Organic nitrogen – molecular regulation of uptake and physiological implications

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DOCTORAL THESIS
Umeå 2025

Acta Universitatis Agriculturae Sueciae
2025:65

Cover: Whole seedlings and root hair detail photo of *Arabidopsis thaliana* grown on either glutamine or nitrate as sole nitrogen source
(Photos: Laura Tünnermann)

ISSN 1652-6880
ISBN (print version) 978-91-8124-049-8
ISBN (electronic version) 978-91-8124-095-5
https://doi.org/10.54612/a.4rg1sqql2a

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Print: Original Tryckeri Umeå 2025

The more you know, the more you know, you don’t know .

Attributed to Aristotle

Organic nitrogen – molecular regulation of uptake and physiological implications

Abstract

Nitrogen (N) is an essential nutrient for plant growth and development. While research has traditionally focused on inorganic N forms, such as nitrate and ammonium, organic N forms, including amino acids, have also been detected in the soil. Notably, plants were shown to take up organic N forms and utilize them as an alternative N source. These findings highlight the significance of organic N nutrition. However, most current knowledge on N uptake mechanisms and N starvation responses is derived from studies on inorganic N. This has left substantial gaps in our understanding of how plants acquire and respond to organic N. The work presented in this thesis addresses some of these gaps by investigating the physiological effects of amino acid nutrition and the molecular regulation of amino acid uptake in Arabidopsis thaliana. The results reveal a distinct organic N-specific root phenotype and show that plants supplied solely with the amino acid glutamine (L-gln) are not N-starved. The second part of the thesis investigates the regulation of the amino acid uptake by focusing on the high-affinity transporter LHT1 (LYSINE HISTIDINE TRANSPORTER 1). Experiments identify the CALCIUM DEPENDENT KINASE1 (CPK1) as an interaction partner of LHT1, suggesting a post-translational regulation of the transporter. Interestingly, uptake assays show that CPK1 negatively influences the amino acid uptake. Further experiments confirmed a negative regulatory effect of CPK1 on LHT1 transport activity. To investigate the role of phosphorylation in regulating LHT1, phospho-mutants of the transporter were generated and analyzed. Two potential phosphorylation sites were identified, with Thr151 being identified as a potential CPK1 target site. This thesis enhances our understanding of how plants respond to organic N and provides deeper insights into amino acid uptake, with a particular focus on the LHT1 transport regulation.

Keywords: *Arabidopsis thaliana*, organic nitrogen, amino acids, glutamine, root phenotype, amino acid transporter, LHT1, post-translational modification, phosphorylation, CPK1

Organiskt kväve – molekylär reglering av upptag och fysiologiska konsekvenser

Sammanfattning

Kväve (N) är ett essentiellt näringsämne för växters tillväxt och utveckling. Forskning har traditionellt fokuserat på oorganiska N-former, såsom nitrat och ammonium, men organiska N-former, inklusive aminosyror, har också påträffats i jorden. Forskningsstudier har visat att växter kan ta upp organiska N-former och använda dem som en alternativ N-källa. Dessa resultat belyser betydelsen av organisk N-näring. Dock bygger den mesta nuvarande kunskapen om N-upptagsmekanismer och svar på N-begränsning på studier av oorganiskt N. Detta har lämnat betydande kunskapsluckor i vår förståelse av hur växter tar upp och reagerar på organiskt N. Det arbete som presenteras i denna avhandling behandlar några av dessa kunskapsluckor genom att undersöka de fysiologiska effekterna av aminosyrabaserad näring samt den molekylära regleringen av aminosyraupptag i Arabidopsis thaliana. Resultaten visar på en tydlig organiskt N-specifikt rotfenotyp och att växter som enbart försörjs med aminosyran glutamin (L-gln) inte är N-begränsade. Den andra delen av avhandlingen undersöker regleringen av aminosyraupptag med fokus på hög-affinitetstransportören LHT1 (LYSINE HISTIDINE TRANSPORTER1). Experiment identifierar CALCIUM DEPENDENT KINASE1 (CPK1) som en interaktionspartner till LHT1, vilket antyder en post-translationell reglering av transportören. Intressant nog visar upptagsförsök att CPK1 påverkar aminosyraupptaget negativt. Ytterligare experiment bekräftade en negativ regulatorisk effekt av CPK1 på LHT1:s transportaktivitet. För att undersöka fosforyleringens roll i regleringen av LHT1 genererades och analyserades fosfo-mutanter av transportören. Två potentiella fosforyleringsställen identifierades, där mina resultat pekade på att Thr151 kan vara en möjlig mål för interaktion för CPK1. Denna avhandling fördjupar vår förståelse av hur växter reagerar på organiskt N och ger nya insikter i aminosyraupptag, med särskilt fokus på regleringen av LHT1-transportören.

Nyckelord: *Arabidopsis thaliana*, organiskt kväve, aminosyror, glutamin, rotfenotyp, aminosyratransportör, LHT1, posttranslationell modifiering, fosforylering, CPK1

Organischer Stickstoff – molekulare Regulation der Aufnahme und physiologische Auswirkungen

Zusammenfassung

Stickstoff (N) ist ein essenzieller Nährstoff für das Wachstum und die Entwicklung von Pflanzen. Während sich die Forschung traditionell auf anorganische N-Formen wie Nitrat und Ammonium konzentriert hat, wurden auch organische N-Formen, darunter Aminosäuren, im Boden nachgewiesen. Es konnte gezeigt werden, dass Pflanzen organische N-Formen aufnehmen und diese als alternative N-Quelle nutzen. Diese Erkenntnisse unterstreichen die Bedeutung der organischen N-Versorgung. Dennoch basiert ein Großteil des derzeitigen Wissens über Mechanismen der N-Aufnahme und Reaktionen auf N-Mangel aus Studien zu anorganischem N. Dies hat erhebliche Lücken in unserem Verständnis darüber hinterlassen, wie Pflanzen organischen N aufnehmen und darauf reagieren. Die hier vorgestellte Arbeit schließt einige dieser Lücken, indem sowohl die physiologischen Effekte der Aminosäureversorgung als auch die molekulare Regulation der Aminosäuretransporter in Arabidopsis thaliana untersucht wurden. Die Ergebnisse zeigen einen deutlichen spezifischen, organischen-N Wurzelphänotyp und belegen, dass Pflanzen, die ausschließlich mit der Aminosäure Glutamin (L-gln) versorgt werden, keinen N-Mangel aufweisen. Der zweite Teil dieser Arbeit untersucht die Regulation der Aminosäureaufnahme mit besonderem Fokus auf den Hochaffinitätstransporter LHT1 (LYSINE HISTIDINE TRANSPORTER1). Dabei konnten Experimente die CALCIUM-DEPENDENT PROTEIN KINASE1 (CPK1) als Interaktionspartner von LHT1 identifizieren, was auf eine posttranslationale Regulation des Transporters hindeutet. Interessanterweise zeigen Aufnahmetests, dass CPK1 die Aminosäureaufnahme negativ beeinflusst. Weitere Experimente bestätigten diesen hemmenden Effekt von CPK1 auf die Transportaktivität von LHT1. Um die Rolle der Phosphorylierung bei der Regulation von LHT1 zu untersuchen, wurden Phospho-Mutanten des Transporters erzeugt und analysiert. Zwei potenzielle Phosphorylierungsstellen konnten identifiziert werden, wobei Thr151 als eine mögliche Zielstelle von CPK1 hervorgehoben wurde. Diese Arbeit erweitert unser Verständnis darüber, wie Pflanzen auf organischen N reagieren, und liefert tiefere Einblicke in die Aminosäureaufnahme - mit besonderem Schwerpunkt auf der Regulation des LHT1-Transporters.

Schlüsselwörter: *Arabidopsis thaliana*, organischer Stickstoff, Aminosäuren, Glutamin, Wurzelphänotyp, Aminosäuretransporter, LHT1, posttranslationale Modifikation, Phosphorylierung, CPK1

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Tünnermann, L.***, Aguetoni Cambui, C.*, Franklin, O., Merkel, P., Näsholm, T., and Gratz, R. (2025). Plant organic nitrogen nutrition: costs, benefits, and carbon use efficiency. New Phytologist, 245: 1018-1028. https://doi.org/10.1111/nph.20285

II. **Tünnermann, L.**, Svensson, E., Antoniadi, I., Zielasek, F., Ivanov, R., Löfstedt, T., Näsholm, T., Gratz, R. A deeper look into the regulation of AtLHT1, a key amino acid transporter for organic nitrogen (manuscript)

III. **Tünnermann, L.**, Colou, J., Näsholm, T., Gratz, R. (2022). To have or not to have: expression of amino acid transporters during pathogen infection. Plant Molecular Biology 109, 413–425. https://doi.org/10.1007/s11103-022-01244-1

IV. **Tünnermann, L.**, Colou, J., Näsholm, T., Löfstedt, T., Gratz, R. Applying nephelometry for the screening of liquid yeast cultures (manuscript)

* Shared first authorship

Papers I and III were published open access

The contribution of Laura Tünnermann to the papers included in this thesis was as follows:

I. Project design, experimental execution, data analysis, contribution to writing and formatting the manuscript, and contribution to review and editing.

II. Project design, experimental execution, data analysis, writing the original draft, and writing the manuscript.

III. Literature research, contribution to writing the original draft, and contribution to review and editing.

IV. Project design, experimental execution, data analysis, and writing the original draft.

List of figures

1. Introduction

1.1 Soil nitrogen forms

Nitrogen (N) is a crucial element for all living organisms, forming essential components such as nucleic acids and amino acids. In plants, N deficiency severely inhibits growth and development. Although N in the form of N2 is one of the most abundant elements in the atmosphere, it is not biologically available to most organisms, including plants (Galloway et al., 2003; Martínez-Espinosa et al., 2011). To cover the plant's demand for N, atmospheric N_2 needs to be converted into bioavailable N forms like ammonia (NH_3), ammonium (NH_4^+), or nitrate (NO_3^-) via, for instance, biological N fixation or the synthetic, energy-consuming Haber-Bosch process (Galloway et al., 2003; Martínez-Espinosa et al., 2011). For a long time, it was believed that N in the soil was predominantly present in its inorganic form (NH_4^+ and NO_3^-), however, bioavailable N exists in both inorganic and organic forms (e.g., amino acids, proteins, peptides, nucleotides, and amino sugars) (Schulten & Schnitzer, 1997). These two forms differ significantly. Inorganic N, especially NO_3^-, is highly soluble, which makes it easily available for plants but also prone to leaching. In contrast, organic N contains additional carbon (C) atoms and is less mobile in the soil, reducing the risk of leaching (Hansen et al., 2000; Paungfoo-Lonhienne et al., 2012). Furthermore, it was shown that organic N is a considerable N source for plants in the soil, particularly in ecosystems like boreal forests, where it represents the dominant N source (Inselsbacher & Näsholm, 2012). However, the mere presence of a N source in the soil does not guarantee its use by plants; they must also possess the ability to take up and metabolize the available N forms. Uptake experiments revealed that plants, in addition to inorganic N, actively absorb amino acids from the soil (Näsholm & Persson, 2001; Näsholm et al., 2009; Cambui et al., 2011), highlighting the importance of organic N nutrition. However, research on organic N remains limited compared to the extensive research on inorganic N.

1.2 Nitrogen acquisition

Nutrient delivery to plant roots is driven by two mechanisms: mass flow and diffusion. Mass flow involves the movement of nutrients toward the root surface along with the transpiration-driven water flow. This mechanism predominantly applies to mobile nutrients, such as soluble inorganic N forms. Diffusion, on the other hand, moves nutrients along a concentration gradient and is especially important for immobile nutrients (Nye, 1977; Tinker & Nye, 2000; McMurtrie & Näsholm, 2018). Modelling studies suggest that mass flow-dominated N acquisition requires low root surface area (McMurtrie & Näsholm, 2018). In contrast, diffusion-driven acquisition relies on a larger root surface area, achieved through the development of root hairs, a branched root system, and the interaction with mycorrhizal fungi. This model aligns with observations of NO_3^- uptake (a mobile nutrient) and phosphorus and potassium uptake (immobile nutrients), which corresponds to low and high root surface areas, respectively (Bates & Lynch, 2001; Jungk, 2001; Bienert et al., 2021). Amino acids, being immobile, are likely acquired via diffusion and thus require greater root investment. Similarly, N deficiency has been shown to increase root surface area, suggesting that plants shift toward a diffusion-based strategy under N-limited conditions (Hermans et al., 2006). This raises the question of whether amino acid availability triggers an N starvation response or if the plant is preparing for the uptake of alternative N sources. Nevertheless, it is important to note that the proliferation of root hair comes at a metabolic cost, requiring additional energy in form of carbohydrates (Zerihun et al., 1998; Franklin et al., 2017).

1.3 Nitrogen uptake

Once N is present in the rhizosphere, it becomes available for uptake by the plant. The uptake of N is orchestrated by a range of specialized membrane transporters, localized in the plasma membrane of root cells. To avoid redundancy and maximize efficiency, most N transporters function within different substrate affinities and expression patterns (Liu et al., 2006; Svennerstam et al., 2011; Tegeder & Masclaux-Daubresse, 2018). Inorganic N is taken up by a distinct set of transport proteins that belong to two major families: the nitrate transporter family (NRT) and the ammonium transporter family (AMT) (Yuan et al., 2007; Fan et al., 2017; Tegeder & Masclaux-Daubresse, 2018). NO_3^- uptake is facilitated by two systems: the low-affinity

transporters (LATS) and high-affinity transporters (HATS) involving NRT1 and NRT2 transporters, respectively (Fan et al., 2017). The uptake of NH_4^+, on the other hand, is mostly mediated by high-affinity members of the AMT family (Loque et al., 2006; Yuan et al., 2007). NH_4^+ uptake is dominated by only three key transporters (AMT1;1, AMT1;2, and AMT1;3), which together facilitate approximately 95% of its total flux.

In contrast to inorganic N forms, organic N can be present in the soil in the form of various amino acids (Näsholm et al., 2009), and its uptake is mediated by several amino acid transporter families within the amino acid/auxin permease (AAAP) superfamily (Tegeder & Masclaux-Daubresse, 2018). These include families such as the auxin transporters (AUXs), the amino acid permeases (AAPs), γ-aminobutyric acid transporters (GATs), proline and glycine betaine transporters (ProTs), and lysine/histidine transporters (LHTs) (Tegeder & Masclaux-Daubresse, 2018; Yao et al., 2020). Each transporter is specialized in transporting specific amino acid groups. Among these, five key transporters have been identified as being actively involved in root amino acid uptake. Two members of the AAP family generally exhibit moderate affinity and broad substrate specificity, covering the uptake of glutamate and neutral amino acids (AAP1) and basic amino acids (AAP5) (Lee et al., 2007; Svennerstam et al., 2008; Svennerstam et al., 2011). In contrast, LHTs are characterized as high-affinity transporters of neutral and acidic amino acids (Hirner et al., 2006; Lee et al., 2007; Forsum et al., 2008; Svennerstam et al., 2011; Ganeteg et al., 2017). In *A. thaliana*, two transporters, LHT1 and AAP5, with complementary affinity spectra, are responsible for the majority of amino acid uptake at low concentrations (10 – 50 μM) (Svennerstam et al., 2011). Additionally, LHT6 contributes to the uptake of acidic amino acids, as well as neutral amino acids, such as glutamine and alanine, while ProT2 facilitates the uptake of proline and glycine betaine into root cells (Rentsch et al., 1996; Perchlik et al., 2014).

1.4 Assimilation and transport, and metabolism

While the uptake of different N forms shares many features, their subsequent assimilation and associated energetic costs vary dramatically. Among the different N sources, NO_3^- possesses the highest assimilation cost due to the requirement for multiple enzymatic reduction steps before it is

incorporated into organic compounds (Zerihun et al., 1998; Franklin et al., 2017). First, NO_3^- is reduced to nitrite via the nitrate reductase in the cytosol, and transported into the plastid where it is reduced to NH_4^+ (Liu et al., 2022). This process can occur in the roots as well as in the mesophyll cells of leaves. The resulting NH_4^+ is then assimilated into amino acids via the glutamine synthetases/glutamine-2-oxoglutarate aminotransferase cycle (GS/GOGAT). In contrast, NH_4^+ taken up directly from the soil bypasses the reductive steps and is assimilated immediately, reducing the energetic cost (Zerihun et al., 1998; Franklin et al., 2017; Liu et al., 2022). Notably, most of the NH_4^+ absorbed into the roots is assimilated here and transported to the shoots in the form of amino acids, in particular glutamine and asparagine (Liu et al., 2022). Even more energy efficient is the assimilation of soil-derived amino acids, such as glutamine and arginine, which can be directly integrated into metabolic pathways, making them the N sources with the lowest assimilation cost (Zerihun et al., 1998; Franklin et al., 2017). Furthermore, amino acids provide an additional source of C, a benefit referred to as "the C bonus" (Franklin et al., 2017). This dual advantage, low assimilation costs, and the C bonus, are predicted to offset the energetic demands associated with increased root biomass under immobile nutrient nutrition. This suggests a feed-forward mechanism, in which the energetic advantage of amino acids promotes greater root investment, leading to an increased uptake of organic N (Figure 1) (Franklin et al., 2017). Beyond root uptake, nearly all N transporter families facilitate N translocation within the plant, including xylem loading, xylem-phloem transfer, as well as import into leaf tissues (Tegeder & Masclaux-Daubresse, 2018). Notably, amino acids are the principal form of remobilized N, especially from senescing tissues, a process triggered by factors such as aging, nutrient deficiency, or pathogen attack (Guiboileau et al., 2013; Havé et al., 2017).

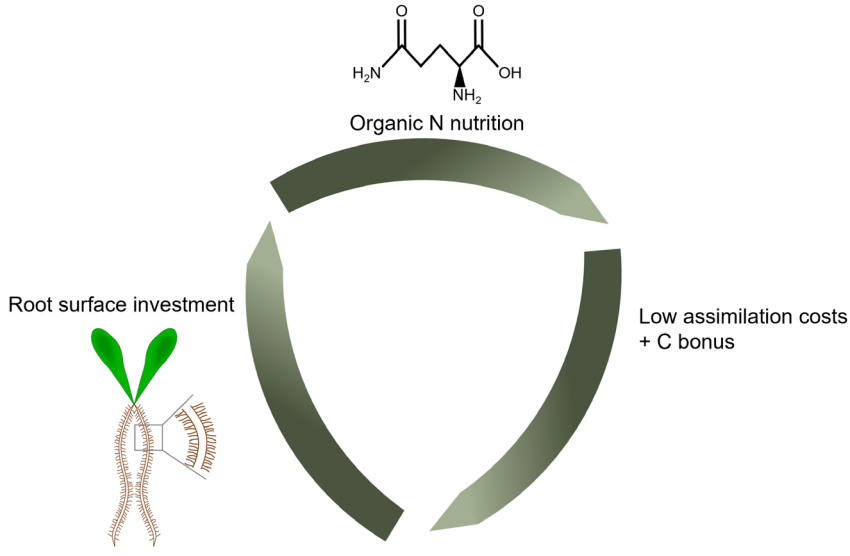

Figure 1: Simplified scheme of the effects of organic N nutrition based on Franklin et al. (2017). It illustrates a proposed feed-forward mechanism in which soil amino acids provide an energetic advantage, in form of low assimilation costs and C bonus, which promotes root surface investment, thereby improving the uptake of organic N.

