24
	Q9M1D0	BGLU16	Beta-glucosidase 16
	Q9C525	BGLU21	Beta-glucosidase 21
	Q9C8Y9	BGLU22	Beta-glucosidase 22
	Q9SR37	BGLU23	Beta-glucosidase 23
	O65399	At1g11820	Glucan endo-1,3-beta-glucosidase 1
	Q9C7U5	At1g66250	Glucan endo-1,3-beta-glucosidase 2
	Q9ZU91	At2g01630	Glucan endo-1,3-beta-glucosidase 3
	Q94CD8	At3g13560	Glucan endo-1,3-beta-glucosidase 4
	Q9M088	At4g31140	Glucan endo-1,3-beta-glucosidase 5
	Q93Z08	At5g58090	Glucan endo-1,3-beta-glucosidase 6
	Q9M069	At4g34480	Glucan endo-1,3-beta-glucosidase 7
	Q6NKW9	At1g64760	Glucan endo-1,3-beta-glucosidase 8
	Q9FHX5	At5g42100	Glucan endo-1,3-beta-glucosidase 10
	Q8VYE5	At4g29360	Glucan endo-1,3-beta-glucosidase 12
	P33157	BG2	Glucan endo-1,3-beta-glucosidase, acidic isoform
	Q8VZJ2	At4g16260	Probable glucan endo-1,3-beta-glucosidase
	F4J270	BG3	Probable glucan endo-1,3-beta-glucosidase
	O82772	BGLU25	Probable inactive beta-glucosidase 25
	P37702	TGG1	Myrosinase 1
	Q8GRX1	TGG4	Myrosinase 4

Q3ECS3	TGG5	Myrosinase 5
Q3E8E5	TGG3	Putative myrosinase 3
Q9FT97	AGAL1	Alpha-galactosidase 1
Q8VXZ7	AGAL3	Alpha-galactosidase 3
Q9SCV9	BGAL3	Beta-galactosidase 3
Q9SCV4	BGAL8	Beta-galactosidase 8
Q9C6W4	BGAL15	Beta-galactosidase 15
Q8GX69	BGAL16	Beta-galactosidase 16
Q9M5J8	PGIP2	Polygalacturonase inhibitor 2
Q9SZW7	At4g30140	GDSL esterase/lipase
Q9FXJ1	At1g28570	GDSL esterase/lipase
Q9FXJ2	At1g28580	GDSL esterase/lipase
Q9LJP2	At3g14220	GDSL esterase/lipase
Q9FPE4	At1g28660	GDSL esterase/lipase
Q9LJG3	ESM1	GDSL esterase/lipase
Q1H583	GLL22	GDSL esterase/lipase 22
Q9C7N5	At1g29660	GDSL esterase/lipase
Q8W4H8	GLL23	Inactive GDSL esterase/lipase-like protein 23
Q42589	LTP1	Non-specific lipid-transfer protein 1
Q9S7I3	LTP2	Non-specific lipid-transfer protein 2
Q9LDB4	LTP6	Non-specific lipid-transfer protein 6
Q9ZPW9	LTP8	Non-specific lipid-transfer protein 8
Q9LZV9	LTP10	Non-specific lipid-transfer protein 10
Q9C7F7	LTPG1	Non-specific lipid transfer protein GPI-anchored 1
Q9LJ86	LTPG5	Non-specific lipid transfer protein GPI-anchored 5
F4I082	LTPG6	Non-specific lipid transfer protein GPI-anchored 6
Q9ZQI8	LTPG11	Non-specific lipid transfer protein GPI-anchored 11
O64864	LTPG13	Non-specific lipid transfer protein GPI-anchored 13
Q1G3I0	LTPG28	Non-specific lipid transfer protein GPI-anchored 28
Q8VYI9	LTPG31	Non-specific lipid transfer protein GPI-anchored 31
Q9FJ62	GDPDL4	Glycerophosphodiester phosphodiesterase
Q9C907	GDPD5	Glycerophosphodiester phosphodiesterase
Q7Y208	GDPDL1	Glycerophosphodiester phosphodiesterase
Q9SZ11	GDPDL3	Glycerophosphodiester phosphodiesterase
O80475	MES8	Methylesterase 8
Q9SU35	AZI1	pEARLI1-like lipid transfer protein 1
A7WM73	HEXO1	Beta-hexosaminidase 1
F4JAU3	P4H2	Prolyl 4-hydroxylase 2
P94078	At3g26720	Alpha-mannosidase
Q8LPJ3	At5g13980	Probable alpha-mannosidase
Q9FKW9	At5g66150	Probable alpha-mannosidase
Q9ZV85	GGH3	Probable gamma-glutamyl hydrolase 3
P57681	FCLY	Farnesylcysteine lyase
Q9FUJ2	CKX4	Cytokinin dehydrogenase 4
Q304B9	NCER2	Neutral ceramidase 2