Plants can synthesize proteinogenic amino acids directly within the plastids (Galili et al., 2016). Until recently, however, the specific transporters mediating amino acid transport across the plastid envelope were poorly understood. Kuhnert et al (2025) identified RETICULATA1 (RE1), a plastid transmembrane transporter responsible for transporting the basic amino acids arginine and lysine (Kuhnert et al., 2025). This study highlights a previously uncharacterized protein family and underscores its role in amino acid transport.

Amino acid biosynthesis in the plastids is strongly intertwined with the photorespiratory pathway, which provides NH_4^+ for N assimilation. Photorespiration is not only an energy-consuming process for the fixation of oxygen, but has a major effect on the N metabolism (Bauwe et al., 2010; Peterhansel et al., 2010; Eisenhut et al., 2019). During this pathway, glycine is converted into serine in the mitochondria, releasing NH_3 as a byproduct. Due to the toxicity of NH_3, it is rapidly protonated to NH_4^+ when entering the cytosol. NH_4^+ is then reassimilated in the plastids through the

GS/GOGAT cycle, leading to the synthesis of glutamate (Bauwe & Kolukisaoglu, 2003; Eisenhut et al., 2019).

Amino acids occupy a central role in plant N metabolism, therefore, understanding the function and regulation of amino acid transporters is essential. Among them, AtLHT1 has emerged as a critical transporter in root amino acid uptake and distribution.

1.5 AtLHT1 – a key amino acid transporter

The *A. thaliana* LHT1 was the first member identified among ten other family members. Initially characterized by Chen and Bush, LHT1's substrate specificity was identified through heterologous complementation in *Saccharomyces cerevisiae* (baker's yeast), where it was shown to transport the amino acids lysine and histidine (Chen & Bush, 1997). However, subsequent in-depth studies both in yeast and *in planta* revealed that LHT1 has a higher affinity for neutral and acidic amino acids, such as glutamine, alanine, asparagine, and glutamic acid (Hirner et al., 2006; Svennerstam et al., 2007; Ganeteg et al., 2017). LHT1 functions as a high-affinity transporter moving amino acids from the apoplast over the plasma membrane into the cell (Svennerstam et al., 2011). Expression analysis using β-Glucuronidase (GUS) reporter lines in *A. thaliana* localized *LHT1* expression at several root tissues, including the root cap and epidermis, indicating its direct role in amino acid soil uptake (Hirner et al., 2006). Additionally, *LHT1* is expressed in mesophyll cells, highlighting a role in internal amino acid transport in source tissues (Hirner et al., 2006). Beyond proteinogenic amino acids, LHT1 also facilitates the transport of non-proteinogenic amino acids like 1-aminocyclopropane-1-carboxylate (ACC), a precursor of the plant hormone ethylene, suggesting its broader physiological significance (Shin et al., 2015).

The analysis of *A. thaliana* T-DNA insertion lines lacking functional LHT1 (e.g., *lht1-1*, *lht1-5*) further revealed its impact on the plant's growth and development (Hirner et al., 2006; Svennerstam et al., 2007). The mutants exhibited severely impaired root amino acid uptake, stunted growth, and early leaf senescence, emphasizing the essential role of LHT1 in amino acid uptake and plant development. However, it is unclear whether LHT1's involvement in leaf senescence is triggered by age-related processes or external factors such as pathogen attacks (Liao et al., 2020). Recent findings

suggest that the *A. thaliana* transcription factor ORESARA1 (ORE1), a central regulator of senescence, may modulate LHT1 gene expression (Liao et al., 2020). However, whether ORE1 directly controls *LHT1* expression and how this regulation affects amino acid and/or ACC transport remains to be clarified.

1.6 The versatile role of amino acid transporters in the plant

Beyond their significance for the plant, amino acids are also vital for microbial communities, including pathogens, creating direct competition between plant and microbes for this resource (Rentsch et al., 2007; Roberts & Jones, 2012; Kuzyakov & Xu, 2013). Paper III presents a literature review on amino acids and their transporters, highlighting their roles in pathogen attacks and underscoring the importance of amino acids for both plant nutrition and resistance. This paper addresses the current gap in literature, as no concise literature review on this topic is available. While some amino acids serve as nutrients for pathogens, others contribute to the plant's defense mechanisms, demonstrating that the control over amino acid uptake and their respective transport system can decide over survival or death (Sonawala et al., 2018; Li et al., 2020; Froschel et al., 2021).

The AAP transporter family, for instance, is suggested to be a negative regulator of plant defense reactions for multiple species (Elashry et al., 2013; Marella et al., 2013). AAP-knockout mutant plants of cucumber, tomato, and Arabidopsis, challenged with biotrophic and hemibiotrophic pathogens, indicated increased resistance (Marella et al., 2013; Berg et al., 2021). In addition, it was found that the transcripts of certain AAP family members increased after pathogen infection, suggesting that an increase in transporter transcript abundance supports the pathogen's survival strategy.

In addition, several studies identified LHT1's involvement in plant responses to various pathogens. LHT1 expression in *A. thaliana* is upregulated during infections by hemibiotrophic bacteria such as *Pseudomonas syringae* and the fungi *Colletotrichum higginsianum* (Liu et al., 2010). Since many biotrophic pathogens rely on apoplastic nutrients (Fatima & Senthil-Kumar, 2015; Wang et al., 2020), it has been hypothesized that LHT1 facilitates the removal of amino acids from the apoplast into the plant cell and thereby limiting the pathogen's access to nutrients (Paper III,

Figure 1a). However, plants lacking functional LHT1 (*lht1-1*) exhibited enhanced resistance against both pathogens, identifying LHT1 as a negative regulator of plant immunity (Liu et al., 2010). The authors hypothesized that the reduced glutamine uptake in the *lht1-1* mutants alters the cellular redox status, increases reactive oxygen species (ROS) accumulation, and activates programmed cell death. This leads to the suggestion that *LHT1* expression is regulated by the pathogen to avoid the induction of subsequent defense mechanisms. However, a more recent study revealed that LHT1 contributes to plant defense against *P. syringae* (Rogan et al., 2024). The authors demonstrated that LHT1 promotes resistance by depleting apoplastic proline, thereby limiting the pathogen's ability to use the nutrient and grow. In contrast, *lht1* loss-of-function mutants failed to deplete proline after infection and showed higher bacterial growth compared to wild-type plants (Rogan et al., 2024). In contrast, necrotrophic pathogens invade plant cells by disrupting the plasma membrane and inducing programmed cell death. These attacks typically trigger the ethylene/jasmonic acid-mediated defense response (Pieterse et al., 2012; Huang et al., 2020). Interestingly, *LHT1* is upregulated during necrotrophic pathogen infection, suggesting a possible contribution to the ethylene-mediated resistance via the transport of ACC (Paper III, Figure 1b) (Farjad et al., 2018; Xiong et al., 2018). Furthermore, *lht1-1* mutants did not display increased resistance after necrotrophy infection, indicating that LHT1's role during infections is pathogen lifestyle-dependent (Liu et al., 2010). Despite these insights, the precise molecular function of LHT1 in plant-pathogen interactions remains poorly understood.

Plants must continuously adapt to a variety of stresses. In this context, the dual role of amino acids, serving as nutrients and defense mechanisms, underscores the importance of precise regulation of amino acid transport within the plant.

1.7 Transport regulation

The regulation of transport proteins is essential for ensuring access to nutrients while maintaining energy efficiency and nutrient homeostasis. Rather than being constitutively expressed, transporters are often regulated in response to environmental cues. For instance, to cope with changing soil N conditions, many inorganic and organic N transporters are regulated at the transcriptional level (Liu & Bush, 2006; Ho et al., 2009). However, although

some information is available on the transcriptional regulation of N transporters, most post-translational studies have focused on inorganic N transporters (Ho et al., 2009; Wu et al., 2019; Qin et al., 2020; Yue et al., 2025), while amino acid transporters remain comparatively understudied. Post-translational regulation includes various potential modifications, such as ubiquitination, methylation, lipidation, or phosphorylation, each affecting a protein differently, and multiple modifications can occur at the same amino acid residue (Walsh et al., 2005; Friso & van Wijk, 2015; Hashiguchi & Komatsu, 2017). Among these, protein phosphorylation is one of the most common. It involves the reversible addition of a phosphate group to specific amino acid residues, introducing a negative charge that can significantly alter the protein's conformation, activity, stability, or subcellular localization, thereby fine-tuning the transporter's function in response to the plant's needs (Friso & van Wijk, 2015; Hashiguchi & Komatsu, 2017; Wu et al., 2019; Qin et al., 2020). A variety of inorganic N transporters were identified phosphorylation targets, and the functional consequences of this modification have been shown. Work by Wu et al. (2019) demonstrated how the NH_4^+ transporter AMT1;3 is regulated in response to external N conditions. During high NH_4^+ concentrations, AMT1;3 is phosphorylated at the threonine residues at positions T464 and T494, leading to transporter inactivation and preventing the uptake of toxic NH_4^+ concentrations. Conversely, in the presence of external NO_3^- conditions, these sites become dephosphorylated, resulting in transporter activation. N-deficient conditions cause phosphorylation only at T494, producing an intermediate activity state. This example illustrates the fine-tuning capacity of phosphorylation, where multiple phosphorylation events can modulate protein activity (Wu et al., 2019). In addition, phosphorylation regulates other functional aspects of N transporters. For instance, the dual-affinity NO_3^- transporter NRT1.1 alters its substrate affinity depending on its phosphorylation status at T101. This modification acts as a molecular switch, shifting NRT1.1 between low and high affinity states, triggered by external NO_3^- concentrations (Wang et al., 1998; Liu et al., 1999; Ho et al., 2009; Sun et al., 2014).

To date, post-translational regulation of organic N transporters has received little attention. As a result, our understanding of how these transporters are regulated at the protein level remains limited. Nevertheless, given their crucial roles in various aspects of plant physiology, it is essential to investigate the mechanisms that control their activity. The well-

documented post-translational modification of inorganic N transporters suggests that amino acid transporters are likely subject to similar regulatory mechanisms. Supporting this, a phosphor-proteomic study identified several amino acid transporters, indicating that phosphorylation could modulate their function (Mergner et al., 2020). However, protein kinases that catalyze amino acid transporter phosphorylation events are largely unknown.

1.8 Calcium-dependent protein kinases

Kinases are essential enzymes mediating post-translational modifications by catalyzing the reversible phosphorylation of target proteins. They are present across all organisms, including plants, where they play critical roles in adjusting to dynamic environmental conditions and responding to biotic and abiotic stresses (Simeunovic et al., 2016; Yip Delormel & Boudsocq, 2019). In Arabidopsis, the CDPK-SnRK (Ca^{2+}-dependent protein kinase—Snf1-related kinase) superfamily has been identified, including the Ca^{2+}-dependent protein kinases (CDPKs or CPKs) (Harper et al., 2004). Within this superfamily, CPKs play a notable role due to their dual function as Ca^{2+} sensors and signal transducers. This unique feature enables CPKs to directly bind Ca^{2+} and respond by translating the signal into downstream phosphorylation events (Harper et al., 2004; Yip Delormel & Boudsocq, 2019). Ca^{2+} is a central second messenger in plant signaling networks. Under resting conditions, cytosolic Ca^{2+} concentrations are low (0.1 – 0.2 mM). However, in response to external stimuli, cytosolic Ca^{2+} concentrations can rapidly increase to 1 – 2 mM (Pittman et al., 2011). The oscillation of the cytosolic Ca^{2+} concentrations is referred to as "Ca^{2+} signature". Each Ca^{2+} signature encodes distinct information, reflecting the stimulus, that can be decrypted by the specific CPK and translated into downstream regulation events, regulating cellular responses (Pittman et al., 2011). Such external stimuli can be soil N conditions (Liu et al., 2020). Studies revealed two CPK family members (CPK28 and CPK32) to respond to external N conditions and regulate the uptake of NO_3^- and NH_4^+, respectively (Qin et al., 2020; Yue et al., 2025).

1.9 CPK structure

The conserved structure of CPKs ensures the binding and decoding of Ca^{2+} signatures. Each CPK comprises four distinct domains: a variable N-terminal domain (VNTD), a Ser/Thr kinase domain, which includes an N-lobe and a C-lobe carrying the active site of the kinase, an autoinhibitory junction domain (JD), and a calmodulin-like regulatory domain (CaMLD) located at the C-terminus (Yip Delormel & Boudsocq, 2019). The CaMLD typically consists of four EF-hand Ca^{2+}-binding motifs, which are grouped into two lobes, a low-affinity N-lobe and a high-affinity C-lobe. Under resting cytosolic Ca^{2+} conditions, the JD acts as a pseudo-substrate by occupying the kinase active site, hence leading to an autoinhibition of the kinase (Harmon et al., 1994; Harper et al., 1994; Huang et al., 1996). The inhibition is further stabilized through interactions between the N-lobe of the CaMLD and the N-lobe of the kinase domain, as well as between the C-lobe of the CaMLD and the JD. Upon increasing cytosolic Ca^{2+} levels, the N-lobe of the CaMLD binds Ca^{2+}, inducing conformational changes in the kinase protein structure, including the backfolding of the JD and CaMLD. These structural changes relieve the autoinhibition, leading to an active conformation through the exposure of the kinase active site and enabling substrate binding.

1.10 CPK1 – a model kinase

CPK1 (previously known as AK1) was the first member of the CPK family identified in *A. thaliana* (Harper et al., 1993; Harper et al., 1994). It has long served as a model for studying Ca^{2+}-dependent activation and the pseudosubstrate regulatory mechanism of CPKs (Huang et al., 1996). In addition to being activated by Ca^{2+}, CPK1 performs autophosphorylation, adding another level of regulation (Durian et al., 2020 B). However, it remains unclear whether CPK1 is also phosphorylated by other upstream kinases. Furthermore, an upstream post-translational regulation of CPK1 in form of dephosphorylation has been identified. The PROTEIN PHOSPHATASE 2A-B (PP2A) negatively regulates the activation status of CPK1 by removing the phosphorylation (Durian et al., 2020 A).

CPK1 is shown to be located at peroxisomes and oil bodies (Coca & San Segundo, 2010). In addition, it was also identified to be N-myristoylated, giving more detailed information about its subcellular localisation and

protein function, since the N-myristoylation functions as a membrane anchor (Coca & San Segundo, 2010).

CPK1 covers diverse regulatory roles, for instance, being involved in the response to both biotic and abiotic stresses (Coca & San Segundo, 2010; Huang et al., 2018). Furthermore, in the last decades, a wide range of CPK1 targets have been identified, underscoring its broad functional significance (Hwang et al., 2000; Giacometti et al., 2012; Durian et al., 2020 B).

1.11 CPK1 involvement in biotic and abiotic stress responses

CPK1 exhibits broad substrate specificity and plays a critical role in enhancing plant resistance and survival under both biotic and abiotic stress conditions (Coca & San Segundo, 2010; Huang et al., 2018; Durian et al., 2020 B). CPK1 was identified in the regulation of pathogen resistance and the salicylic acid (SA) signaling pathways (Coca & San Segundo, 2010; Gao & He, 2013). Overexpression of *CPK1* enhanced the resistance to both bacterial pathogens, such as *Pseudomonas syringae*, and fungal pathogens, including *Fusarium oxysporum* and *Botrytis cinerea*. It also induced the expression of the SA-related defense genes. The study highlighted that in *A. thaliana*, the presence of CPK1 is essential for effective immune responses, as plants lacking CPK1 (*cpk1-1*) demonstrated significantly reduced survival rates during pathogen attacks (Coca & San Segundo, 2010). Additional studies have shown that CPK1 is involved in multiple stages of the plants' defense mechanisms. One key regulatory mechanism is CPK1 deactivation through dephosphorylation by the protein phosphatase PP2A-B'γ (Durian et al., 2020 A). This interaction serves as a fine-tuning mechanism to prevent premature activation of defense responses and SA signaling, thereby balancing plant immunity according to developmental and environmental cues (Durian et al., 2020 A; Mhamdi, 2020). CPK1 also contributed to ROS-mediated defense signaling. It interacts with and phosphorylates RBOHD (Respiratory Burst Oxidase Homolog D, NADPH oxidase), which produces ROS, in response to pathogen detection. This phosphorylation enhances an oxidative burst, which is a rapid accumulation of ROS that serves as an early defense response against pathogen invasion (Gao et al., 2013; Gao & He, 2013).

Beyond its role in plant immunity, CPK1 has been identified as a positive regulator of leaf senescence (Durian et al., 2020 B). In this work, the authors showed that CPK1 phosphorylates the transcription factor ORE1, increasing and promoting the expression of senescence-related genes that ultimately lead to programmed cell death. However, the exact signals that activate CPK1 and lead to the subsequent phosphorylation of ORE1 are still unknown.

1.12 CPKs and nitrogen uptake

Previous studies have demonstrated the involvement of CPK family members in the regulation of inorganic N uptake (Qin et al., 2020; Yue et al., 2025). For instance, CPK32 positively regulates the NH_4^+ transporter AMT1;1 through phosphorylation (Qin et al., 2020). Loss-of-function experiments showed that in the absence of CPK32, AMT1;1 lost its transport activity. Additionally, *CPK32* expression was responsive to external N availability and was upregulated under N starvation conditions (Qin et al., 2020). Another study identified that CPK28 enhances NO_3^- uptake by phosphorylating NRT2.1 (Yue et al., 2025). Similar to *CPK32*, the expression of *CPK28* was also influenced by external N levels.

The discovery that several inorganic N transporters are regulated through phosphorylation raises two key questions: Is organic N uptake regulated similarly, and if so, is a member of the CPK family involved in these regulatory events? LHT1 seems to be a strong candidate for such regulation due to its wide range of functions, including amino acid uptake, stress responses, and plant development. Moreover, CPK1, a kinase involved in leaf senescence and plant defense responses, shares functional overlap with LHT1 in these pathways, however, its role in nutrient uptake remains unknown. A strong indication supporting this came from a membrane protein interaction study in *A. thaliana* (Chen et al., 2012). This study proposed 132 potential interaction partners for LHT1. After data extraction and applying stringent filtering criteria, including an increased repetition threshold, we narrowed these down to four candidate interactors: CNGC13 (Cyclic Nucleotide-Gated Channels), ABCG10 (ATP Binding Cassette Transporter), NHX8 (Sodium-Proton Exchanger), and CPK1. Notably, the identification of CPK1 as one of the four possible interactors suggests that LHT1 may be post-translationally modified by this kinase. This finding supports the

hypothesis that organic N transporters, like their inorganic counterparts, are modulated by CPK family kinases.