	Q9M5J9	PGIP1	Polygalacturonase inhibitor 1
	Q8L7S6	HEXO3	Beta-hexosaminidase 3
	Q8VXX5	SKS1	Monocopper oxidase-like protein
	Q9SU40	SKU5	Monocopper oxidase-like protein
	Q39119	At4g32940	Vacuolar-processing enzyme gamma-isozyme
	Q8VZ56	AMY1	Alpha-amylase 1
	Q9SG80	ASD1	Alpha-L-arabinofuranosidase 1
	Q8VZR2	ASD2	Alpha-L-arabinofuranosidase 2
	Q8GW72	FUC1	Alpha-L-fucosidase 1
	O65355	GGH2	Gamma-glutamyl hydrolase 2
	Q67XZ3	CWINV3	Beta-fructofuranosidase, insoluble isoenzyme
	O81030	GULLO5	L-gulonolactone oxidase 5
Cell wall	P24806	XTH24	Xyloglucan endotransglucosylase/hydrolase protein 24
	Q9ZSU4	XTH14	Xyloglucan endotransglucosylase/hydrolase protein 14
	Q9SMP1	XTH11	Probable xyloglucan endotransglucosylase/hydrolase protein 11
	Q9FKL9	XTH12	Probable xyloglucan endotransglucosylase/hydrolase protein 12
	Q38910	XTH23	Probable xyloglucan endotransglucosylase/hydrolase protein 23
	Q9S7Y7	XYL1	Alpha-xylosidase 1
	Q9FGY1	BXL1	Beta-D-xylosidase 1
	Q9FLG1	BXL4	Beta-D-xylosidase 4
	Q9LXA8	BXL6	Probable beta-D-xylosidase 6
	Q8L7W8	FUC95A	Alpha-L-fucosidase 2
	Q9SB37	PMEI7	Pectinesterase inhibitor 7
	Q9SI74	PMEI10	Pectinesterase inhibitor 10
	Q940J8	PAE7	Pectin acetylesterase 7
	Q9FH82	PAE11	Pectin acetylesterase 11
	Q9C5M8	At4g24780	Probable pectate lyase 18
	Q9SRX4	PME7	Probable pectinesterase/pectinesterase inhibitor 7
	O48711	PME12	Probable pectinesterase/pectinesterase inhibitor 12
	O22149	PME17	Probable pectinesterase/pectinesterase inhibitor 17
	O22256	PME20	Probable pectinesterase/pectinesterase inhibitor 20
	Q94CB1	PME25	Probable pectinesterase/pectinesterase inhibitor 25
	Q9LXK7	PME32	Probable pectinesterase/pectinesterase inhibitor 32
	Q8RXK7	PME41	Probable pectinesterase/pectinesterase inhibitor 41
	Q9SMY7	PME44	Probable pectinesterase/pectinesterase inhibitor 44
	Q9LXD9	PME51	Probable pectinesterase/pectinesterase inhibitor 51
	Q93ZA3	At1g30760	Monolignol oxidoreductase 13
	O64743	MEE23	Monolignol oxidoreductase 23
	Q93V72	PDCB4	PLASMODESMATA CALLOSE-BINDING PROTEIN 4
	Q9FM65	FLA1	Fasciclin-like arabinogalactan protein 1
	Q9SU13	FLA2	Fasciclin-like arabinogalactan protein 2
	Q9SNC3	FLA4	Fasciclin-like arabinogalactan protein 4
	Q9SIL7	FLA6	Fasciclin-like arabinogalactan protein 6
	Q9SJ81	FLA7	Fasciclin-like arabinogalactan protein 7

	O22126	FLA8	Fasciclin-like arabinogalactan protein 8
	Q9ZWA8	FLA9	Fasciclin-like arabinogalactan protein 9
	Q9LZX4	FLA10	Fasciclin-like arabinogalactan protein 10
	Q8LEJ6	FLA11	Fasciclin-like arabinogalactan protein 11
	Q9FFH6	FLA13	Fasciclin-like arabinogalactan protein 13
	Q66GR0	FLA17	Fasciclin-like arabinogalactan protein 17
	Q93W32	FLA18	Fasciclin-like arabinogalactan protein 18
	Q9FN39	ENODL1	Early nodulin-like protein 1
	Q9T076	ENODL2	Early nodulin-like protein 2
	Q6NLD7	ENODL8	Early nodulin-like protein 8
	Q9SK27	ENODL14	Early nodulin-like protein 14
	Q9SZ51	ENODL15	Early nodulin-like protein 15
	Q9STZ8	ENODL19	Early nodulin-like protein 19
Cell cycle	O22824	FH2	Formin-like protein 2
	O23373	FH3	Formin-like protein 3
	Q93V74	CYCLASE1	Cyclase-like protein 1
	Q94JT5	CYCLASE2	Cyclase-like protein 2
	Q94LA9	CYCLASE3	Cyclase-like protein 3
	Q00874	DRT100	DNA damage-repair/toleration protein
	P42813	RNS1	Ribonuclease 1
	P42815	RNS3	Ribonuclease 3
Proteins processing	P29402	CNX1	Calnexin homolog 1
	O04151	CRT1	Calreticulin-1
	Q38858	CRT2	Calreticulin-2
	Q9FZP1	At5g34940	Heparanase-like protein 3
	Q9LS40	ASPG1	Protein ASPARTIC PROTEASE IN GUARD CELL 1
	Q9LEW3	AED1	Aspartyl protease
	O04496	AED3	Aspartyl protease
	Q8S9J6	At5g10770	Aspartyl protease family protein
	Q8VYV9	APF1	Aspartyl protease family protein 1
	Q9LM66	XCP2	Cysteine protease
	Q9LT78	RD21C	Probable cysteine protease
	Q9LT77	RDL2	Probable cysteine protease
	Q9FMH8	RD21B	Probable cysteine protease
	Q93VC9	CATHB2	Cathepsin B-like protease 2
	Q94K85	CATHB3	Cathepsin B-like protease 3
	Q9LMU2	KTI5	Kunitz trypsin inhibitor 5
	Q9M8Y9	KTI7	Kunitz trypsin inhibitor 7
	Q8RXD5	KTI4	Kunitz trypsin inhibitor 4
	Q0WNE5	KTI6	Kunitz trypsin inhibitor 6
	Q8H166	ALEU	Thiol protease aleurain
	Q8VYL3	APA2	Aspartic proteinase A2

	O65390	APA1	Aspartic proteinase A1
	P43297	RD21A	Cysteine proteinase 21
	Q84WT8	CYS4	Cysteine proteinase inhibitor 4
	Q41916	CYS5	Cysteine proteinase inhibitor 5
	O04529	2MMP	Metalloendoproteinase
	Q8L7B2	SCPL20	Serine carboxypeptidase-like 20
	Q9LSV8	SCPL21	Serine carboxypeptidase-like 21
	Q9ZQQ0	SCPL26	Serine carboxypeptidase-like 26
	Q949Q7	SCPL29	Serine carboxypeptidase-like 29
	Q56WF8	SCPL48	Serine carboxypeptidase-like 48
	Q9M9Q6	SCPL50	Serine carboxypeptidase-like 50
	Q9STL4	CEP2	KDEL-tailed cysteine endopeptidase
	Q9XI01	PDIL1-1	Protein disulfide isomerase-like 1-1
	Q9SRG3	PDIL1-2	Protein disulfide isomerase-like 1-2
	Q8VX13	PDIL1-3	Protein disulfide isomerase-like 1-3
	Q9FF55	PDIL1-4	Protein disulfide isomerase-like 1-4
	Q66GQ3	PDIL1-6	Protein disulfide isomerase-like 1-6
	O22263	PDIL2-1	Protein disulfide-isomerase like 2-1
	O48773	PDIL2-3	Protein disulfide-isomerase 2-3
Other	Q94BT2	AIR12	Auxin-induced in root cultures protein 12
	Q9LZR8	At5g03700	PAN domain-containing protein
	Q9SIH2	DOT1	Glycine-rich protein
	Q9SL15	GRP3	Glycine-rich protein 3
	Q9LSB4	NAI2	TSA1-like protein
	Q9ZVA1	At1g78820	EP1-like glycoprotein 1
	Q9LRJ9	CRRSP38	Cysteine-rich repeat secretory protein 38
	Q9LV60	CRRSP55	Cysteine-rich repeat secretory protein 55
	B9DFI7	At1g26850	Probable methyltransferase
	Q94F20	DGR2	Protein DUF642 L-GALACTONO-1,4-LACTONE-RESPONSIVE GENE 2
	O81417	SRPP	Protein SEED AND ROOT HAIR PROTECTIVE PROTEIN
	P43082	HEL	Hevein-like preproprotein
	Q07488	BCB	Blue copper protein (Blue copper-binding protein)
	O80517	At2g44790	Uclacyanin-2 (Blue copper-binding protein II)
	Q9FIG6	DIR1	Dirigent protein 1
	O22960	TBL37	Protein trichome birefringence-like 37
	Q8VY22	TBL38	Protein trichome birefringence-like 38
	O65660	PLAT1	PLAT domain-containing protein 1
	Q9SIE7	PLAT2	PLAT domain-containing protein 2
	Q93XX5	At5g67130	PI-PLC X domain-containing protein
	Q9FF98	ML3	MD-2-related lipid-recognition protein 3
	Q9SSG3	HIPL1	HIPL1 protein

Table D.3. Overrepresentation of biological processes of root exudate proteins specific to high nutrients condition. Processes related to interactions with other organisms are highlighted in blue.