2. Objectives

Nitrogen, traditionally recognized in the forms of NO_3^- and NH_4^+, has long been considered essential for plant growth and development. However, research over the past decades has revealed a more complex picture of nitrogen nutrition. The presence of organic nitrogen and especially of amino acids in soil, the demonstration of plant amino acid uptake in the field, and the identification of transporters mediating uptake of organic nitrogen marked a turning point in the research. These findings have reshaped our understanding of organic nitrogen nutrition. This thesis and the underlying studies build on that foundation and aim to contribute to a deeper understanding of organic nitrogen nutrition and the molecular regulation of amino acid uptake by:

1) Investigating how different nitrogen sources affect plant phenotype as well as nitrogen and carbon allocation (**Paper I**).

2) Uncovering the mechanisms that regulate AtLHT1-mediated amino acid uptake (**Paper II**).

3) Reviewing the roles of amino acids and transporters beyond root uptake, with AtLHT1 illustrating their function in plant defense (**Paper III**).

4) Introducing nephelometry as a method to conduct and analyze *Saccharomyces cerevisiae* growth experiments, with the potential to characterize AtLHT1 from different plant species (**Paper IV**).

3. Materials and methods

The methods used in Papers I and II are standard plant physiological and molecular experiments. The exact descriptions and protocols can be found in the materials and methods section of the respective paper. Additional experiments shown in this thesis are based on the experiments performed in Paper II. Here, the focus is on additional details of key methods (Papers I and II) and the application of nephelometry for monitoring and analyzing yeast growth (Paper IV).

3.1 A. thaliana

Plant experiments conducted in Papers I and II utilized the model organism *Arabidopsis thaliana*. The ecotype Col-0 was used as wild-type and hence as control in experiments performed with *A. thaliana* mutants. To gain an understanding of amino acid uptake, the following mutant and overexpression lines of *LHT1* were used: T-DNA insertion knockout line *lht1-5* (SALK_115555) (Svennerstam et al., 2007), overexpression line *35S:LHT1-1* and *35S:LHT1-2* (Forsum et al., 2008). To gain an understanding of the impact of the kinase CPK1 on amino acid uptake, the T-DNA insertion lines *cpk1-1* (SALK_096452) and *cpk1-2* (SALK_080155) were used. Both lines (*cpk1-1* and *cpk1-2*, Alonso et al., 2003) were received from the Salk Institute Genome Analysis Laboratory. The seeds were screened for a T-DNA insertion, and homozygosity was confirmed using PCR. However, RT-qPCR confirmed that *cpk1-1* is a true null-mutant (Gao et al., 2013) while *cpk1-2* is a knockdown mutant, indicating reduced gene expression (Paper II, Supplemental Figure 3b).

3.2 *A. thaliana* growth systems

Most plant experiments were conducted in sterile vertical square Petri dishes containing N-free ½ Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) following established protocols (Svennerstam et al., 2007). Depending on the treatment, the medium was supplemented with either NO_3^-, as an inorganic N source, or L-glutamine (L-gln), as an organic N form. L-gln was selected as a representative organic N source due to its growth-promoting effects (Forsum et al., 2008). N was provided as L-gln, and NO_3^-

in concentrations of 1.5 mM and 3 mM, respectively, to provide an equivalent total N concentration of 3 mM. This sterile and controlled growth system enabled a precise analysis of organic N nutrition. Sterile conditions were crucial for the understanding of how the N source and concentration affect plant growth, phenotype, and gene expression, while avoiding interference from soil and soil microorganisms with respect to amino acid uptake.

Amino acid uptake can be studied using various approaches. One effective method involves using D-isomers of amino acids, such as D-alanine (D-ala). D-ala is toxic to plants and inhibits growth, serving as a selective marker and indirect indicator of amino acid uptake efficiency (Erikson et al., 2004; Forsum et al., 2008). The benefit of this experiment is the straightforward analysis, which relies on visible phenotypic changes. In this study*, A. thaliana* mutants were initially germinated and grown on N-free ½ MS media supplemented with 3 mM NO_3^-. Seven-day-old seedlings were then transferred to N-free ½ MS media supplemented with 3 mM NO_3^- and 10 mM D-ala (Paper II, Figure 2). This transfer protocol was established after multiple rounds of optimization, which showed that seedlings need to germinate on NO_3^- before exposure to toxic D-amino acids to ensure consistent and measurable responses. In addition, the final D-ala concentration was set significantly higher than typical amino acid levels (e.g., 1.5 mM L-gln) to generate a clearer phenotypic distinction between the tested lines and controls.

A second, more direct method to measure amino acid uptake involves ^{15}N and ^{13}C dually-labeled L-gln (Paper II, Figure 3). This approach allows precise tracing of N uptake into root and shoot tissues (Svennerstam & Jämtgård, 2022). Plants were germinated and grown for 21 days on N-free ½ MS medium supplemented with 3 mM NO_3^-. For the uptake experiment, a 50 µM solution of dually-labeled L-gln was prepared. The seedling roots were incubated in the solution for 15 min, while the shoots were covered with aluminum foil to prevent direct contact. This ensured that any ^{15}N detected in the shoots resulted from uptake exclusively through the roots. A key strength of this method is the use of soil-relevant amino acid concentration.

3.3 Phospho-mimetic

The synthesis of phospho-mutants is a useful approach to understand how a potential phosphorylation affects proteins such as the amino acid transporter LHT1. To identify potential phosphorylation sites, I used the *in silico* prediction tool PhosPhAt[1] (v. 4) (Paper II, Supplemental Figure 6a). The selection of potential phosphorylation sites was restricted to sites within the cytosolic domain of the transmembrane transporter, as phosphorylation typically occurs in accessible cellular regions. To determine these domains, I predicted the structure of LHT1 using the in silico prediction tool for protein transmembrane helices (TMHMM v. 2.0[2]) (Paper II, Supplemental Figure 4a). The focus was specifically on serine and threonine residues, as CPK1 is a Ser/Thr kinase.

Phospho-mimetics provide an indirect and artificial method of analyzing potential protein phosphorylation events. This approach relies on the structural similarity between certain amino acids, such as glutamic acid (E), and the phosphorylated form of, for instance, threonine (T) or serine (S) (Chen & Cole, 2015). Glutamic acid has a negatively charged side chain that mimics the negative charge introduced by a phosphorylation (Thorsness & Koshland, 1987). Based on this principle, the predicted phosphorylation sites in LHT1 T251 and T255 were substituted with glutamic acid to mimic a constitutive phosphorylation. This substitution approximates the impact of a potential phosphorylation without requiring the actual kinase. In contrast, phospho-dead mutants are designed to introduce a non-phosphorylated scenario by replacing the target amino acid with a structurally similar amino acid that lacks a negative charge, in this case, alanine (A).

It is important to note the limitations of this system. Alanine substitutions remove the hydroxymethyl side chain present in serine residues, potentially altering the biochemical properties of the protein (Chen & Cole, 2015). Similarly, while glutamic acid introduces a negative charge to mimic phosphorylation, it only contributes one negative charge compared to the two introduced by a phosphate group. Moreover, glutamic acid differs in size and

[1] https://phosphat.uni-hohenheim.de/

[2] https://services.healthtech.dtu.dk/services/TMHMM-2.0/

conformation from a phosphorylated residue. These differences can impact the protein and the experimental outcome (Chen & Cole, 2015).

However, the use of phospho-mutants has many advantages and is an accepted approach within the scientific community (Li et al., 2017; Gratz et al., 2019). Notably, the substituted amino acids, glutamic acid and alanine, are not kinase targets and thereby avoid unintended phosphorylation. This allows a controlled comparison between the effects of mimicked phosphorylation and the absence of phosphorylation on the protein. Additionally, the approach can be performed *in vitro* as well as *in vivo* and helps to get a functional understanding of the outcomes of potential protein phosphorylation.

3.4 Nephelometry

Paper IV focuses on the development and optimization of a nephelometry-based method for yeast experiments.

Paper IV aimed to adapt established yeast growth experiments, such as yeast complementation, to liquid-based systems in response to the limitations inherent in classic agar-based experiments. Specifically, difficulties in the detection of subtle growth differences between constructs or different yeast mutants. Previous studies demonstrated the transition of yeast experiments to liquid assays using spectrophotometry and identified several advantages, including real-time monitoring of the organism's growth, precise quantification, and improved sensitivity, in addition to reduced experimental time, labor, and costs (Diaz-Camino et al., 2003; Donnard et al., 2014; Bracher et al., 2018).

Spectrophotometry is a widely accepted and validated approach for microbial growth analysis, however, the exploration and implementation of alternative methods, such as nephelometry, are essential for further advancing experimental capabilities.

Previous studies have demonstrated the benefits of nephelometry over spectrophotometry in experiments involving filamentous fungi and algae, organisms that grow non-homogeneously in liquid cultures (Joubert et al., 2010; Calmes et al., 2020). Nephelometric measurements were shown to be more stable and robust compared to spectrophotometer results, due to its distinct measurement principle (Calmes et al., 2020). Nephelometry measures the turbidity of a sample by detecting the scattered light, rather than

quantifying light absorption, as in spectrophotometry. Yeast, however, is growing mostly homogenously in liquid cultures, and spectrophotometry has long been the standard for its analysis (Diaz-Camino et al., 2003; Donnard et al., 2014). However, in solutions containing particles, such as algae or fungi, light is not primarily absorbed but instead scattered. As a result, spectrophotometry measurements of these samples reflect a loss of transmitted light caused by scattering rather than a true increase in light absorption by the dissolved solutes (Fouda et al., 2006; Seyfarth et al., 2008; Joubert et al., 2010; Calmes et al., 2020).

Paper IV validated nephelometry as a viable alternative for yeast growth assays and compared classic spectrophotometry and nephelometry by analyzing the performance and accuracy in quantifying yeast growth experiments.

The yeast complementation assay tested constructs expressing AtLHT1 and PtrLHT1.2 (Hirner et al., 2006; Gratz et al., 2020). Yeast transformed with an empty vector (pDRf1:ccdB) served as a negative control. To assess transporter activity, yeast was grown in N-free growth medium supplemented with 3 mM L-citrulline (L-cit), which can only be utilized if the transporter is functional. For evaluating yeast viability, the medium was supplemented with 10 mM ammonium sulfate. As an additional control, a non-supplemented N-free medium was used to confirm the absence of background growth. In the spectrophotometer approach, yeast cultures were incubated in 50 ml flasks, and the optical density was measured twice a day. In contrast, the nephelometry-based assay was performed in a 96-well plate format, and measurements were taken every 10 min. Blank controls were included in both methods and measured at each time point to correct for background signal. A second normalization step involved subtracting the negative control values (pDRf1:ccdB grown on L-cit) from the test samples grown on L-cit, accounting for any non-specific background growth. For the quantitative analysis of the growth curves, we identified one key parameter: maximum slope time, demonstrating the time point of the highest growth rate. This parameter enabled a more sensitive and precise comparison of construct performance, revealing differences between AtLHT1 and PtrLHT1.2 that remained undetected in previous agar-based experiments.

4. Results and discussion

This thesis is based on the results and theories presented in Papers I, II, III, and IV. Here, key results from these papers are presented and discussed, with an attempt to connect the findings and to gain a deeper understanding of organic N nutrition and amino acid uptake regulation. Further information and results can be found in the corresponding papers.

4.1 Optimized nutrient uptake through organic nitrogen root phenotype

Research on N has traditionally focused on inorganic N forms such as NO_3^- or NH_4^+. Most of our current understanding of N uptake mechanisms and N-starvation responses are based on studies involving these N forms. However, there is a growing need to address the knowledge gaps related to organic N forms, particularly their uptake and physiological effects on plants.

Paper I aimed to investigate the impact of organic N, specifically amino acids, on plant phenotype. To evaluate the effects of organic N on plant development, we used a split root growth system for *A. thaliana* plants, where the roots of a single seedling were divided between two growth compartments. Each root side had access to N-free ½ MS medium, either supplemented with solely L-gln (L-gln/L-gln), solely NO_3^- (NO_3^-/NO_3^-), or both (L-gln/NO_3^-) (Paper I, Figure 1). Plants supplied exclusively with L-gln (L-gln/L-gln) showed a significant increase in root biomass compared to plants grown solely on NO_3^- (NO_3^-/NO_3^-) (Paper I, Figure 3). Notably, plants with access to both N sources (L-gln/NO_3^-) exhibited changes in the root biomass. The root side exposed to L-gln developed significantly higher biomass than the side with access to NO_3^-, highlighting that a plant can distinguish between different N forms and allocate growth accordingly. While the use of different N sources had strong effects on the root biomass, the shoots were less affected. Furthermore, detailed phenotypic analysis revealed a substantial increase in root hair growth for L-gln-grown seedlings (Paper I, Figure 4, 5), indicating a strong allocation of biomass to the root system under L-gln nutrition.

This phenotype is reminiscent of responses typically observed when immobile nutrients like phosphorus or potassium are available, where uptake depends on an enhanced root surface area due to diffusion-driven uptake (Bates & Lynch, 2001; Jungk, 2001; Bienert et al., 2021). However, previous studies also showed that N-starved plants develop a similar root phenotype (Ågren & Ingestad, 1987; Hermans et al., 2006). This brings into focus whether plants grown on L-gln-supplemented media exhibit a phenotype characteristic of immobile nutrient acquisition or are instead experiencing N starvation. To address this point, we measured the N concentration of these plants (Paper I, Table 1). Plants grown with access to L-gln, either solely (L-gln/L-gln) or in a mix (L-gln/NO_3^-), demonstrated equally high N concentration in the shoots compared to solely NO_3^--grown plants. The roots, however, exhibited reduced N concentrations in plants grown solely on L-gln or a mixed medium, compared to NO_3^--grown plants, which is due to the increased root biomass of these plants. These results highlight that plants with access to L-gln are not N-starved. Assessing the C concentration of the seedlings revealed opposite results. Despite the greater root biomass, which would be expected to increase C demand, plants supplied with L-gln (L-gln/L-gln or L-gln/NO_3^-) showed an even higher C concentration, keeping the increased root biomass of L-gln-grown plants in mind. This aligns with the previously proposed "C bonus" model for organic N sources, which highlights two benefits: the lower assimilation cost of amino acids and the simultaneous direct uptake of C atoms with each amino acid molecule (Zerihun et al., 1998; Franklin et al., 2017). The next step was to experimentally verify the contribution of L-gln-derived C to the total organ C by using ^{15}N and ^{13}C dually-labeled L-gln (Paper I, Figure 6a, c). The data are presented as a regression of excess ^{13}C in the tissue versus excess ^{15}N. The ratio of these two isotopes in one L-gln molecule is 2.5. This theoretical value is shown with the dashed line, representing non-metabolized L-gln that remained in the tissue. A slope lower than 2.5 would indicate a loss of ^{13}C due to L-gln catabolism. The experimental data demonstrated slopes of 1.02 in shoots and 1.08 in roots, indicating that 41% and 43% of the C acquired as L-gln, respectively, remain in the tissue. These findings highlight that L-gln-derived C is a major contributor to the organ C content of these plants.

These findings support the idea that plants exhibit a distinct organic N phenotype, characterized by traits commonly associated with the acquisition of immobile nutrients. These results further suggest that there are similarities

between the organic N phenotype and the phenotype of N-stressed plants, which raises the question of whether part of the N stress phenotype is an acclimatization to gain N not through mass flow but through diffusion. This raises the question of how plants can perceive and respond to the presence of amino acids.

Given the established regulatory complexity of inorganic N transporters, I aimed to investigate whether similar mechanisms could exist for amino acid uptake. There are several reasons why such regulation would be expected. First, multiple layers of regulation have already been demonstrated for inorganic N transporters in plants (Ho et al., 2009; Wu et al., 2019; Qin et al., 2020; Yue et al., 2025). Second, evidence from mammalian systems shows that amino acid uptake is subject to tight control (Rosario et al., 2016). Third, if amino acids and other forms of organic N represent relevant N sources for plants (Näsholm & Persson, 2001; Näsholm et al., 2009), their uptake would likely be strictly regulated to ensure efficient use of resources. Consequently, determining whether amino acid uptake is regulated, and at which level, has direct implications for evaluating the physiological importance of organic N assimilation in plants. Therefore, in Paper II, I explored potential regulatory mechanisms of the key amino acid transporter LHT1 at multiple levels, including the possibility of post-translational regulation.

4.2 CPK1 – A potential key interaction partner of LHT1

Post-translational phosphorylation is a key mechanism regulating the activity and affinity of several inorganic N transporters (Ho et al., 2009; Wu et al., 2019; Qin et al., 2020; Yue et al., 2025), suggesting that amino acid transporters may be subject to similar control. Among the refined list of four proposed interaction partners of LHT1 by Chen et al., we identified the calcium-dependent protein kinase 1 (CPK1), which stood out due to its role in post-translational regulation (Chen et al., 2012). Notably, the CPK family is shown to regulate diverse nutrient transporters, including manganese, arsenic, and inorganic N (NRT2.1, AMT1;1), suggesting a possible regulatory parallel for LHT1 (Ji et al., 2017; Qin et al., 2020; Fu et al., 2022; Yue et al., 2025). To assess the potential regulatory effects of CPK1 on the amino acid uptake, the protein-protein interaction with LHT1 needed to be confirmed. Bimolecular fluorescence complementation (BiFC) experiments

verified the full-length protein interaction *in planta* by reconstituted YFP fluorescence (Paper II, Figure 4a). To further specify where the interaction takes place, we performed an *in silico* analysis (TMHMM version 2.0) to identify cytosolic domains of the transmembrane protein (Paper II, Supplemental Figure 4a). Six cytosolic domains were identified. Each domain was then tested for its interaction with CPK1 in a yeast two-hybrid (Y2H) experiment, where we identified domains 1, 2, and 4 to interact with CPK1 (Paper II, Figure 4b). Subsequent BiFC experiments confirmed the interaction between CPK1 and LHT1 cytosolic domains 1 and 2 (Paper II, Figure 4c).

These results provided strong evidence for a direct interaction between LHT1 and CPK1, leading to the hypothesis that CPK1 regulates LHT1 activity, thereby affecting amino acid uptake.