GO biological process complete	Fold Enrichment	p value	FDR
L-ascorbic acid metabolic process	26.02	2.04E-04	3.14E-02
lignin biosynthetic process	25.38	1.55E-06	7.15E-04
lignin metabolic process	23.13	2.34E-07	2.60E-04
xyloglucan metabolic process	20.47	4.90E-07	3.40E-04
phenylpropanoid metabolic process	13.01	1.20E-06	6.04E-04
phenylpropanoid biosynthetic process	11.83	6.75E-05	1.34E-02
hemicellulose metabolic process	10.86	2.01E-05	5.07E-03
pectin catabolic process	10.62	1.13E-04	2.08E-02
secondary metabolic process	10.3	5.29E-08	7.33E-05
cell wall macromolecule metabolic process	9.91	3.49E-07	2.77E-04
secondary metabolite biosynthetic process	9.55	1.86E-04	2.94E-02
cell wall polysaccharide metabolic process	8.99	1.38E-05	4.03E-03
glucan metabolic process	7.67	1.06E-05	3.26E-03
pectin metabolic process	7.39	1.71E-04	2.96E-02
galacturonan metabolic process	7.35	1.77E-04	2.88E-02
protein folding	6.61	3.13E-04	4.13E-02
cell wall organization or biogenesis	6.55	1.02E-09	2.81E-06
polysaccharide metabolic process	6.55	3.36E-08	6.21E-05
cell wall organization	6.31	1.42E-05	3.93E-03
carbohydrate catabolic process	6.05	1.70E-04	3.05E-02
response to oxidative stress	5.95	3.21E-07	2.96E-04
carbohydrate metabolic process	5.52	3.21E-12	1.78E-08
defense response to symbiont	5.42	3.32E-04	4.28E-02
external encapsulating structure organization	5.2	6.35E-05	1.30E-02
response to external biotic stimulus	2.75	2.32E-04	3.48E-02
response to other organism	2.75	2.32E-04	3.39E-02
response to biotic stimulus	2.75	2.39E-04	3.39E-02
involved in interspecies interaction between organisms	2.72	2.64E-04	3.66E-02
catabolic process	2.5	3.07E-04	4.15E-02
response to stress	2.29	9.36E-07	5.19E-04

Table D.4. The list of high nutrients specific proteins in *A. thaliana* root exudates.

	Protein	Gene	Description
Lectin	Q9LSR8	LRK19	L-type lectin-domain containing receptor kinase I.9
	O64782	SD129	G-type lectin S-receptor-like serine/threonine-protein kinase
LRR-RLK	Q9FPJ5	LRR1	Leucine-rich repeat protein 1
	Q6NQP4	LRR2	Leucine-rich repeat protein 2
	Q9S7I6	RPK2	LRR receptor-like serine/threonine-protein kinase
	C0LGE4	Y1124	Probable LRR receptor-like serine/threonine-protein kinase
	C0LGN2	Y3148	Probable LRR receptor-like serine/threonine-protein kinase
	C0LGG3	Y5182	Probable LRR receptor-like serine/threonine-protein kinase
	Q9FZB1	Y5188	Probable LRR receptor-like serine/threonine-protein kinase
	Q9SI06	Y5573	Putative LRR receptor-like serine/threonine-protein kinase
	Q9FN94	RLK0	Receptor-like protein kinase
	Q70CT4	RLP8	Receptor-like protein 8
	O22187	Y2232	Probable receptor-like protein kinase
	F4HQ22	LRL24	Probable receptor-like serine/threonine-protein kinase
	Q9SLI6	RLP8X	Putative receptor-like protein 8
CRK	Q0PW40	CRK13	Cysteine-rich receptor-like protein kinase 13
	Q8LPI0	CRK	Putative cysteine-rich receptor-like protein kinase
Other receptor	Q9FKT1	LLG1	LORELEI-like-GPI-anchored protein 1
Subtilisin	Q9LUM3	SBT15	Subtilisin-like protease SBT1.5
Chitinase	O22842	CHI61	Endochitinase
	O24598	CHI58	Endochitinase
Biotic stress	O80995	DEF14	Defensin-like protein 14
	Q9FI23	DEF16	Defensin-like protein 16
	Q9FI22	DEF17	Defensin-like protein 17
	Q8RYE7	DF196	Defensin-like protein 196
	Q94JR6	DF206	Defensin-like protein 206
	Q3E8R5	DF207	Defensin-like protein 207
	O80994	DEF15	Putative defensin-like protein 15
	Q9LEA7	GL18	Germin-like protein subfamily 1 member 8
	Q9LUZ6	TBL44	Powdery mildew resistance protein 5
Abiotic stress	F4JMJ1	HSP7R	Heat shock 70 kDa protein 17
Phosphatase	Q8H129	PPA3	Purple acid phosphatase 3
	Q8VYZ2	PPA8	Purple acid phosphatase 8