4.3 Regulation of gene expression by L-gln in root tissue

Previous studies have shown that the expression of kinases, such as *CPK28* and *CPK32*, is regulated by N availability (Qin et al., 2020; Yue et al., 2025), suggesting that kinase gene expression is modulated at an additional regulatory layer, activated under specific nutrient conditions. Therefore, it was crucial to investigate whether *CPK1* expression is influenced by the N source and availability. To assess this regulatory mechanism for CPK1, gene expression analysis of *A. thaliana* Col-0 roots was conducted. The plants were grown on high (1.5 mM L-gln or 3 mM NO_3^-) N concentrations or low (0.5 mM L-gln or 1 mM NO_3^-) N concentrations, representing a total of 3 mM or 1 mM N, respectively. The results revealed a significant increase in *LHT1* expression under low L-gln compared to high L-gln concentration (Paper II, Figure 1a). These results are consistent with its known function as a high-affinity transporter and align with previous expression data (Hirner et al., 2006; Svennerstam et al., 2011). In contrast, *CPK1* expression was significantly downregulated under low L-gln conditions. Interestingly, neither *LHT1* nor *CPK1* expression responded to changes in the NO_3^- concentration, suggesting that their regulation is specific to L-gln.

Overall, *LHT1* and *CPK1* displayed opposing L-gln-dependent expression patterns, which, together with their interaction, indicate that they

may form a regulatory module in which CPK1 modulates LHT1 activity in an L-gln-dependent manner.

4.4 Amino acid uptake is negatively regulated by CPK1

To assess the role of CPK1 in amino acid uptake, *cpk1* knock-out and knock-down mutants (*cpk1-1* and *cpk1-2*, respectively), as well as *LHT1* overexpression lines (OE) (*35S: LHT1-1* and *35S: LHT1-2*) and the *lht1* knock-out line (*lht1-5*) were included in two different uptake studies. In the first study, the effect of toxic D-ala on plant growth was studied (Paper II, Figure 2). Here, the root biomass is shown as a percentage of Col-0 (Figure 2). Consistent with previous studies, the *lht1-5* mutant, which is deficient in amino acid uptake, showed increased root biomass (587%), suggesting reduced uptake of the toxic amino acid. In contrast, *LHT1-OE* lines exhibited significantly reduced biomass (*LHT1-1* = 36%, *LHT1-2* = 22.5% of Col-0), consistent with enhanced amino acid uptake. Notably, cpk1 mutants also showed a pronounced reduction in root biomass (*cpk1-1* = 58%, *cpk1-2* = 48% of Col-0), similar to *LHT1-OE* lines. This suggests that a loss of CPK1 function leads to increased amino acid uptake, identifying CPK1 as a negative regulator of the uptake process. While these findings indicate a clear role for CPK1 in modulating amino acid uptake, the precise mechanism remains unclear. In particular, it is not known whether this regulation occurs via an alternative pathway, however, it is possible that the regulation occurs through direct interaction with LHT1 and hence steers a change in LHT1's activity.

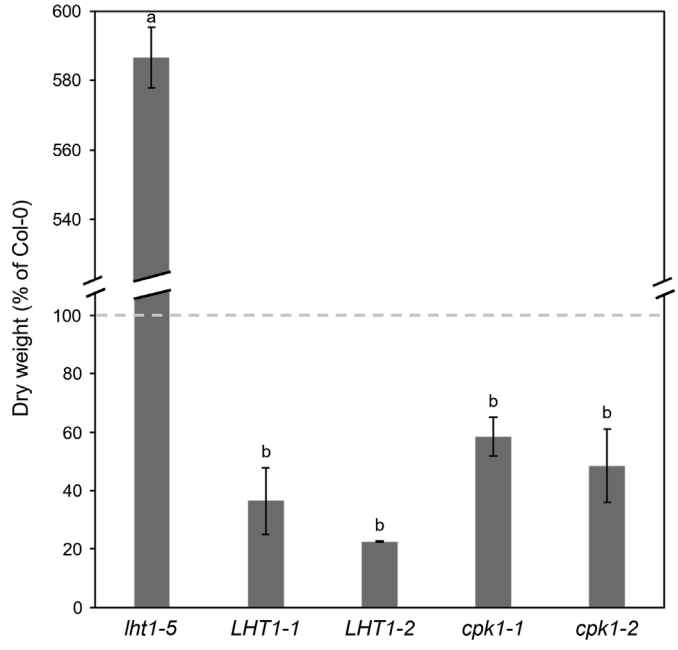

Figure 2: Growth of *A. thaliana* T-DNA insertion lines *lht1-5*, *cpk1-1*, and *cpk1-2* and overexpression lines *LHT1-1* and *LHT1-2*. Plants were grown on N-free ½ MS medium supplemented with 3 mM NO_3^- for 7 days and then transferred to ½ MS medium containing 3 mM NO_3^- + 10 mM D-ala for an additional 14 days. Plants had a total age of 21 days. Bars show mean biomass as a percentage of the biomass of Col-0 ± SE, n=3. The Col-0 biomass corresponds to a mean dry weight of 0.06 mg. The horizontal dashed line indicates the biomass of Col-0. Different lowercase letters indicate significant differences between mutant and overexpression lines ($P \leq 0.05$, one-way ANOVA and Tukey's post hoc test).

To obtain more detailed and quantitative insight into the role of CPK1 in amino acid uptake, a second experiment was conducted using ^{15}N-labeled L-gln (Paper II, Figure 3). As expected, *lht1-5* showed significantly reduced uptake compared to Col-0, while *LHT1-OE* exhibited an increased uptake, consistent with their known transport capacities. Notably, *cpk1-1* and *cpk1-2* mutants also displayed a significant increase in ^{15}N L-gln uptake, confirming the phenotypic response in the D-ala assay (Paper II, Figure 2a). These results further support the finding that CPK1 negatively regulates amino acid uptake. The experiment was conducted using a L-gln concentration of 50 µM, which is within the known affinity range of LHT1 (Svennerstam et al., 2011). Other root amino acid transporters, such as AAP1

or AAP5, operate at different concentrations or target different amino acid groups, respectively. Given these observations, CPK1 likely contributes to the amino acid uptake through the regulation of LHT1.

4.5 CPK1 negatively affects LHT1's activity

The identification of CPK1 as a negative regulator of amino acid uptake in *A. thaliana* provides an important step toward understanding the regulatory mechanisms governing amino acid transmembrane transport. However, to gain deeper insight, it is essential to determine whether the known interaction between LHT1 and CPK1 influences LHT1's uptake activity directly.

To address this, yeast complementation co-expression assays were performed using the amino acid uptake-deficient yeast mutant strain 22574d (Paper II, Figure 5) (Jauniaux et al., 1987). This strain has previously been used to test the functionality of heterologously expressed amino acid transporters, such as LHT1 (Hirner et al., 2006; Gratz et al., 2021). In this system, a N-free yeast growth medium supplemented with 3 mM L-cit served as a readout for functional transport: Yeast expressing functional *LHT1* is able to grow under these conditions (Gratz et al., 2020).

The co-expression of *LHT1* and *CPK1* in this yeast mutant inhibited the growth compared to the sole expression of *LHT1*, indicating a loss of transport activity (Paper II, Figure 5). In contrast, the co-expression of *LHT1* with a kinase-dead version of CPK1 (*CPK1_D274A*) (Durian et al., 2020 B), which lacks the ability to phosphorylate target proteins, restored the growth. The results confirm the negative effect of CPK1 on LHT1 activity and demonstrate its dependence on the kinase function. These findings strengthen the evidence for the role of CPK1 as a negative regulator of amino acid uptake, specifically by modulating LHT1 transport activity. Collectively, we demonstrated that CPK1 interacts with LHT1 and suppresses LHT1-mediated amino acid uptake in yeast. Moreover, the observed L-gln-dependent expression pattern in roots is consistent with such a regulatory role, highlighting CPK1 as a key component in the control of amino acid acquisition. It is therefore hypothesized that CPK1 phosphorylates LHT1 to fine-tune its activity in organic N uptake.

4.6 Phospho-mimicking reveals a regulatory mechanism for LHT1 activity

Protein phosphorylation can have diverse regulatory effects. The phosphorylation of NRT1.1, for instance, alters its affinity, whereas the phosphorylation of AMT1.3 directly affects its activity (Ho et al., 2009; Sun et al., 2014; Wu et al., 2019). To investigate whether phosphorylation modulates the activity of LHT1, phospho-mutant variants of LHT1 (*LHT1m*) were generated and analyzed (Paper II, Figure 6). In silico predictions, using the phospho-site prediction tool PhosPhAt (version 4), identified two threonine residues, at positions T251 and T255, located within the cytosolic domains of LHT1 as potential phosphorylation sites (Paper II, Supplemental Figure 6a). To assess the functional relevance of these sites, the target residues were mutated to either glutamic acid (*LHT1m_T251E*, *LHT1m_T255E*) or to alanine (*LHT1m_T251A*, *LHT1m_S254A_T255A*) to create phospho-mimic or phospho-dead mutants, respectively. These *LHT1m* variants were expressed in the amino acid uptake-deficient yeast strain 22574d to assess their impact on transporter function. Variants that enhance LHT1 activity are expected to restore yeast growth, which was evaluated using N-free medium supplemented with 3 mM L-cit (Paper II, Figure 6). The results revealed that mimicking phosphorylation at T251 (*LHT1m_T251E*) reduced yeast growth compared to wild-type LHT1, indicating that phosphorylation at T251 negatively impacts LHT1 activity. In contrast, a phospho-mimic mutation at T255 (*LHT1m_T255E*) did not affect the transport function, with transport activity remaining similar to that of LHT1 wild-type. The corresponding phospho-dead mutants showed contrasting effects on the growth. *LHT1m_T251A* indicated wild-type-like growth pattern, indicating preserved transport activity. In contrast, the double mutation at S254 and T255 (*LHT1m_S254A_T255A*) showed reduced transport activity.

These findings indicated that different phosphorylation sites of LHT1 may have distinct regulatory roles. Such multi-site regulation is not uncommon, for instance, phospho-regulation of AMT1;3 occurs at two different amino acid residues depending on external N conditions (Wu et al., 2019). Given that CPK1 is a serine/threonine kinase and negatively regulates LHT1 activity, T251 emerges as a potential phosphorylation target site responsible for the observed repression of amino acid transport.

Taken together, the results presented in Paper II reveal that LHT1 is subject to multiple layers of regulation, both at the transcriptional and post-translational levels. We identified a protein-protein interaction with CPK1, which acts as a negative regulator of amino acid root uptake and LHT1 transport activity. Although the trigger for this regulatory response remains unknown, it appears to be linked to external amino acid availability, as indicated by the L-gln-dependent expression of both *LHT1* and *CPK1* genes and the role of LHT1 as a key amino acid transporter in roots. Phosphomimic experiments further showed that phosphorylation can have a residue-dependent effect on LHT1 function, enhancing activity in some variants while impairing it in others. Together, these results underscore the importance of an amino acid sensing mechanism in plants to finely regulate this essential process.

5. Conclusion and outlook

5.1 Identification of a regulatory mechanism for the uptake of amino acids

The results presented in this thesis, together with Papers I and II, describe a stepwise approach showing that CPK1 negatively regulates LHT1-mediated root amino acid uptake in *A. thaliana* roots while looking at the molecular responses of plants to organic N presence:

1) Protein interaction - Confirmation of LHT1 and CPK1 protein interaction in yeast and *in planta*.

2) Transcriptional regulation - Identification of an L-gln-dependent, opposing gene expression pattern of *LHT1* and *CPK1 in planta*.

3) Uptake regulation - Highlighting that CPK1 reduces amino acid uptake *in yeast*.

4) Transport activity - CPK1 negatively affects the transport activity of LHT1 in yeast and *in planta*.

The Ca_2^+-activation mechanism of the CPK family is well characterized (Harmon et al., 1994; Harper et al., 1994; Huang et al., 1996; Liese & Romeis, 2013; Yip Delormel & Boudsocq, 2019). Furthermore, it is known that the presence of external inorganic N can trigger distinct Ca_2^+ signatures that activate calcium-dependent kinases (Riveras et al., 2015). However, whether external organic N conditions, such as amino acids, obtain similar Ca_2^+ responses remains unclear. In our study, we found that *CPK1* expression is down-regulated by low L-gln concentrations, supporting the hypothesis of a negative regulator of L-gln uptake (Paper II, Figure 1). At low external L-gln levels, plants require elevated expression and activity of high-affinity transporters, such as LHT1, to cope with low nutrient availability. In this context, negative regulators like CPK1 should be inactivated to allow efficient uptake. This leads to the following hypothesis (Figure 3): high external L-gln concentrations may induce a specific Ca_2^+ signature that activates CPK1. Once activated, CPK1 phosphorylates LHT1, thus reducing

its activity and maintaining a high-affinity uptake system. Conversely, low L-gln concentrations might create a Ca_2^+ signature, which is not recognized by CPK1, preventing its activation and downregulating its gene expression, thereby allowing LHT1 to remain active. The regulation of amino acid uptake in response to external L-gln levels, as well as the observed root biomass allocation, points towards the possibility that plants possess specific amino acid sensors. For the plant to adjust uptake accordingly, it must be able to detect both the presence and concentration of external amino acids, similar to the known NO_3^- transporter NRT1.1 (Wang & Crawford, 1996; Ho et al., 2009; Sun et al., 2014).

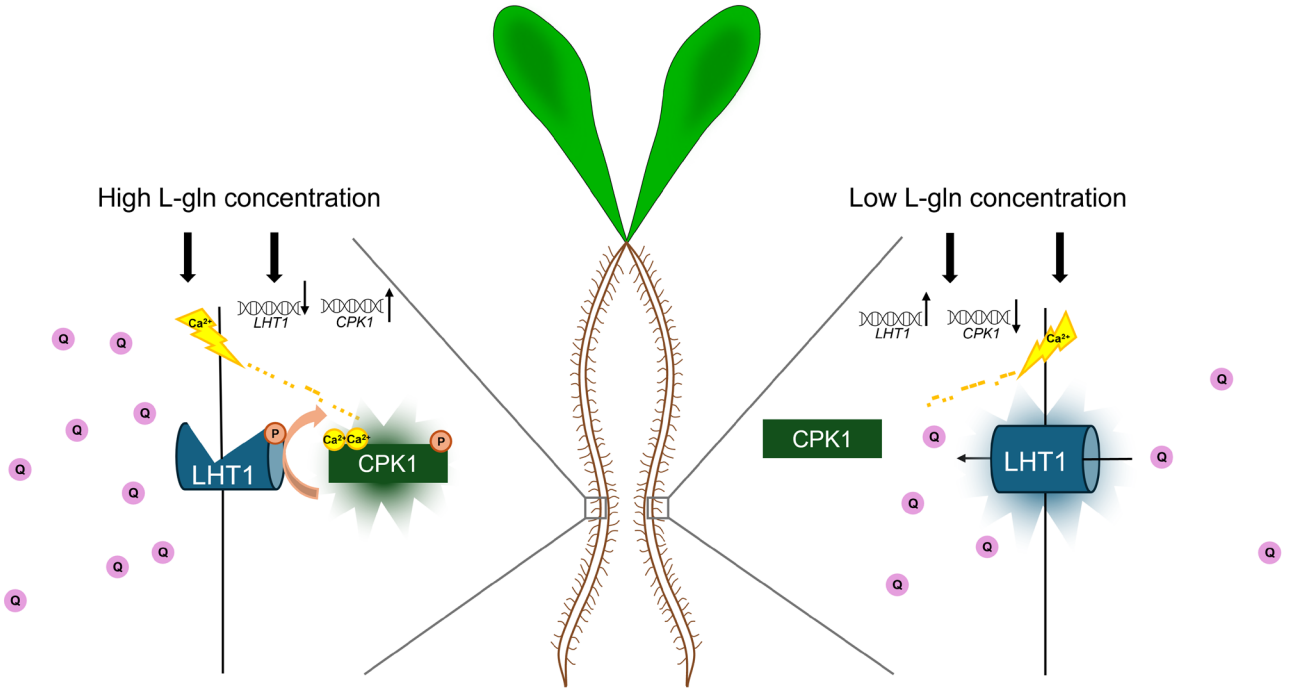

Figure 3: Proposed model for the L-gln-dependent regulation of LHT1. At high external L-gln (Q, purple) concentrations (left side of the figure), a specific Ca_2^+ signature (yellow) is generated that activates the kinase CPK1. Activated CPK1 phosphorylates (P, orange) the amino acid transporter LHT1. High L-gln levels also modulate LHT1 and CPK1 transcript levels, increasing *CPK1* expression while decreasing *LHT1* expression, resulting in an opposing expression pattern. At low external L-gln concentrations (right side of the figure), a different Ca_2^+ signature is generated, which is not recognized by CPK1. As a result, LHT1 is not phosphorylated and remains active. Low L-gln also promotes *LHT1* gene expression, while downregulating *CPK1* expression levels.

5.2 Exploring organic nitrogen sensing in plants

Inorganic N-sensing mechanisms are well known in plants (Ho et al., 2009). NRT1.1, for instance, not only facilitates uptake of nitrate but also acts as a sensor, a so-called transceptor (Wang et al., 1998; Ho et al., 2009; Sun et al., 2014). This inorganic N transceptor is well understood and tightly regulated, particularly at the post-translational level, to fine-tune N uptake under varying environmental conditions and contributes significantly to high-affinity NO_3^- uptake (Wang et al., 1998; Okamoto et al., 2003; Ho et al., 2009; Sun et al., 2014). NRT1.1 can bind NO_3^-, which leads to allosteric changes in the transporter and affects the accessibility of Thr101 for phosphorylation. In the absence of NO_3^-, multiple processes occur simultaneously. The transporter is phosphorylated at Thr101 by CIPK23 (Ródenas & Vert, 2021). This phosphorylation acts as a switch to a high-affinity transport mode (Ho et al., 2009; Sun et al., 2014). In addition, it causes structural changes by shifting NRT1.1 from a homodimeric to a monomeric state and promoting its interaction with Cyclic Nucleotide-Gated Channels 15 (CNGC15) to form a transceptor-channel complex (Sun et al., 2014; Wang et al., 2021). This interaction inhibits the c influx and affects downstream signaling pathways. On the other hand, in the availability of NO_3^-, NRT1.1 binds the molecule, inducing conformational changes that disrupt the transceptor-channel interaction and enable a NO_3^- specific NO_3^- influx. Additionally, NRT1.1 is dephosphorylated, shifting from monomeric to a homodimeric status and from a high- to a low-affinity status. This example highlights the importance and complexity of N transceptors and their regulation.

In contrast, amino acid sensors in plants are not known, however, they have been identified in mammalian systems (Hyde et al., 2007; Dinkeloo et al., 2018). This leads to the question, whether plant amino acid transporters could serve as organic N sensors. Among the amino acid transporters, AtLHT1 stands out as a key high-affinity transporter, significantly affecting the uptake of a broad range of amino acids, similar to NRT1.1 (Svennerstam et al., 2011).