	Q9SIV9	PPA10	Purple acid phosphatase 10
	Q9LJU7	PPA18	Purple acid phosphatase 18
	Q9LXI7	PPA20	Probable purple acid phosphatase 20
	Q9LXI4	PPA21	Purple acid phosphatase 21
	O23244	PPA25	Purple acid phosphatase 25
	Q949Y3	PPA26	Bifunctional purple acid phosphatase 26
Alkaloid	Q9FZC5	FOX2	Berberine bridge enzyme-like 4
	Q9SA88	BBE11	Berberine bridge enzyme-like 11
	Q9FKV2	BBE23	Berberine bridge enzyme-like 23
Peroxidase	Q9SJZ2	PER17	Peroxidase 17
	Q9LDA4	PER38	Peroxidase 38
	Q43731	PER50	Peroxidase 50
	Q9FLC0	PER52	Peroxidase 52
	Q96509	PER55	Peroxidase 55
	Q9FKA4	PER62	Peroxidase 62
	Q96511	PER69	Peroxidase 69
	Q9FJZ9	PER72	Peroxidase 72
Cell wall	O80731	PAE3	Pectin acetylesterase 3
	Q9SR23	PAE4	Pectin acetylesterase 4
	Q9SR22	PAE5	Pectin acetylesterase 5
	B9DFR3	PAE9	Pectin acetylesterase 9
	Q9SFF6	PAE12	Pectin acetylesterase 12
	Q940Q1	PLY1	Probable pectate lyase 1
	Q9FXD8	PLY5	Probable pectate lyase 5
	Q9LJ42	PLY10	Probable pectate lyase 10
	Q93Z25	PLY22	Probable pectate lyase 22
	O81415	PME39	Probable pectinesterase/pectinesterase inhibitor 39
	Q9LZT4	EXLA1	Expansin-like A1
	Q9SVE5	EXLA2	Expansin-like A2
	Q9LZT5	EXLA3	Expansin-like A3
	F4HWQ8	CVIF1	Cell wall / vacuolar inhibitor of fructosidase 1
	Q9FNQ2	PDCB1	PLASMODESMATA CALLOSE-BINDING PROTEIN 1
	Q9SD84	PDCB2	PLASMODESMATA CALLOSE-BINDING PROTEIN 2
	Q9FKK7	XYLA	Xylose isomerase
	Q9M0D1	XTH19	Xyloglucan endotransglucosylase/hydrolase protein 19
	Q9FI31	XTH20	Xyloglucan endotransglucosylase/hydrolase protein 20
	Q38857	XTH22	Xyloglucan endotransglucosylase/hydrolase protein 22
	Q8LER3	XTH7	Probable xyloglucan endotransglucosylase/hydrolase protein 7
	O80803	XTH17	Probable xyloglucan endotransglucosylase/hydrolase protein 17
	Q9M0D2	XTH18	Probable xyloglucan endotransglucosylase/hydrolase protein 18
	Q39131	ENL17	Early nodulin-like protein 17
	Q8L868	E1311	Glucan endo-1,3-beta-glucosidase 11

	Q9ZQG9	E1314	Glucan endo-1,3-beta-glucosidase 14
	Q9FN05	PSL5	Probable glucan 1,3-alpha-glucosidase
Metabolism	Q9SCW1	BGAL1	Beta-galactosidase 1
	Q9SCV0	BGA12	Beta-galactosidase 12
	Q67XN2	BGL08	Beta-glucosidase 8
	Q9FZE0	BGL40	Beta-glucosidase 40
	O80690	BGL46	Beta-glucosidase 46
	O80689	BGL45	Beta-glucosidase 45
	O81032	GGLO6	Probable L-gulonolactone oxidase 6
	Q8H1H9	CAOG1	Amine oxidase
	Q6NQ66	GGLO2	L-gulonolactone oxidase 2
	Q8LPL3	ASO	L-ascorbate oxidase
	Q9FNA3	NAGLU	Alpha-N-acetylglucosaminidase
	Q8RXE1	GAUT5	Probable galacturonosyltransferase 5
	Q0WL80	UGGG	UDP-glucose:glycoprotein glucosyltransferase
	Q8VXY9	UAH	Ureidoglycolate hydrolase
	Q39176	ERLI1	Lipid transfer protein
	Q9LLR7	NLTP3	Non-specific lipid-transfer protein 3
	Q8L7Y9	NPC1	Non-specific phospholipase C1
	Q8RW93	LBP65	Putative lipid-binding protein
	Q93YW8	GDL65	GDSL esterase/lipase
	Q9C7N4	GDL15	GDSL esterase/lipase
	Q9MAA1	GDL49	GDSL esterase/lipase
	Q9SJB4	GDL34	GDSL esterase/lipase
	Q38894	GDL13	GDSL esterase/lipase
	Q9FIA1	GDL87	GDSL esterase/lipase
	Q9FMP3	DPYS	Dihydropyrimidinase
Cell cycle	Q38798	CALX2	Calnexin homolog 2
	Q9C9G4	ENDO2	Endonuclease 2
	P42814	RNS2	Ribonuclease 2
Protein processing	Q8H0X6	CYT6	Cysteine proteinase inhibitor 6
	Q9SQX6	SCP7	Serine carboxypeptidase-like 7
	Q8RUW5	SCP8	Serine carboxypeptidase-like 8
	Q2V465	SCP11	Serine carboxypeptidase-like 11
	O81009	SCP12	Serine carboxypeptidase-like 12
	A3KPF5	PDI15	Protein disulfide isomerase-like 1-5
	Q38935	FK151	Peptidyl-prolyl cis-trans isomerase
	Q8LDP4	CP19D	Peptidyl-prolyl cis-trans isomerase
	Q9SP02	CP20A	Peptidyl-prolyl cis-trans isomerase
	Q38936	FK152	Peptidyl-prolyl cis-trans isomerase
	Q9M0Z6	MSRB3	Peptide methionine sulfoxide reductase

	A0A1P8B760	AMI4G	Probable amidase
	O04373	ILL4	IAA-amino acid hydrolase
Other	Q9ZSJ6	GRP3S	Glycine-rich protein 3 short isoform
	O48848	GRP23	Glycine-rich protein 23
	Q9LRL2	CRR25	Putative cysteine-rich repeat secretory protein 25
	Q9LJX8	VPED	Vacuolar-processing enzyme delta-isozyme
	O64758	VSR5	Vacuolar-sorting receptor 5
	Q681K2	ATS3A	Embryo-specific protein
	Q9FKH6	B561P	Cytochrome b561 and DOMON domain-containing protein
	Q9LTM3	C71BK	Cytochrome P450 71B20
	Q9LTM4	C71BJ	Cytochrome P450 71B19
	Q9SZ28	STGL1	Stigma-specific STIG1-like protein 1
	Q9FLQ0	STGL2	Stigma-specific STIG1-like protein 2
	P46689	GASA1	Gibberellin-regulated protein 1
	Q9ZV89	TBL42	Protein trichome birefringence-like 42
	O48850	VA725	Vesicle-associated membrane protein 725
	Q8LG89	BABL	Basic blue protein
	Q9FVX1	GRXC3	Glutaredoxin-C3
	Q8LFQ6	GRXC4	Glutaredoxin-C4
	Q67YC9	Y4141	Uncharacterized protein
	Q9FZD0	CBX8D	Carbohydrate-binding X8 domain-containing protein

Table D.5. Overrepresentation of biological processes of root exudate proteins specific to low nutrients condition. None of the processes found was related to interactions with other organisms.

GO biological process complete	Fold Enrichment	p value	FDR
cell wall disassembly	> 100	5.29E-05	1.13E-02
cell wall modification involved in abscission	> 100	5.29E-05	1.09E-02
ceramide catabolic process	67.67	3.66E-04	4.83E-02
xylan catabolic process	44.41	3.95E-05	9.51E-03
hemicellulose catabolic process	44.41	3.95E-05	9.11E-03
cell wall polysaccharide catabolic process	41.8	4.78E-05	1.06E-02
cell wall macromolecule catabolic process	28.71	1.12E-05	3.66E-03
xylan metabolic process	19.41	6.06E-06	2.58E-03
hemicellulose metabolic process	18.54	1.50E-09	2.09E-06
xyloglucan metabolic process	15.53	1.31E-04	1.91E-02