The findings of a distinct L-gln-induced phenotype and the ability of plants to distinguish between N sources with two sides of their root system (Paper I) led to further studies to better understand the role of amino acids and to analyze the potential of an organic N-sensing mechanism. The hypothesis of an amino acid-sensing mechanism was supported with an

additional growth experiment (Figure 4). *A. thaliana* plants were grown on N-free ½ MS medium supplemented with varying ratios of L-gln and NO_3^-, maintaining a total N concentration of 3 mM. Surprisingly, the addition of only 25% L-gln to 75% NO_3^- led to a significant increase in root biomass compared to plants with access to 100% NO_3^-. The displayed root biomass investment would only be beneficial if the plant is able to sense the availability of the preferred N source, otherwise, allocating resources to the root system despite sufficient inorganic N would be inefficient.

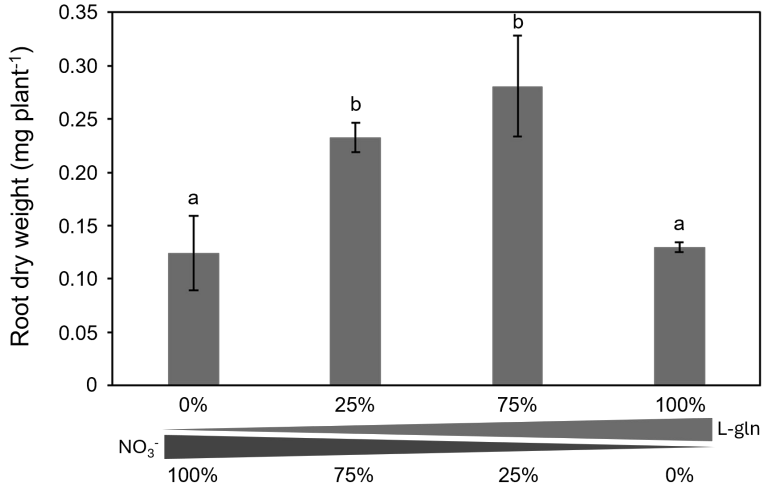

Figure 4: Root biomass of 21-day-old *A. thaliana* seedlings grown on mixtures of L-gln and NO_3^-. Plants were grown on N-free ½ MS medium supplemented with varying ratios of L-gln and NO_3^-, maintaining a total N concentration of 3 mM. Bars represent mean ± SE, n=3. Different lowercase letters indicate significant differences between the N ratios ($P \leq 0.05$, one-way ANOVA and Tukey's post hoc test).

Furthermore, this observation suggests that the C-bonus alone may not fully explain the response, pointing instead to the involvement of a sensing mechanism. This suggests that, together with the presence of amino acid transceptors in mammalian systems, a similar transceptor mechanism may also apply to *A. thaliana*. Despite ongoing research, identifying amino acid transceptors in plants remains challenging. Dinkeloo et al. emphasized that it is not sufficient to identify a correlation between transporter activity and a nutrient signal, but also to demonstrate that the transporter is the origin of the signal. While the presented data do not conclusively demonstrate a

transceptor's role for LHT1, they provide important indications supporting this possibility. For instance, a hallmark of transceptors is that their expression increases under low availability of the corresponding nutrient. Consistent with this, I observed elevated *LHT1* expression under low L-gln concentrations as well as NO_3^- availability (Paper II, Figure 1), which together reflect conditions of organic N deprivation. Furthermore, the analysis of the *A. thaliana* interactome study identified CNGC13 as a potential interactor of LHT1 (Chen et al., 2012). Multiple CNGC family members have already been identified to be involved in NO_3^- sensing (CNGC15) and root hair development (CNGC5, 6, and 9) (Wang et al., 2021; Zhu et al., 2025). These findings support the hypothesis that LHT1 may function as more than just a transporter, potentially acting as a hub for interacting proteins that might facilitate the sensing of external amino acid levels and regulate the plant's response and uptake. However, to fully understand the molecular mechanism behind potential amino acid sensing, more experiments need to be conducted.

5.3 Differential regulation of LHT1 in roots and shoots

The main focus in this thesis was on the root amino acid uptake, however, previous studies have shown that LHT1 is expressed not only in root tissues but also in mesophyll cells, suggesting it may serve multiple functions (Hirner et al., 2006).

The broad expression pattern of *LHT1* highlights its significance, as few N transporters operate across multiple tissue types. While LHT1 is well established in facilitating amino acid soil uptake, its role in the shoots remains less defined, however, it has been linked to leaf senescence and pathogen responses (Hirner et al., 2006; Svennerstam et al., 2007; Liu et al., 2010; Rogan et al., 2024). Similarly, CPK1 is known to be involved in leaf-specific processes such as senescence and pathogen defense responses, and was recently shown to play a role in root hair development (Coca & San Segundo, 2010; Durian et al., 2020 A; Zhu et al., 2025). These overlapping functional roles suggest that the interaction between LHT1 and CPK1 may be of significance in the different plant tissues, with potentially different functions in roots and shoots, and should be part of future research in order to fully understand how amino acids affect plants and the role of LHT1 (Dinkeloo et al., 2018).

Supporting gene expression analysis using Col-0 plants grown on high (1.5 mM L-gln or 3 mM NO_3^-) N or low (0.5 mM L-gln or 1 mM NO_3^-) N concentration revealed differential regulation patterns (Figure 5). As previously described in roots, low L-gln conditions led to increased *LHT1* expression in shoots, compared to high concentrations. However, in contrast to the root response, high NO_3^- concentrations caused a nearly fivefold increase in *LHT1* expression in shoots.

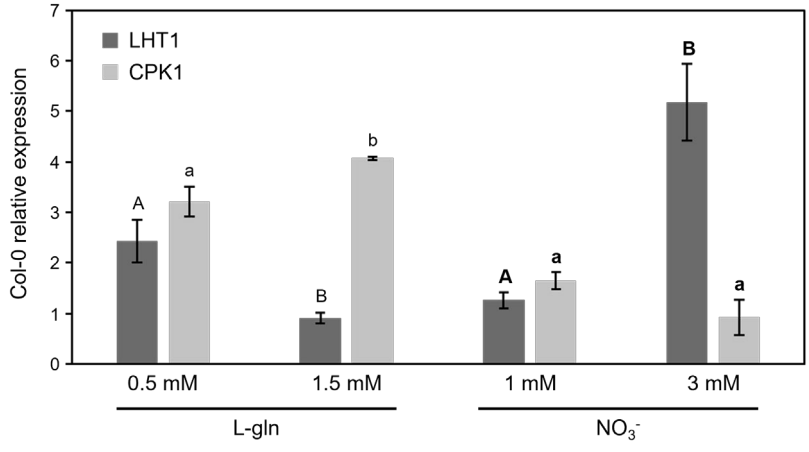

Figure 5: Growth of *A. thaliana* Col-0 plants on N-free ½ MS medium supplemented with either high (3 mM N in form of 1.5 mM L-gln or 3 mM NO_3^-) or low (1 mM N in form of 0.5 mM L-gln or 1 mM NO_3^-) concentrations of L-gln and NO_3^-. Bars represent relative gene expression of *AtLHT1* (dark grey) and *AtCPK1* (light grey) in shoots. Actin (*AtACT2*) and Ubiquitin (*AtULP*) served as reference genes. Bars represent mean values ± SE, n=4. Gene expression was analyzed using one-way ANOVA followed by Student's t-test ($P \leq 0.05$). Different capital and lowercase letters indicate statistically significant differences between high and low L-gln concentrations for LHT and CPK1, respectively. Different bold capital and lowercase letters indicate statistically significant differences between high and low NO_3^- concentrations for LHT and CPK1, respectively.

These findings suggest that LHT1 is subject to distinct regulatory controls in roots and shoots, potentially reflecting tissue-specific functional roles. The increased expression might be due to increased NO_3^- assimilation and amino acid synthesis, and transport to the shoot tissue at external NO_3^- conditions (Chen & Bush, 1997; Hirner et al., 2006; Liu & Bush, 2006; Muratore et al., 2021). Interestingly, *CPK1* expression in shoots is not influenced by L-gln concentrations and does not exhibit an expression pattern that opposes *LHT1* under these conditions. However, shoot expression of *CPK1* is sensitive to

NO_3^- levels, showing a decrease under high NO_3^- concentrations. These results suggest that, in shoots, both *LHT1* and *CPK1* are regulated by a different N source compared to roots and display an opposing expression pattern. This may indicate a distinct functional relationship between LHT1 and CPK1 in shoot tissues, possibly involving different regulatory triggers. The regulation of LHT1 in mesophyll cells could therefore serve a different physiological role than in roots. However, whether the amino acid transport activity in shoot tissues is affected remains to be tested.

5.4 Leaf senescence

In the shoot, one of LHT1's physiological roles was identified as contributing to leaf senescence (Svennerstam et al., 2007). *A. thaliana* mutants lacking functional LHT1 (*lht1-5*) display an early leaf senescence phenotype, suggesting that impaired amino acid import can trigger the activation of senescence programs in leaves. CPK1 has been identified as a positive regulator of leaf senescence (Durian et al., 2020 B), promoting this process by phosphorylating the key senescence transcription factor ORE1, enhancing ORE1's activity, and leading to the upregulation of senescence-related genes. Notably, a recent study suggested that ORE1 may directly regulate the transcription of LHT1 (Liao et al., 2020), highlighting another potential regulatory level of LHT1. To investigate whether ORE1 influences amino acid uptake, ^{15}N-labeled L-gln uptake assays were performed using the *A. thaliana ore1-1* mutant line. The results showed no significant difference in amino acid uptake in roots compared to wild-type plants (Figure 6), suggesting that ORE1 might not affect amino acid uptake in roots under the tested conditions.

Interestingly, LHT1 and CPK1 appear to have opposing roles in shoot tissue, similar to the root regulation of LHT1. While CPK1 promotes senescence through activation of downstream genes, LHT1 may act as a negative regulator. The early senescence observed in *lht1-5* mutants implies that adequate amino acid import via LHT1 is necessary to delay or prevent senescence, suggesting that LHT1 contributes to maintaining leaf vitality under normal growth conditions.

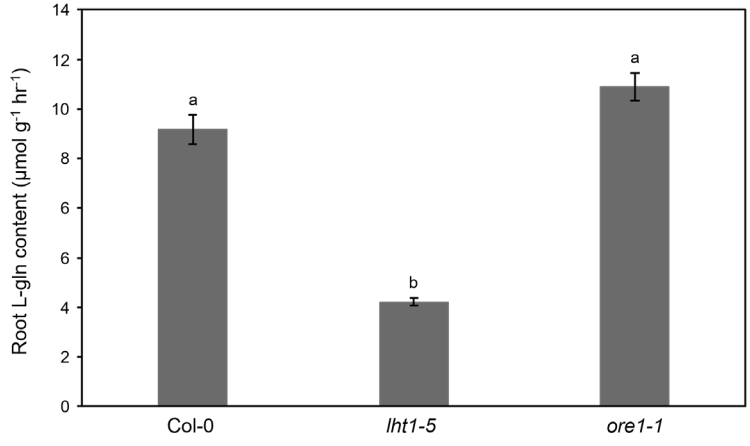

Figure 6: Root L-gln content of Col-0 and T-DNA insertion lines *lht1-5* and *ore-1-1*. Seedlings were grown on N-free ½ MS medium supplemented with 3 mM NO_3^- for 21 days. The uptake experiment was conducted with a solution of 50 µM L-gln, and the seedlings were incubated in it for 15 min. Bars represent mean L-gln concentration ± SE, n=5. Different lowercase letters indicate significant differences between lines ($P \leq 0.05$, one-way ANOVA and Tukey's post hoc test).

This thesis aimed to deepen our understanding of amino acid uptake, examining its effects from whole-plant growth to molecular regulation. It provides novel insights into organic N nutrition and uncovers previously unknown regulatory mechanisms involving the key amino acid transporter LHT1 and the protein kinase CPK1. Importantly, this work contributes to the ongoing debate of organic N plant nutrition. If amino acids were insignificant in ecosystems and throughout evolution, plants would gain no fitness advantage from regulating amino acid uptake. However, this thesis demonstrates that amino acid uptake is subject to regulation through mechanisms similar to inorganic N uptake regulation, suggesting that amino acids are important N sources.

At the same time, these discoveries exposed new questions. Future studies may explore the potential function of LHT1 as a transceptor and additionally test the hypothesis of whether the presence of amino acids might trigger its post-translational regulation. This includes investigating whether the presence or absence of amino acids induces specific Ca^{2+} signatures.

Identifying additional LHT1 interaction partners, such as CNGC13, and understanding their involvement in the amino acid transport will also be important next steps. To improve plant growth and resilience, the function of LHT1 during biotic and abiotic stress in shoot tissues should be investigated in more detail. Finally, homologues of LHT1 have been identified in important crops, such as rice and tomato. Understanding the regulatory mechanisms of amino acid transport in these crops could enable strategies to enhance yields and reduce losses caused by, for instance, pathogens.

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Popular science summary

Nutrients are as important for plants as they are for us humans. One of the most important nutrients is nitrogen, and it exists in two different chemical forms. The first is inorganic nitrogen, like nitrate and ammonium. The second is organic nitrogen, which includes amino acids. For a long time, scientists focused on inorganic nitrogen, and most of what we know about how plants take up nitrogen is based on this form. However, inorganic nitrogen has disadvantages, it can easily leach through the soil into the groundwater, causing environmental problems. In recent years, researchers discovered the importance of organic nitrogen forms. They found that plants can absorb amino acids from the soil and that they could serve as an alternative to inorganic nitrogen. One major advantage of organic nitrogen is that it contains carbon. This carbon delivers additional energy for the plant, which means organic nitrogen is not only nutritious but also an energy source.

The research field of organic nitrogen is underdeveloped compared to that of inorganic nitrogen, and many of details remain unknown. This PhD thesis is looking at some of the open questions, focusing on how organic nitrogen affects root growth and how the uptake of organic nitrogen is regulated.

The first part focuses on the effect of the amino acid glutamine on plant growth. Experiments showed that when glutamine was provided for the plant, its roots developed a unique shape; they were bigger and grew more root hairs. Interestingly, similar root growth has been observed when plants are nitrogen-deficient. Normally, such changes would help the plant to take up more of the missing nutrient from the soil. However, measurements revealed that the glutamine-grown plants were not nitrogen-deficient, identifying a specific glutamine-related root type.

The second part of this work looks at how plants take up amino acids. Special proteins in the roots, called transporters, move amino acids from the soil into the plant. It is known that the activity of inorganic nitrogen transporters can be adjusted. This means that the transporters can be turned on or off. This work looks into one particular amino acid transporter, the Lysine Histidine Transporter1 (LHT1), to understand whether and how the transporter´s activity is adjusted. Experiments identified another protein, Calcium Dependent Kinase1 (CPK1), that was shown to interact with LHT1. Furthermore, it was shown that the interaction between CPK1 and LHT1

negatively influences root amino acid uptake of the plant and the activity of the transporter LHT1. This means that CPK1 is reducing the amino acid uptake under specific conditions. Additional experiments pinpointed two amino acid positions in the LHT1 protein where such an adjustment on the activity could take place.

In addition, this work also highlights the role of amino acids and their transporters beyond nutrition. For instance, amino acids are important components in plant-pathogen interactions. On the one hand, plants may use amino acids as a part of their defense system. On the other hand, pathogens try to steal these valuable nutrients. Because of this, amino acid transporters can play an important role in whether the plant survives a pathogen infection or the pathogen gains the upper hand.

Thus, this work provides new insights into the influence that amino acid nutrition has on the plant and demonstrates how the amino acid uptake is regulated.

Populärvetenskaplig sammanfattning

Näringsämnen är lika viktiga för växter som de är för oss människor. Ett av de viktigaste näringsämnena är kväve, och det förekommer i två olika kemiska kategorier. Den första är oorganiskt kväve, såsom nitrat och ammonium. Den andra är organiskt kväve, som inkluderar aminosyror. Under lång tid har forskare främst fokuserat på oorganiskt kväve, och det mesta vi vet om hur växter tar upp kväve bygger på studier av oorganiskt kväve. Men oorganiskt kväve har nackdelar – det kan lätt lakas ur jorden och hamna i grundvattnet, vilket orsakar miljöproblem. Under de senaste åren har forskare upptäckt betydelsen av organiskt kväve. De fann att växter kan ta upp aminosyror från jorden och att dessa kan fungera som alternativ till oorganiskt kväve. En fördel med organiskt kväve är att det innehåller kol. Detta kol ger extra energi till växten, vilket innebär att organiskt kväve inte bara är ett näringsämne utan också en energikälla.

Forskningsfältet kring organiskt kväve är mindre utvecklat än för oorganiskt kväve, och många av dess funktioner är fortfarande okända. Denna doktorsavhandling undersöker några av de öppna frågorna, med fokus på hur organiskt kväve påverkar rotutvecklingen och hur upptaget av organiskt kväve regleras.

Den första delen av arbetet fokuserar på effekten av aminosyran glutamin på växters tillväxt. Experiment visade att när växterna fick glutamin utvecklade deras rötter en särskild form. Rötterna hos dessa växter blev större, mer förgrenade och utvecklade fler rothår. Intressant nog observerades ett liknande rotmönster hos växter som lider av kvävebrist. Normalt skulle sådana förändringar hjälpa växten att ta upp mer av det saknade näringsämnet från jorden. Men mätningar visade att de växterna som odlats på glutamin inte hade kvävebrist, vilket pekar på en specifik glutaminrelaterad rotutveckling.

Den andra delen av arbetet undersöker hur växter tar upp aminosyror. Speciella proteiner i rötterna, så kallade transportörer, flyttar aminosyror från jorden in i växten. Det är känt att aktiviteten hos transportörer för oorganiskt kväve kan justeras, det vill säga att de kan slås på eller av. Mitt arbete har fokuserats på en specifik aminosyratransportör, LHT1 (LYSINE HISTIDINE TRANSPORTER1), för att förstå om och hur transportörens aktivitet regleras. Experimenten identifierade ett annat protein, CPK1 (CALCIUM DEPENDENT KINASE1), som interagerar med LHT1.

Dessutom visade resultaten att CPK1 påverkar växtens aminosyraupptag negativt och minskar aktiviteten hos LHT1. Det innebär att CPK1 begränsar aminosyraupptaget under vissa förhållanden. Ytterligare experiment pekade ut två positioner i LHT1-proteinet där en sådan justering av aktiviteten kan ske.

Dessutom belyser detta arbete aminosyrornas och aminosyratransportörernas roller utöver deras funktion som näringsämnen. Till exempel är aminosyror viktiga komponenter i växt-patogeninteraktioner. Å ena sidan kan växter använda aminosyror som en del av sitt försvarssystem. Å andra sidan försöker patogener stjäla dessa värdefulla näringsämnen. Därför kan aminosyratransportörer spela en avgörande roll för om växten överlever en infektion eller om patogenen får övertaget.

Sammanfattningsvis ger detta arbete nya insikter i hur aminosyrabaserad näring påverkar växter och visar hur upptaget av aminosyror regleras.