cell wall polysaccharide metabolic process	14.62	1.83E-09	2.03E-06
cell wall macromolecule metabolic process	12.53	8.11E-09	7.49E-06
polysaccharide catabolic process	12.38	2.94E-07	2.04E-04
pectin catabolic process	12.08	6.11E-05	1.21E-02
cell wall modification	9.42	1.02E-05	3.53E-03
pectin metabolic process	8.41	8.41E-05	1.46E-02
plant-type cell wall organization	8.41	8.41E-05	1.41E-02
galacturonan metabolic process	8.36	8.69E-05	1.42E-02
cell wall organization or biogenesis	8.33	1.37E-12	7.58E-09
protein localization to membrane	8.22	3.71E-04	4.78E-02
polysaccharide metabolic process	7.98	6.78E-10	1.25E-06
cell wall organization	7.97	5.57E-07	3.09E-04
external encapsulating structure organization	7.9	4.33E-08	3.43E-05
carbohydrate catabolic process	7.86	8.78E-06	3.24E-03
plant-type cell wall organization or biogenesis	7.35	4.08E-06	2.06E-03
cell wall biogenesis	7.09	6.31E-05	1.21E-02
response to oxidative stress	6.25	5.35E-07	3.30E-04
carbohydrate metabolic process	5.53	6.21E-11	1.72E-07
catabolic process	3	1.66E-05	4.83E-03

Table D.6. The list of low nutrients specific proteins in *A. thaliana* root exudates.

	Protein	Gene	Description
LRR	O22476	BRI1	Protein BRASSINOSTEROID INSENSITIVE 1
	Q9LZV7	PXC2	Leucine-rich repeat receptor-like protein kinase
	C0LGF5	RGI5	LRR receptor-like serine/threonine-protein kinase
	Q9LFS4	NIK1	Protein NSP-INTERACTING KINASE 1
	C0LGW2	PAM74	Probable LRR receptor-like serine/threonine-protein kinase
	C0LGD7	Y1684	Probable LRR receptor-like serine/threonine-protein kinase
	C0LGU1	Y5374	Probable LRR receptor-like serine/threonine-protein kinase
	C0LGL4	Y2289	Probable LRR receptor-like serine/threonine-protein kinase
	P45434	SSRA	Signal sequence receptor subunit alpha
	O81905	SD18	Receptor-like serine/threonine-protein kinase
	Q94C77	RPKL	Receptor protein kinase-like protein
	P43298	TMK1	Receptor protein kinase
	Q9SNA3	Y3463	Putative receptor-like protein kinase

	Q9FMD7	Y5659	Probable inactive receptor kinase
	Q9LVI6	RLK902	Probable inactive receptor kinase
CRK	Q9CAL3	CRK2	Cysteine-rich receptor-like protein kinase 2
	Q9ZP16	CRK11	Cysteine-rich receptor-like protein kinase 11
	Q8L710	CRK17	Cysteine-rich receptor-like protein kinase 17
	O65472	CRK12	Putative cysteine-rich receptor-like protein kinase 12
Other receptor	O80977	VSR3	Vacuolar-sorting receptor 3
	Q56ZQ3	VSR4	Vacuolar-sorting receptor 4
	Q84MC0	UGPI4	Uncharacterized GPI-anchored protein
Subtilisin	Q8L7I2	SBT36	Subtilisin-like protease SBT3.6
Biotic stress	Q9FZ27	GL22	Germin-like protein subfamily 2 member 2
	Q9M263	GL24	Germin-like protein subfamily 2 member 4
	Q9SFF9	GL17	Germin-like protein subfamily 1 member 7
	P92997	GL113	Germin-like protein subfamily 1 member 13
	Q9FID0	GL114	Germin-like protein subfamily 1 member 14
	Q9FIC9	GL115	Germin-like protein subfamily 1 member 15
	Q9FIC6	GL117	Germin-like protein subfamily 1 member 17
	Q9FL89	GL119	Germin-like protein subfamily 1 member 19
	Q9FMB0	GL19	Putative germin-like protein subfamily 1 member 9
	Q9ZNS4	PRB1	Pathogenesis-related protein 1
	Q2V2Q8	DF208	Defensin-like protein 208
Phosphatase	Q9C510	PPA6	Purple acid phosphatase 6
	O48840	PPA13	Purple acid phosphatase 13
Alkaloid	Q9LPC3	BBE1	Berberine bridge enzyme-like 1
	Q9FKU9	BBE25	Berberine bridge enzyme-like 25
Peroxidase	P0DI10	PER1	Peroxidase 1
	Q67Z07	PER2	Peroxidase 2
	O23044	PER3	Peroxidase 3
	Q96520	PER12	Peroxidase 12
	O80822	PER25	Peroxidase 25
	Q93V93	PER44	Peroxidase 44
	Q9FG34	PER54	Peroxidase 54
	O22862	PER26	Probable peroxidase 26
	Q9FLV5	PER61	Probable peroxidase 61
Metabolism	Q8VYF9	SRGT1	Peptidyl serine alpha-galactosyltransferase
	Q93Z16	RPN2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2
	Q8RX86	AGAL2	Alpha-galactosidase 2