Acknowledgements

Four years of PhD studies. Four long, exhausting, but also happy, successful, and rewarding years. Years filled with people who helped, taught, and supported me. People who were there for me when I needed them. Here, I would like to thank you all. This journey would not have been possible without you!

First and foremost, I would like to thank my main supervisor, **Torgny**. You welcomed me into your group almost 6.5 years ago, when I was working on my master's thesis, which is when I first became fascinated by nitrogen and its uptake. But it wasn't just the project that made me want to continue, it was also your exceptional way of supervising and the supportive atmosphere you fostered in the group. I am very grateful for the opportunities I received and for your constant support and encouragement. You guided me in developing my own ideas, creating a project I can identify with, and, most importantly, you encouraged me to believe more in myself and my work.

Regina, where do I begin to thank you? Our time together goes so far back that I can't even remember when our relationship as supervisor and student turned into a wonderful friendship that helped me through the ups and downs of my PhD. You were always there for me, with valuable advice and many hours at the whiteboard. And even beyond work, you always had an open ear and time for me. Thank you for reminding me to celebrate even the small successes. You made me the scientist I am today, and I am glad to know I can always count on you.

Ioanna, thank you for your constant support and help throughout the years as my co-supervisor. I deeply appreciate that you always had time for my big and small problems. Thank you for all the fika, lunches, and dinner we had together, which helped me forget the stress and the work for a moment.

Ellen, you were always there when two hands in the lab were just not enough, thank you! It is always fun to work with you, have brunch, just talk, or watch a movie (that we never managed), however, you stole my window seat on the plane, and I cannot forget that :) I enjoyed working with you and watching you develop more confidence in your work and your start as a PhD student. Even though I was not able to get you interested in LHT1, I wish you good luck and all the best for your PhD project and your future.

Justine, I am so happy that we met! You made working in the lab definitely more fun. However, you enjoyed scaring me a little too much… You showed me not to take everything too seriously and not to forget the fun at work and in life.

Thank you, **Tommy**, for an amazing collaboration on multiple projects. It would have been so much harder without your help and input. I am also very glad that we discovered common interests outside of work (JM).

I would also like to thank the **C/N Group** for every meeting and discussion we had. Thank you for your valuable input, interesting ideas, and support over the years. Even though the topics and projects in this group are so broad, I never had the impression that this was a disadvantage, rather, the opposite. I would also like to thank my **co-authors** on the different papers for your input and feedback, it would not have worked without you. Thanks to **Ulrika** for your help throughout the years. And special thanks to **Rumen** for the interesting ideas and the support you offered since my master's project. Thank you, **Sonja**, for your help in the lab in the last months of my PhD, and **Özer**, for your support and help, especially in these last crazy weeks.

Tinkara, this PhD would not have been the same without you! I am glad we could do this amazing journey together, and I really miss you in Umeå. It is crazy to think about all the things we did together, from organizing seminars and a conference to traveling to Svalbard and Hawaii. You were always there for me, to hear my complaints (a lot of them), to support me, and for fika. It is weird to think about not working together anymore, but I am excited to see what the future holds for us! I wish you all the best and good luck with everything you will do.

Marie, ich bin so froh, dass wir uns damals in der Ersti-Woche getroffen haben! Die Blaubeermuffins vor dem Mathetutorium haben diese langen Tage und die ersten zwei Semester definitiv einfacher gemacht. Auch wenn sich unsere Wege danach getrennt haben (zumindest an der Uni), haben wir uns nie aus den Augen verloren, und ich bin so dankbar für deine Freundschaft und Unterstützung in den letzten Jahren.

An dieser Stelle möchte ich auch meiner **Familie** danken. Ohne euch wäre ich nicht hier, wo ich heute bin. Ich bin unendlich dankbar für eure Unterstützung, Hilfe und euer Verständnis in den letzten Jahren. Auch wenn ich etwas weiter weggezogen bin, als uns allen vielleicht lieb war, hatte ich nie das Gefühl mich von euch zu entfernen. Die Carepakete und all die kleinen Gesten in den letzten Jahren haben mir das Heimweh genommen und gezeigt, dass ihr immer für mich da seid.

Finally, **Jonhan**, Thank You - a lot! I know that the past four years have been as exhausting for you as they were for me. However, your unlimited support and trust in me during that time never declined, and that means a lot to me. Thank you for always being there to listen to me after a long day, and for offering advice. You helped me through every single part of this PhD, comforting me when things were not going well, encouraging me to keep going, and celebrating small and big milestones with me.

Thank you all!

I

Research

New Phytologist

Plant organic nitrogen nutrition: costs, benefits, and carbon use efficiency

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Received: *26 September 2024*
Accepted: *30 October 2024*

New Phytologist (2025) **245:** 1018–1028
doi: 10.1111/nph.20285

Key words: amino acids, *Arabidopsis thaliana*, carbon use efficiency, glutamine, organic nitrogen, root hair.

Summary

- Differences in soil mobility and assimilation costs between organic and inorganic nitrogen (N) compounds would hypothetically induce plant phenotypic plasticity to optimize acquisition of, and performance on, the different N forms. Here we evaluated this hypothesis experimentally and theoretically.
- We grew Arabidopsis in split-root setups combined with stable isotope labelling to study uptake and distribution of carbon (C) and N from L-glutamine (L-gln) and NO_3^- and assessed the effect of the N source on biomass partitioning and carbon use efficiency (CUE).
- Analyses of stable isotopes showed that 40–48% of C acquired from L-gln resided in plants, contributing 7–8% to total C of both shoots and roots. Plants grown on L-gln exhibited increased root mass fraction and root hair length and a significantly lower N uptake rate per unit root biomass but displayed significantly enhanced CUE.
- Our data suggests that organic N nutrition is linked to a particular phenotype with extensive growth of roots and root hairs that optimizes for uptake of less mobile N forms. Increased CUE and lower N uptake per unit root growth may be key facets linked to the organic N phenotype.

Introduction

Plants have evolved a range of adaptions for optimizing acquisition of mineral nutrients. Thus, plants experiencing low-nitrogen (N) availability are characterized by a high-root mass fraction, increased root branching and increased root surface area through an extensive production of root hairs, and in relevant cases, symbiotic interactions with mycorrhizal fungi. These features of N-starved plants are well known from both old (Brouwer, 1962; Ågren & Ingestad, 1987) and more recent (Hermans *et al.*, 2006) studies and molecular cues underpinning such plant responses have been described (Kiba & Krapp, 2016). Based on data from 77 studies and 129 species, Reynolds & D'Antonio (1996) observed that in the majority of the cases, the root biomass ratio increased with decreased nitrogen availability. It has been assumed that such phenotypic characteristics will increase the fitness of plants in low-N environments through enhancing the ability to acquire the limiting resource – N (Brouwer, 1962). An increase in root hair density and/or -length is reported in a range of studies for nutrients that are relatively immobile in soil such as phosphorus and potassium (Gahoonia *et al.*, 1997; Gahoonia & Nielsen, 1998; Bates & Lynch, 2001; Jungk, 2001; Bienert *et al.*, 2021), but also for nutrients with higher mobility like inorganic N when occurring at low concentrations (Bhat *et al.*, 1979; Foehse & Jungk, 1983; Ewens & Leigh, 1985; Saengwilai *et al.*, 2021).

Plant N acquisition is mainly governed by two processes: diffusion (movement of N molecules through the soil water driven by a concentration gradient) and mass flow (transport together with soil water) (Nye, 1977; Tinker & Nye, 2000; McMurtrie & Näsholm, 2018). With decreasing N supply rates, concentrations of N in the soil solution decreases and hence the relative contribution of mass flow decreases. Consequently, plant responses to low-N supply should be aimed towards optimization for N acquisition via diffusion and this is mainly accomplished through an increase in root surface area. A model describing plant optimization for N acquisition (McMurtrie & Näsholm, 2018) points to the possibility that mass flow is enhanced when the internal spacing of roots (i.e. the mean distance between roots of the same plant) is large, and when the total root surface area is low. Thus, optimization of mass flow-driven N acquisition will predictably lead to lower N acquisition via diffusion. This suggests a trade-off between plant optimization for diffusive and mass flow-mediated N acquisition.

Plant acquisition of organic N should therefore primarily be governed by diffusion while acquisition of inorganic N, in particular, NO_3^-, should be governed by mass flow.

*These authors contributed equally to this work.

From the above one may conclude that for both low-N supply and for a dominance of organic N, plant fitness is linked to characteristics that optimizes N acquisition via diffusion, and hence phenotypic shifts associated with low-N availability should overlap with those related to organic N nutrition.

In nonmycorrhizal plants, the abundance and length of root hairs are pivotal for the total root surface area (Jungk, 2001; Smith & De Smet, 2012). As discussed above, root hair growth is highly responsive to the supply of immobile nutrients such as phosphate and potassium. Following the same logic, we can infer that root hair growth should also be responsive to immobile N forms. Root and root hair proliferation are dependent on photosynthetically derived carbohydrates but the actual costs in terms of energy and carbon (C) is strongly dependent on the source of N acquired by roots. Thus the biochemical cost for assimilation of different N forms varies and is substantially higher for NO_3^- compared to NH_4^+ (Bloom *et al.*, 2003). The difference is even greater comparing NO_3^- and organic N such as the amino acids glutamine and arginine (Zerihun *et al.*, 1998; Franklin *et al.*, 2017). Here, the difference originates both from the lower energetic requirements for reduction and assimilation of N but also from the extra C derived from uptake of organic N. A model based on these differences in biochemical costs of assimilation predicts a significant increase in root mass fraction linked to organic N nutrition (Franklin *et al.*, 2017). This would provide a feed-forward mechanism by which the lower costs for assimilation and the C bonus from organic N uptake enables a larger root surface investment that, in turn, enhances organic N nutrition.

Carbon use efficiency (CUE), the ratio of photosynthesis to respiration, is a critical factor for the global carbon budget and a key parameter in global vegetation models. It is well known that plant CUE is influenced by nutrient, in particular N, availability (Vicca *et al.*, 2012) but to what extent plant use of organic or inorganic N may affect plant CUE has not been investigated. However, the above-described differences in C costs pertaining to uptake and assimilation of different N forms would theoretically also influence plant CUE. Analysing the potential impact of organic vs inorganic N nutrition on plant CUE may hence provide important information for the development of new global C models.

Here, we theoretically (through modelling) and experimentally analysed the expected effects of different N forms on plant growth and C and N allocation. We grew *Arabidopsis thaliana* (Arabidopsis) axenically to investigate how root : shoot allocation, root hair formation, and CUE compares between the two N sources NO_3^- and L-gln. We hypothesized that plants grown on the organic N source would display a reduced N uptake per root mass, increased root biomass and -surface area, and an increase in root mass fraction. We used stable isotope labelling (^{13}C and ^{15}N) to quantify uptake and distribution of N and C sources by plants, enabling assessment of the role of C uptake for the development of an organic N phenotype and enabling calculation of effects of organic N uptake on plant CUE.

Materials and Methods

Plant material and growth conditions

In all experiments *A. thaliana* (L.) *Heynh. Col-0* (wild-type, WT) plants were used. The seeds were surface sterilized and stratified for 48 h at 4°C. Unless stated otherwise, all plant experiments have been performed using half-strength N-free Murashige & Skoog medium (MS) (Murashige & Skoog, 1962), supplemented with 3 mM N in form of either 1.5 mM L-gln or 3 mM KNO_3. 1% agar (Duchefa Biochemie, RV Haarlem, the Netherlands) was added after buffering the pH to 5.8 using 7.7 mM 2-(*N*-morpholino)ethanesulfonic acid (MES). The medium was free of sucrose. Potassium was compensated for in the L-gln treatment with addition of KCl equivalent to that in the KNO_3 treatment.

Experiment 1: the split-root experiment

Seeds were germinated on vertical plates filled with half-strength MS medium supplemented with 3 mM KNO_3 and 0.5% sucrose. The plants were grown for 14 d under short-day conditions with an 8 h : 16 h, day : night rhythm (Photosynthetic Photon Flux Density (PPFD) = 200 µmol m^{-2} s^{-1}). The primary roots of these 14 d old seedlings were cut to stimulate lateral root development, enabling the establishment of plants in the split-root system so that similar root biomass would be present in the two root compartments. After seven additional days of growth, the 21 d old seedlings were transferred to the horizontal-plate, split-root system and cultivated for additional 14 d.

For the split-root system, Petri dishes with two identical separate compartments were filled with half-strength N-free MS medium (Fig. 1). Each compartment of the Petri dish was supplemented with 3 mM N in form of 1.5 mM L-gln or 3 mM KNO_3 (L-gln/NO_3^-). The medium was free of sucrose. Petri dishes filled exclusively with one N source, 1.5 mM Gln or 3 mM KNO_3 were used as reference treatments (L-gln/L-gln or NO_3^-/NO_3^-) (Supporting Information Fig. S1). The in total 35 d old plants had root hair length evaluated during the experiment and were then harvested, dried at 60°C and prepared for biomass, N and C concentration.

Experiment 2

Seeds were germinated and plants were grown on horizontal plates containing half-strength MS medium supplemented with 3 mM KNO_3 and 0.5% sucrose for 21 d. Then the seedlings were transferred to four section Petri dishes containing either 1.5 mM universally labelled L-gln (^{15}N and ^{13}C; 10 atom% excess of each) or a mixture of universally labelled L-gln (1.5 mM) + KNO_3 (3 mM) (Fig. 2). A filtered (0.22 µm) air input was connected to each plate in order to avoid respired $^{13}CO_2$ to accumulate inside the system. Furthermore, control seedlings grown on nonlabelled L-gln were also included in each plate to account for re-fixation of respired $^{13}CO_2$. This experiment lasted either 1, 3 or 6 d and consisted of 13 biological replicates for the reference treatments

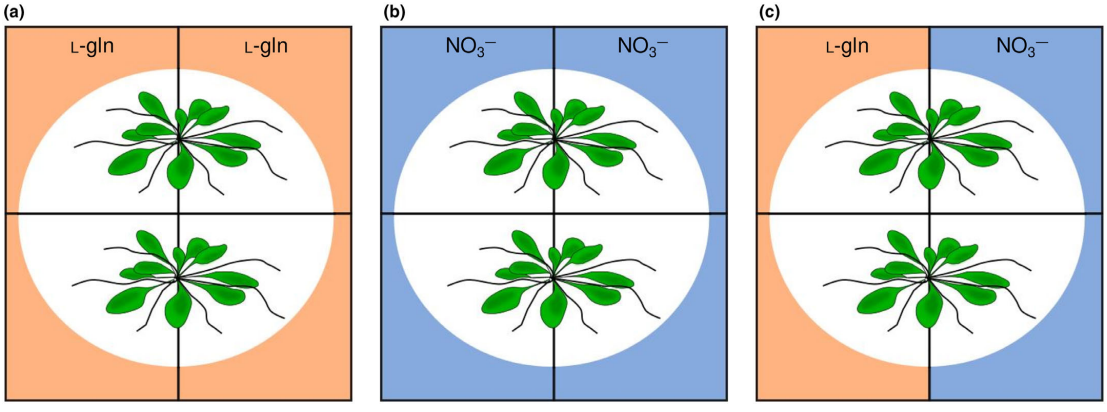

Fig. 1 Setup of experiment 1. Shoots were positioned on the middle rib of the plate and roots were divided equally between two growth compartments in which N was supplied as either: (a) 1.5 mM L-gln on both sides, (b) as 3 mM NO_3^- on both sides or (c) as 1.5 mM L-gln on one side and 3 mM NO_3^- on the other side. Results shown in Figs 3–5 and in Table 1 are derived from this experimental system.

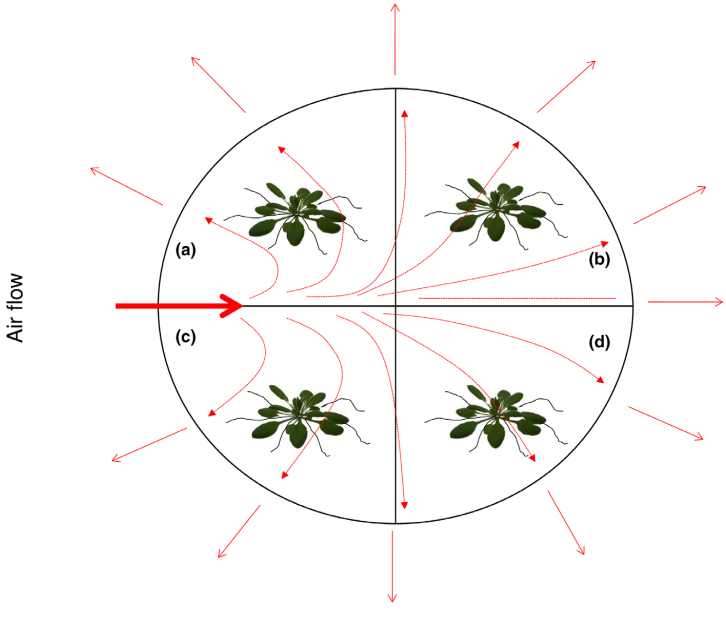

Fig. 2 Setup of experiment 2. ^{13}C, ^{15}N labelling to study uptake of C and N from L-gln. Seedlings precultivated on vertical plates and moved to plates with air flow. N was supplied as (a) 1.5 mM $U^{15}N_2{}^{13}C_5$ – L-gln (10 atom% enrichment) or on (b) 1.5 mM $U^{15}N_2{}^{13}C_5$ – L-gln + 3 mM NO_3^-. Control seedlings grown on (c) nonlabelled L-gln or on (d) nonlabelled L-gln + NO_3^-, to account for re-fixation of respired $^{13}CO_2$ were included in each plate. Results shown in Figs 6–8 and Supporting Information Figs S1 and S2 are derived from this experimental system.

and 26 biological replicates for the L-gln/NO_3^- treatment, each biological treatment including 60 technical replicates.

Measurements of N, ^{15}N, C and ^{13}C

For N and C analyses the samples were ground and homogenized. Analyses were conducted using an Elemental Analyzer – Isotope Ration Mass Spectrometer (EA-IRMS) (EA: Flash EA 2000, IRMS: DeltaV, both from Thermo Fisher Scientific, Waltham, MA, USA) (Werner *et al.*, 1999).

Root hair length measurement

Root hair development was analysed during the course of the organic and inorganic split-root experiment. Pictures of roots were taken 7 d after the transfer to the split-root growth system

(28 d old plants), using a Leica DC300 digital camera coupled to a Leica MZ95 stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). From each Petri dish compartment, 1–2 pictures of representative areas were taken. Only pictures of 28 d old seedlings were taken due to the high density of roots and root hairs in some treatments in later stages of the experiment. 600–2000 root hairs were measured per treatment using the program ImageJ 1.43 (http://imagej.nih.gov/ij/, Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). For this, 18–20 biological replicates have been analysed, each consisting of 60 technical replicates per treatment.

Calculations and data analysis

Isotopic data (atom% ^{13}C and atom% ^{15}N) was used to calculate the fraction of plant tissue C and plant tissue N derived from L-gln, considering the label intensity (10 atom% excess for ^{13}C and ^{15}N). All data were analysed using the Jmp Pro 16.0.0 software performing a one-way ANOVA followed by Tukey's *post hoc* test to evaluate the significance. Bars marked with different letters indicate significant differences at P-value ≤ 0.05.

Estimation of the effects of N form on N assimilation costs and carbon use efficiency

Carbon use efficiency of biomass growth (CUE) is equal to the fraction of C taken up (C_u) that remains in the biomass (C_b), that is $CUE = C_b/C_u$. CUE was calculated based on the $^{13}C:{}^{15}N$ ratio in biomass compared to L-gln molecules (C : N for L-gln = 0.47 g C g^{-1} N). Assuming all N taken up remains in the biomass, the observed $CUE_o = {}^{13}C:{}^{15}N$ biomass/$^{13}C:{}^{15}N$ L-gln.

To quantify the effect of N assimilation carbon costs on CUE, we modelled CUE as a function of the different assimilation costs of C, organic N, and inorganic N (C_c, oN_c, iN_c, respectively) and their contribution to biomass (C_b, oN_b, iN_b, respectively). The total C cost of biomass growth (C_{ctot}) is the sum $C_{ctot} = C_c\ C_b + oN_c\ oN_b + iN_c\ iN_b$. The net C ending up in biomass is $C_b = C_u - C_{ctot}$, which is combined with the expression $CUE = C_b/C_u$ to yield the equation for modelled CUE_m as a function of assimilation costs:

$$CUE_m = C_b/(C_c\ C_b + oN_c\ oN_b + iN_c\ iN_b + C_b) \qquad \text{Eqn 1}$$

The N assimilation costs were estimated by fitting CUE_m to CUE_o. For this we also need an estimate of the C assimilation cost C_c. The overall cost per plant C assimilation in biomass including associated N assimilation and other processes (the growth respiration), has been estimated to 0.43 (Choudhury, 2001). Because C_c is the cost excluding N assimilation costs, it must be lower than 0.43 and we assumed that $C_c = 0.2$. Smaller or larger C_c slightly affects the estimated average assimilation costs of the two N forms, but not the relative difference between their assimilation costs, which is our main interest here. To be able to use linear fitting (lm function in R software) we made oN_c and iN_c linear coefficients by transforming Eqn 1 to $(1/CUE_o - C_c - 1)\ C_b = oN_c\ oN_b + iN_c\ iN_b$.

Results

Organic N causes changes in plant phenotype

To test whether plant available organic or inorganic N forms affect a plant's phenotype differently, *A. thaliana* was grown in a split-root system, where the roots had access to either only L-gln (L-gln/L-gln), only NO_3^- (NO_3^-/NO_3^-) or both (L-gln/NO_3^-) (Fig. 1).

The root biomass of plants differed significantly between the N sources (Fig. 3). Plants grown solely on organic N (L-gln/L-gln) demonstrated a higher root biomass compared to plants with access to NO_3^-. However, seedlings with access to both N sources (L-gln/NO_3^-) showed opposing responses, the root side with access to NO_3^- had significantly higher biomass compared to the root side exposed to L-gln. In addition to that, plants grown solely on L-gln did not significantly differ in shoot biomass between the different N treatments. However, plants grown on both N sources (L-gln/NO_3^-) displayed significantly higher shoot biomass compared to plants grown solely on NO_3^- (Fig. 3). The shoot biomass production was influenced by the availability of L-gln and also the total biomass was enhanced by the availability of L-gln.

Plants grown exclusively on L-gln, developed elongated root hairs compared to plants that only had access to inorganic N (Fig. 4a,b). Plant roots with access to both N sources (L-gln/NO_3^-) exhibited similar responses as roots that had access to a single N source only (Fig. 4c,d). However, the root development was not affected by the corresponding N source on the other root side. Root hair length measurements confirmed these observations (Fig. 5). Seedlings grown solely on L-gln (L-gln/L-gln) displayed significantly increased root hair length compared to seedlings grown on NO_3^- as the sole N source (L-gln/L-gln = 0.45 ± 0.05 mm, NO_3^-/NO_3^- = 0.17 ± 0.01 mm; Fig. 5). A positive effect of L-gln on root hair length was also visible for plants having access to both N sources (L-gln/NO_3^-): The root side with access to organic N had significantly longer root hairs compared to the side which had access to NO_3^- (L-gln side = 0.41 ± 0.01 mm, NO_3^- side = 0.22 ± 0.01 mm). These results demonstrated that plants develop a unique root phenotype with increased root hair length when exposed to organic N (Fig. 4).

Organic N plants display similar N status but higher C concentration

Analysis of N and C contents of plants in the split-root experiment revealed that those grown on L-gln (L-gln/L-gln), or mixtures of L-gln and NO_3^- (L-gln/NO_3^-) had shoot-N concentrations equally high as those grown on NO_3^- only (Table 1). However, root N concentrations were lower for plants grown on L-gln and in the mixed N treatment, roots supplied L-gln displayed lower N concentrations than those supplied NO_3^- (Table 1). Carbon concentrations followed the opposite pattern, being higher for all treatments and both organs for plants supplied L-gln. Interestingly, in the mixed N treatment, roots supplied NO_3^- also exhibited increased C concentrations (Table 1).

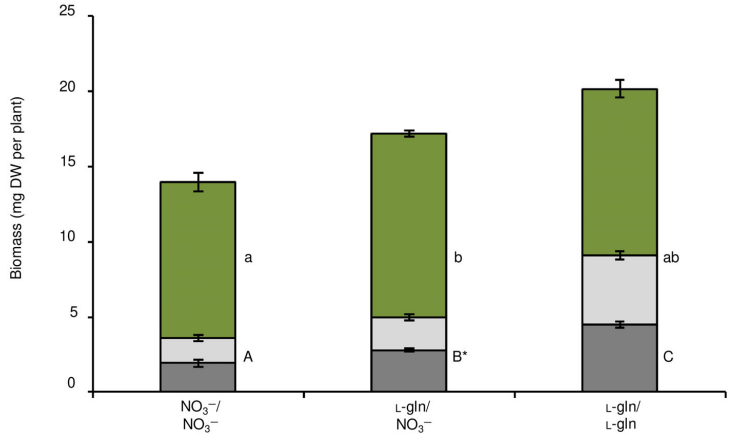

Fig. 3 Shoot and root biomass of *Arabidopsis thaliana* plants grown on axenic split-root systems. Roots were divided equally between two growth compartments containing agar media with N administered either as 3 mM nitrate in both root compartments (NO_3^-/NO_3^-), $n = 5$, as 3 mM nitrate in one of the root compartments and 1.5 mM L-gln in the other compartment (L-gln/NO_3^-), $n = 10$, or as 1.5 mM L-gln (L-gln/L-gln) in both root compartments, $n = 5$. Green, upper part of the bars correspond to shoots, light grey, middle part of the bars to L-gln root compartment in the NO_3^-/L-gln treatment and lower grey part of the bars correspond to roots in the NO_3^- compartment in the L-gln/NO_3^- treatment. Bars represent average ± SE. Statistical significance was calculated using one-way ANOVA and Tukey *post hoc* test. Different lower-case and upper-case letters indicate significant differences at *P*-value ≤ 0.05 in shoot and root biomass between treatments, respectively. The * indicates a statistical difference in root biomass between root compartments in the L-gln/NO_3^- treatment. DW, dry weight.

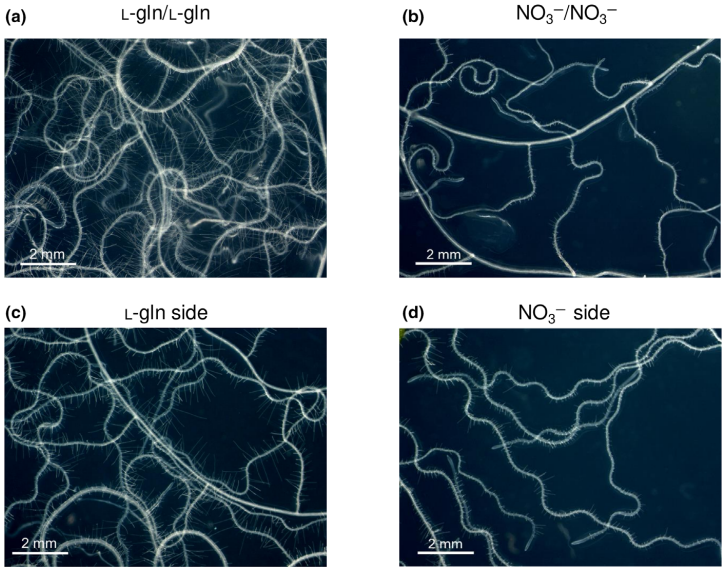

Fig. 4 Root systems of *Arabidopsis thaliana* plants grown in split-root systems with N supplied as (a) 1.5 mM L-gln supplied on both sides (L-gln/L-gln), (b) 3 mM NO_3^- on both sides (NO_3^-/NO_3^-), as or as N supplied as (c) 1.5 mM L-gln on one side and supplied (d) 3 mM NO_3^- on the other side. Pictures were taken using a Leica DC300 digital camera coupled to a Leica $MZ9_5$ stereomicroscope.

Stable isotope labelling shows that organic N contributes to plant C

We traced the uptake and partitioning of C and N from organic N, growing Arabidopsis on ^{13}C, ^{15}N labelled L-gln ($U^{15}N_2U^{13}C_5$-L-gln) either as the sole N source (Fig. 6a,c) or in a 50 : 50 (moles of N) mixture with NO_3^- (Figs 2, 6b,d). The ^{15}N abundance in the growth medium was 10 atom% and in agreement with this, the slope of the regression line total plant N vs excess ^{15}N for shoots and roots of plants growing on labelled

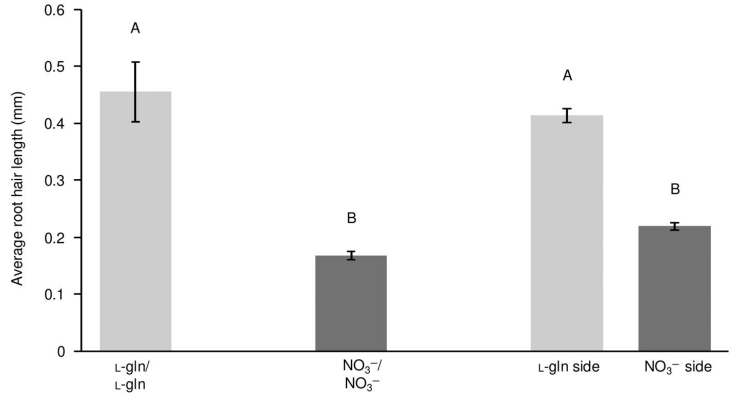

Fig. 5 Root hair length of *Arabidopsis thaliana* plants with N supplied as 1.5 mM L-gln supplied on both sides (L-gln/L-gln), 3 mM NO_3^- on both sides (NO_3^-/NO_3^-), as or as N supplied as 3 mM NO_3^- on one side and 1.5 mM L-gln supplied on the other side. Pictures of roots from 18 to 20 plants of each treatment were analysed and root hair length was measured using the program ImageJ. Values indicate average ± SE ($n = 18$–20). Statistical significance was calculated using one-way ANOVA and Tukey *post hoc* test. Different capital letters indicate statistical differences at *P*-value ≤ 0.05 in root hair length between N treatments.

Table 1 Nitrogen and carbon concentrations of shoots and roots of *Arabidopsis thaliana* plants grown in split-root systems.

Treatment	Organ	N concentration (% DW)	C concentration (% DW)	No. of replicates
NO_3^-/NO_3^-	Shoot	6.37 (0.09) A	35.01 (0.74) A	5
	Root	4.54 (0.05) a	38.14 (0.21) a	5
L-gln/L-gln	Shoot	6.62 (0.13) A	39.23 (0.33) B	5
	Root	3.14 (0.11) b	40.24 (0.21) b	5
L-gln/NO_3^-	Shoot	6.54 (0.07) A	35.61 (0.49) A	10
	Root L-gln side	3.24 (0.10) b	39.99 (0.36) b	10
	Root NO_3^- side	4.15 (0.24) a	39.92 (0.31) b	10

Roots were divided equally between two growth compartments containing agar media. Nitrogen (3 mM) was supplied to roots either exclusively as nitrate (NO_3^-/NO_3^- treatment) or exclusively as glutamine (L-gln/L-gln treatment) or as nitrate in one of the root compartments and on the other (L-gln/NO_3^- treatment). Values represent mean ± SE, $n = 5$–10. Different letters indicate significant differences (*P*-value ≤ 0.05) between treatments for shoots (upper-case letters) and roots (lower-case letters). DW, dry weight.

L-gln was 0.098 and 0.102 (Fig. S1a,b, respectively). The corresponding slope for shoots and roots of plants growing on an equimolar N mixture of labelled L-gln and NO_3^- was 0.06 and 0.08 respectively, that is higher than 0.05 which would correspond to identical uptake rates of NO_3^- and L-gln (Fig. S2a,b, respectively), suggesting a higher rate of acquisition of L-gln than of NO_3^-.

Regression analysis of excess ^{13}C vs excess ^{15}N content in plants grown on 1.5 mM $U^{15}N_2U^{13}C_5$-L-gln or a mixture of 0.75 mM, $U^{15}N_2U^{13}C_5$-L-gln (10 atom%) and 1.5 mM NO_3^- showed that between 41% and 43% of the C acquired from uptake of L-gln remained in the tissues (Fig. 6).

The costs and benefits of organic vs inorganic N: N assimilation costs, carbon use efficiency, and N uptake

Carbon use efficiency of biomass growth increased with the fraction of N that was taken up as L-gln relative to NO_3^- and the difference was mainly explained by the difference in N assimilation costs between N forms (Fig. 7). As the influence of pre-experimental differences between plants declined over time, the correlation between CUE and N form increased. After 6 d of growth, the lower N assimilation cost of L-gln compared to NO_3^- explained as much as 89% of the difference in CUE. The estimated N assimilation costs were 2.63 ± 0.10 g C g^{-1} N for L-gln and 4.56 ± 0.25 g C g^{-1} N for NO_3^-. N taken up at a given root biomass was *c.* 20% higher for plants growing on the mixed N than for those growing on L-gln only (Fig. 8).

Discussion

Changes in root : shoot ratios and root morphology in response to N supply is a well-documented phenomenon (Hermans *et al.*, 2006; Lynch *et al.*, 2023). The general view is that plants adjust biomass allocation between above- and belowground structures to optimize acquisition of the limiting resource; the functional balance or functional equilibrium hypothesis (Brouwer, 1962; Poorter *et al.*, 2012). Root phenotypic responses to N supply have been extensively studied in Arabidopsis, but most of these studies have concerned mineral N and in particular NO_3^-. From these reports, it was concluded that increasing N supply leads to a reduction in the root mass fraction, reduced lateral root formation and fewer active root tip meristems (Jia & von Wirén, 2020). Through the series of experiments described here, we challenge the view that N supply rates is the single determinant of root morphology and architecture and propose that the source of N (inorganic or organic) available to plants exerts a strong influence on plant phenotype.

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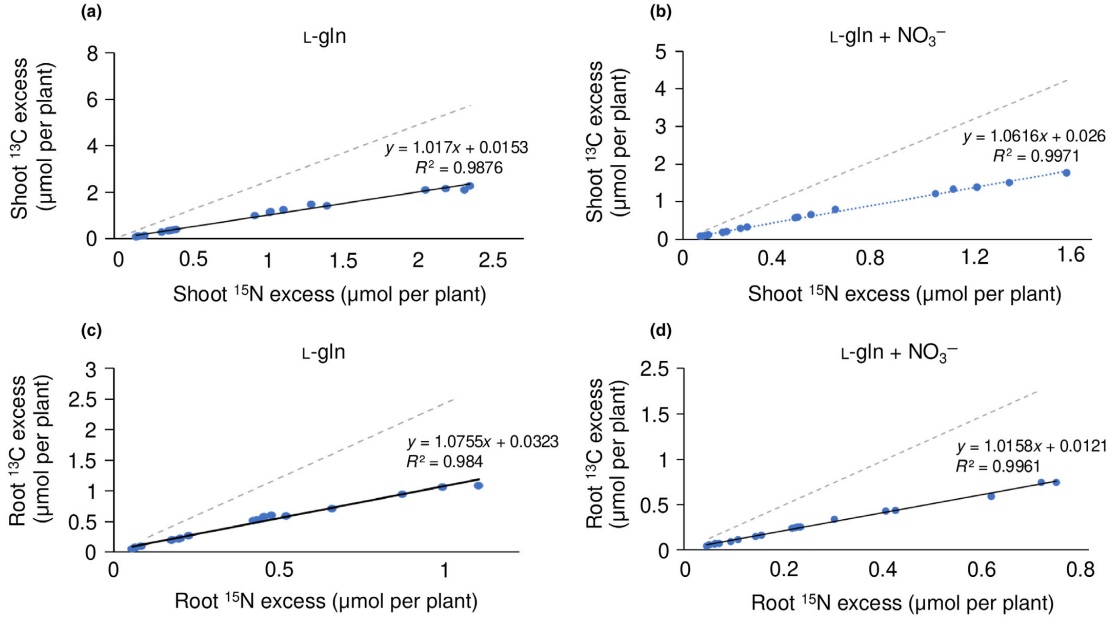

Fig. 6 Regression analysis of excess ^{13}C vs excess ^{15}N content in *Arabidopsis thaliana* plants grown on 1.5 mM $U^{15}N_2U^{13}C_5$-L-gln (10 atom%; a, c) or a mixture of 0.75 mM, $U^{15}N_2U^{13}C_5$-L-gln (10 atom%) and 1.5 mM nitrate (nonlabelled; b, d). Dotted lines with slope 2.5 indicate theoretical regressions corresponding to all ^{13}C acquired through uptake of L-gln remaining in tissues. Regression equations (a) $y = 1.017x + 0.015$ ($R^2 = 0.99$); (b) $y = 1.062x + 0.03$ ($R^2 = 1.0$); (c) $y = 1.075x + 0.03$ ($R^2 = 0.99$); (d) $1.016 + 0.012$ ($R^2 = 1.0$). Slopes correspond to (a) 41%; (b) 42%; (c) 43% and (d) 41% of carbon derived from uptake of L-gln remaining in plant biomass.