	Q9M2B9	CAOG2	Amine oxidase
	Q93ZI4	BGL10	Beta-glucosidase 10
	Q9FK75	GDL82	GDSL esterase/lipase
	Q3ECP6	GDL22	GDSL esterase/lipase
	Q8H965	NPC6	Non-specific phospholipase C6
	Q9XFS7	NLTP5	Non-specific lipid-transfer protein 5
	O64865	LTG14	Non-specific lipid transfer protein GPI-anchored 14
	Q9LJ85	LTG20	Non-specific lipid transfer protein GPI-anchored 20
	Q0WVZ1	Y3330	Putative metallophosphoesterase
Cell wall	Q9SRX3	GUN1	Endoglucanase 1
	O49296	GUN4	Endoglucanase 4
	Q9CAC1	GUN8	Endoglucanase 8
	O81416	GUN17	Endoglucanase 17
	Q9SZ90	GUN18	Endoglucanase 18
	Q9SUS0	GUN20	Endoglucanase 20
	Q9SVJ4	GUN22	Endoglucanase 22
	Q8GY58	GUN23	Endoglucanase 23
	Q8LDW9	XTH9	Xyloglucan endotransglucosylase/hydrolase protein 9
	P93046	XTH31	Xyloglucan endotransglucosylase/hydrolase protein 31
	Q8L9A9	XTH8	Probable xyloglucan endotransglucosylase/hydrolase protein 8
	Q9FKL8	XTH13	Putative xyloglucan endotransglucosylase/hydrolase protein 13
	F4J6T7	XYL2	Putative alpha-xylosidase 2
	Q9LXD6	BXL3	Beta-D-xylosidase 3
	Q9SGZ5	BXL7	Probable beta-D-xylosidase 7
	O23038	PME8	Probable pectinesterase 8
	O81301	PME40	Probable pectinesterase/pectinesterase inhibitor 40
	Q9FF77	PME47	Probable pectinesterase/pectinesterase inhibitor 47
	Q9FJU9	E1313	Glucan endo-1,3-beta-glucosidase 13
	Q949Z1	PGLR4	Polygalacturonase
	Q8RY29	ADPG2	Polygalacturonase
	O23147	ADPG1	Polygalacturonase
	Q43866	INV1	Beta-fructofuranosidase
	O49603	CVIF2	Cell wall / vacuolar inhibitor of fructosidase 2
	A0A1P8B8F8	XYN5	Endo-1,4-beta-xylanase 5
	O23547	EXLB1	Expansin-like B1
	F4HQM3	NCER1	Neutral ceramidase 1
	F4KHQ8	NCER3	Neutral ceramidase 3
	Q9C5C2	BGL37	Myrosinase 2
	Q9SCV3	BGAL9	Beta-galactosidase 9
	Q9T0F7	GXM2	Glucuronoxylan 4-O-methyltransferase 2
	Q9LQ32	GXM3	Glucuronoxylan 4-O-methyltransferase 3
	Q9LJU1	ENL09	Early nodulin-like protein 9
	Q8LC95	ENL13	Early nodulin-like protein 13
	Q9FT45	FLA15	Fasciclin-like arabinogalactan protein 15

	Q8RWC5	FLA16	Fasciclin-like arabinogalactan protein 16
	Q9C891	DIR20	Dirigent protein 20 (AtDIR20)
Protein processing	Q5XF51	3MMP	Metalloendoproteinase
	F4HVZ1	CATB1	Cathepsin B-like protease 1
	Q8RWQ9	ALEUL	Thiol protease aleurain-like
	O65493	XCP1	Cysteine protease
	O04084	SCP31	Serine carboxypeptidase-like 31
	Q9LEY1	SCP35	Serine carboxypeptidase-like 35
	Q67Y83	SCP51	Serine carboxypeptidase-like 51
Other	Q940S0	TMN2	Transmembrane 9 superfamily member 2
	Q9ZPS7	TMN3	Transmembrane 9 superfamily member 3
	F4KIB2	TMN8	Transmembrane 9 superfamily member 8
	Q9C5N2	TMN9	Transmembrane 9 superfamily member 9
	F4JRE0	TMN12	Transmembrane 9 superfamily member 12
	Q8RWM6	P24D5	Transmembrane emp24 domain-containing protein
	Q94F08	HIPL2	HIPL2 protein
	Q9FHD5	CRR57	Cysteine-rich repeat secretory protein 57
	Q9FHD4	CRR58	Cysteine-rich repeat secretory protein 58
	Q8GUJ2	PDLP5	Plasmodesmata-located protein 5
	Q6NPM5	ATS3B	Embryo-specific protein
	Q9FZA2	AGP31	Non-classical arabinogalactan protein 31
	O80844	PMTG	Probable methyltransferase
	Q0WRW8	B561K	Cytochrome b561 and DOMON domain-containing protein