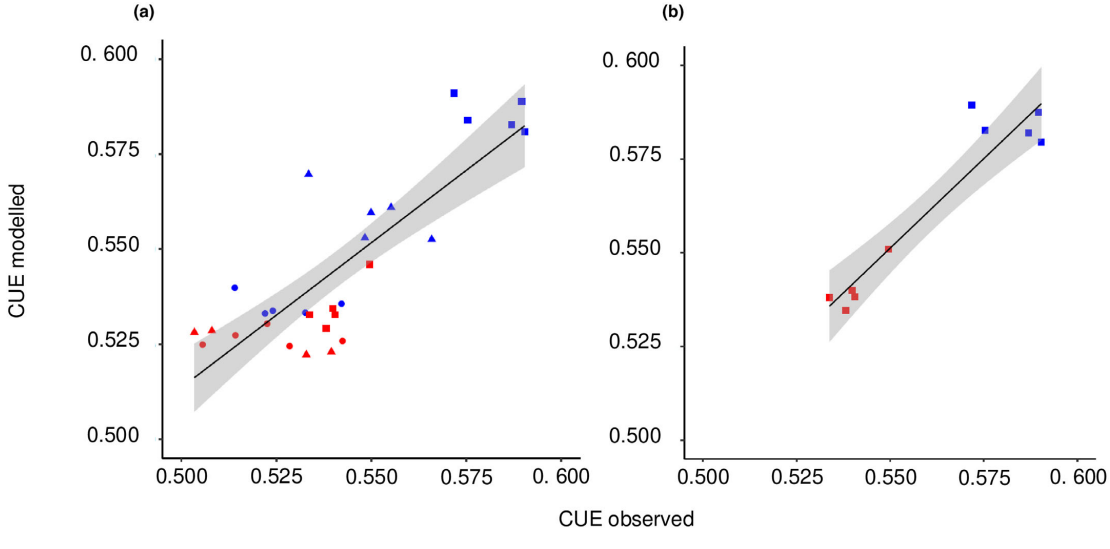

Fig. 7 Carbon use efficiency (CUE) modelled based on N assimilation costs vs observed CUE for *Arabidopsis thaliana* plants grown on 1.5 mM L-gln (blue symbols) or mixed L-gln and NO_3^- (0.75 and 1.5 mM, respectively; red symbols). The growing times were 1 d (circles), 3 d (triangles) and 6 d (squares). (a) All observations, $R^2 = 0.66$, (b) only observations at day 6, $R^2 = 0.89$. The shaded areas indicate a 95% confidence band of the mean.

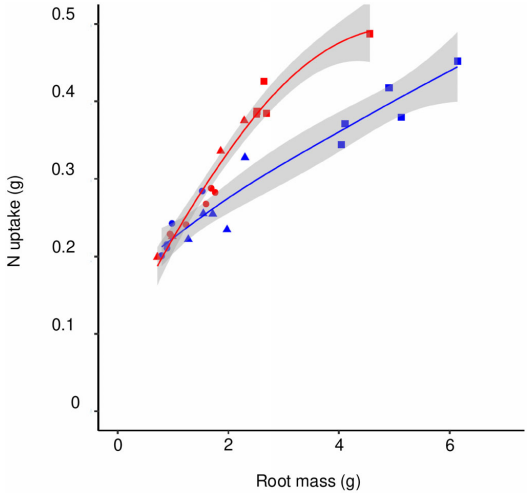

Fig. 8 N uptake vs root mass for *Arabidopsis thaliana* plants grown on 1.5 mM L-gln (blue symbols) or mixed L-gln and NO_3^- (0.75 and 1.5 mM, respectively; red symbols), for 1 d (circles), 3 d (triangles) and 6 d (squares). The shaded area indicates a 95% confidence band of the mean.

Growing Arabidopsis from seeds on vertical plates and then transferring them to horizontal plates, with split-root setups (Fig. 1), revealed that growth of roots was significantly enhanced by L-gln, leading to a higher total plant biomass (Fig. 3). Our results also show that a key facet of root morphology; root hair length, responds strongly to the supply of L-gln. While the abundance of root hairs was similar between roots exposed to L-gln and NO_3^-, root hairs were nearly three times longer for plants grown on L-gln vs plants grown on NO_3^- and *c.* 2 times longer for roots in the split-root setup supplied L-gln vs roots supplied NO_3^- (Figs 4, 5). This corresponds to a significant increase in root surface area, a characteristic that would also have a strong fitness value for growth on organic N and for survival under drought (Choi & Cho, 2019). Our estimate of root hair length for NO_3^--treated roots is similar to those reported for low- and intermediate NO_3^- concentration treated Arabidopsis Col-0 reported by De Pessemier *et al.* (2022). The average root hair length of roots exposed to L-gln in our study was twice that reported by these authors, illustrating the strength of the phenotypic response to the organic N source. A recent study reported on various aspects of L-gln nutrition of Arabidopsis: enhanced stress responses and disease resistance but also a significant increase in lateral root density compared with plants grown on NO_3^- (Lia *et al.*, 2024). While there are differences between our experimental system and that of Lia *et al.* in that they used higher L-gln concentrations (5 mM vs 1.5 mM) and grew plants on vertical plates rather than horizontal plates and for a shorter period (12 d vs 35 d), the notion of an increase in lateral root initiation is in line with our main hypothesis; that L-gln, as a source of organic N, promotes root proliferation (Fig. 3). The importance of expansion of root surface area for uptake of immobile nutrients has been verified using root hair mutants with short (Gahoonia *et al.*, 2001) and long (Zhang *et al.*, 2018) root hairs. Future studies may hence use a similar approach to test the role of root hairs for acquisition of immobile organic N sources. Here, we note that the phenotypic response of Arabidopsis to an organic N source is like the well-documented response to immobile phosphorus and potassium (Gahoonia *et al.*, 1997; Gahoonia & Nielsen, 1998; Bates & Lynch, 2001; Jungk, 2001; Bienert *et al.*, 2021).

A key question is to what extent the root morphological response to L-gln documented in our study is calid also for (1) other organic N sources and (2) for other plants, in particular mycorrhizal plants. A study using Arabidopsis and *Hakea actites* (*Proteaceae* nonmycorrhizal) reported increased root length when plants were grown in axenic culture and supplied with a complex organic N source (the protein Bovine Serum Albumin; BSA; Paungfoo-Lonhienne *et al.*, 2008). Increased root hair length in response to presence of BSA in the root medium was reported for Arabidopsis (Lonhienne *et al.*, 2014). Thus, at the two ends of the complexity spectra of organic N (single amino acid; L-gln; the current study and large protein 583 amino acids) plants react with increased root surface area through increased root length and increased length of root hairs. Regarding the generality of response, in particular mycorrhizal plants, a study by Gruffman *et al.* (2012) reported increased root mass fraction as well as increased frequency of mycorrhizal root tips for conifer seedlings (*Pinus sylvestris* and *Picea abies*) when these were cultivated with the amino acid L-arg as a nitrogen source.

Following absorption of organic N, endogenous metabolism will lead to that a fraction of the acquired C is lost via respiration while the rest is incorporated into biomass. The instantaneous metabolism of L-gln was shown to produce L-glu, L-asp and GABA, leading to a 15% loss of C acquired from L-gln over a time course of 120 min (Svennerstam & Jämtgård, 2022). Here, we show that between 57 and 59% of the C acquired from uptake of L-gln was lost through respiration, independently of tissue and independently if N was administered as L-gln only or as a mixture of N compounds (Fig. 6). The concentration of tissue C derived from L-gln uptake can be estimated as the product of tissue N concentration × slope of the regression excess ^{13}C vs excess ^{15}N (Fig. 6). For shoots and roots of plants grown on L-gln this amounts to 6.7% and 3.4% of DW respectively. This means that 17.2% and 8.4% of shoot and root C was derived from uptake of L-gln.

A long-standing debate within the field of N nutrition is whether inorganic N, in particular NO_3^-, is the preferred N source for plants (Harrison *et al.*, 2007) and that organic N would be of importance only at low-inorganic N availabilities. The results from our experiment (Figs S1, S2), with N available as mixtures of inorganic and organic N, the opposite result was achieved. Thus, slopes of regression lines for shoots and roots of plants growing on an equimolar N mixture of labelled L-gln and NO_3^- was 0.06 and 0.08 respectively (Fig. S2a,b, respectively). Re-calculated, this equals the fraction of N derived from L-gln

was 60% for shoots and 80% for roots L-gln than of NO_3^-. This shows that L-gln was the preferred N source under the growth conditions used in the experiment. Also, our data illustrates that roots, to a higher extent than shoots, used N absorbed as L-gln for growth.

A fundamental difference between inorganic and organic N is the C savings and C bonus connected to plant use of organic N. Franklin *et al.* (2017) developed a model based on the differences in C costs for different N sources and suggested this extra C to drive a shift towards an increased root mass fraction. In the current study, the contribution of L-gln-derived C to total organ C was assessed on plant grown on $U^{15}N_2U^{13}C_5$-L-gln. The ratio of the two isotopes ^{13}C to ^{15}N in L-gln, and hence in the growth media was 2.5. This means that if absorbed L-gln was not metabolized by the plant following uptake, we would expect the ratio of excess ^{13}C and excess ^{15}N to equal 2.5 in plants and that any deviation from 2.5 would be due to losses of ^{13}C via catabolism of L-gln. The slopes of regressions of excess ^{13}C vs excess ^{15}N provides information here, and results showed similar slopes for both roots and shoots and for both plants grown on L-gln and plants grown on a combination of L-gln and NO_3^- (Fig. 6). Recalculated, these slopes (1.02–1.08; Fig. 6) correspond to 40.8–43.2 of the C acquired as L-gln remaining in tissues. The ^{13}C data was also used to calculate the fraction of plant C that was derived from L-gln uptake. For plants grown on L-gln, as the sole N source, *c.* 10% of root C was derived from L-gln at the final harvest (10 d after plants had been moved to the labelled source). These results show that uptake of L-gln made a significant contribution to plant C and to root C.

Carbon use efficiency is a key metric describing the efficiency by which photosynthetically derived C is converted to biomass C (Manzoni *et al.*, 2018). For small plants, assimilation of inorganic N, in particular NO_3^-, may constitute a significant C cost (Bloom *et al.*, 2003) and this would hence also potentially affect plant CUE. The carbon bonus of organic N nutrition, as described by Franklin *et al.* (2017) and further demonstrated here, pertains both to the C acquired through uptake of organic N but also to the C savings derived from not having to reduce NO_3^- to NO_2^- via nitrate reductase and further to NH_4^+ via nitrite reductase as well as the assimilation of NH_4^+ via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway. In line with this reasoning, we show here that CUE of small Arabidopsis plants was positively correlated with the degree of L-gln assimilation (Fig. 7). Moreover, measured CUE was well predicted based on differences in C costs for assimilation between L-gln and NO_3^-, explaining 89% of the difference after 6 d of growth (Fig. 7). In addition, the validity of the estimated N assimilation costs was supported by their similarity to estimates based on the underlying biochemical reactions, that is 2.16 and 5.81 (Zerihun *et al.*, 1998). These results indicate that N assimilation costs are decisive for CUE of small plants and are clearly affected by the differences in assimilation costs of different N sources.

While substantial C savings may come from uptake of organic N, their lower mobility also incurs C costs. Acquisition of N is chiefly through mass flow and diffusion, the former primarily of importance for N forms that occur in substantial amounts in the soil solution, mainly NO_3^-. Our data suggest root N uptake per unit root mass to be higher for NO_3^- than for L-gln (Fig. 8), implying a higher C cost per N uptake for organic than inorganic N, which is only partly alleviated by the longer root hairs. This effect would be further aggravated under conditions allowing for mass flow (McMurtrie & Näsholm, 2018), which would not occur in the test system used here but which would limit the benefits of longer root hairs.

We conclude that strong differences in soil mobility may have exerted a selection pressure for plant plasticity in root allocation and root architecture not only linked to the availability of N but also to the chemical composition of available N. This plasticity is manifested through increases in root mass fraction, root branching and extension of root surface area through root hairs, enabling enhanced uptake of compounds of lower mobility. At the same time, these exact responses result in lower rates of mass flow-mediated N gain, suggesting plants face a trade-off between acquisition of less mobile and mobile N sources.

The demand for higher root surface areas to optimize uptake of organic N incurs an additional C cost for plants, potentially reducing growth rates. However, the substantially lower cost for N assimilation of organic N, leading to a higher CUE, alleviates this negative effect.

Overall, our results show that in a whole plant perspective, the two key differences between organic and inorganic N, (1) a lower N uptake per unit root biomass and (2), a lower N assimilation cost leading to higher CUE, make a higher root mass fraction an inevitable consequence for the plant to maintain a balanced N : C ratio during growth.

Acknowledgements

The authors acknowledge support from the Knut and Alice Wallenberg Foundation (nos. 2015.0047 and 2018.0259) and The Kempe Foundations as well as The Swedish University of Agricultural Sciences (T4F, Bio4Energy) for support.

Competing interests

TN declares a competing interest as he owns shares in, and works part time for, the company Arevo AB that develops, produces, and markets organic fertilizers. RG also declares a competing interest as she is also employed by Arevo AB. All other authors declare that the research was performed without any conflicting commercial or financial relationships and hence declare no conflict of interest.

Author contributions

LT, CAC, TN and RG designed the project. LT, CAC, PM and RG performed the experiments. LT, CAC, OF, PM, TN and RG analysed the data. LT, CAC and TN wrote the initial draft with input from all other authors. All co-authors provided feedback and revised the manuscript. LT and CAC contributed equally to this work.

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Data availability

All data used in this study are uploaded to a data repository and can be accessed at doi: 10.5281/zenodo.13740411.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Regression analysis of excess ^{15}N vs total N contents of 10 atom% excess $U^{15}N_2U^{13}C_5$-L-gln grown *Arabidopsis thaliana* plants.

Fig. S2 Regression analysis of excess ^{15}N vs total N contents of *Arabidopsis thaliana* plants grown on a mixture of 0.75 mM 10 atom% excess $U^{15}N_2U^{13}C_5$-L-gln and 1.5 mM nonlabelled NO_3^-.

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Plant Molecular Biology (2022) 109:413–425
https://doi.org/10.1007/s11103-022-01244-1

REVIEW

To have or not to have: expression of amino acid transporters during pathogen infection

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Received: 28 September 2021 / Accepted: 16 January 2022 / Published online: 1 February 2022

Abstract
The interaction between plants and plant pathogens can have significant effects on ecosystem performance. For their growth and development, both bionts rely on amino acids. While amino acids are key transport forms of nitrogen and can be directly absorbed from the soil through specific root amino acid transporters, various pathogenic microbes can invade plant tissues to feed on different plant amino acid pools. In parallel, plants may initiate an immune response program to restrict this invasion, employing various amino acid transporters to modify the amino acid pool at the site of pathogen attack. The interaction between pathogens and plants is sophisticated and responses are dynamic. Both avail themselves of multiple tools to increase their chance of survival. In this review, we highlight the role of amino acid transporters during pathogen infection. Having control over the expression of those transporters can be decisive for the fate of both bionts but the underlying mechanism that regulates the expression of amino acid transporters is not understood to date. We provide an overview of the regulation of a variety of amino acid transporters, depending on interaction with biotrophic, hemibiotrophic or necrotrophic pathogens. In addition, we aim to highlight the interplay of different physiological processes on amino acid transporter regulation during pathogen attack and chose the LYSINE HISTIDINE TRANSPORTER1 (LHT1) as an example.

Keywords Amino acids · Amino acid transporter · Lysine histidine transporter (LHT) · Organic nitrogen · Pathogen defense · Ethylene signaling

Availability of nitrogen orchestrates plant pathogen resistance

A proper plant nitrogen (N) nutrition is warranted by the uptake of inorganic and organic N sources. Organic N such as proteins, peptides or amino acids (AAs) are taken up via specific root transporters (Paungfoo-Lonhienne et al. 2008; Nasholm et al. 2009; Tegeder and Rentsch 2010; Inselsbacher and Näsholm 2012; Tegeder and Masclaux-Daubresse 2018; Gratz et al. 2021) that have multiple functions within a plant (Yang et al. 2020; Yao et al. 2020). AAs represent an important storage and transport form of organic N and are precursors for protein synthesis. AAs are especially important for the development of roots, leaves, and seeds (Rentsch et al. 2007; Tegeder and Masclaux-Daubresse 2018), which makes AA transport systems a key component for plant development. Not only plants but also the microbial community relies on the availability of AAs, and it is not surprising that both compete for this N source (Roberts and Jones 2012; Kuzyakov and Xu 2013; Wilkinson et al. 2014). We identified the need of a concise survey highlighting the role of AA transporters (AATs) during pathogen infection due to the fact that literature mostly focusses on the influence of inorganic N on plant resistance (Ballini et al. 2013; Huang et al. 2017; Farjad et al. 2018; Sun et al. 2020).

Amino acid pools and fluxes are, however, dependent on N supply and the absolute majority of studies reporting on N effects on pathogen resistance have focused on comparisons of the inorganic N sources nitrate (NO_3^-) and ammonium (NH_4^+). In addition to reviewing the links between AATs and pathogen resistance, we therefore also performed a literature search aiming to compare effects of nitrate and ammonium addition on the plant's ability to resist

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pathogens that differ in their nutrition strategy (Table 1, Supplementary Tables 1–3). Especially the different nutrient acquisition strategies by different pathogens such as biotrophic, hemibiotrophic as well as necrotrophic pathogens are important in this context. Biotrophic pathogens exhibit specialized feeding structures that allow nutrient retrieval from living cells. Hemibiotrophic microbes, however, first colonize the living cell but then transition into a necrotrophic phase. Necrotrophs obtain their nutrients from killed cells (Spanu and Panstruga 2017). Within biotrophic pathogens, the presence of different inorganic N sources led to strong and opposing effects: addition of NO_3^- reduced plant resistance in the majority of analyzed cases (11 out of 15 cases). Interestingly, not only the presence but also the rate of NO_3^- addition influenced defense responses of plants (Ding et al. 2021). Tomato plants infected with the biotroph *Ralstonia solanacearum*, for instance, demonstrated less disease lesions when grown on 1 mM compared to 7 mM NO_3^- (Ding et al. 2021). Interestingly, the presence of NH_4^+ as N source, though, demonstrated an opposing trend: in 10 out of 14 cases elevated plant resistance was found (Table 1, Supplementary Table 1). Plant resistance against hemibiotrophic pathogens seems not to display any clear response to different inorganic N sources and both positive and negative effects of NO_3^- and NH_4^+ addition have been reported (Table 1, Supplementary Table 2). Concentration-related effects such as reduced disease lesions were observed for tomato plants after infection with *Pseudomonas syringae* (*P. syringae*), when plants were grown on 1 mM compared to 7 mM NO_3^- (Ding et al. 2021). In 9 out of 15 cases NO_3^- led to a positive immune response such as increased resistance or hypersensitive response during necrotrophic attack (Table 1, Supplementary Table 3). Similar to biotrophic and hemibiotrophic infection, plant responses after necrotrophic interaction seem to dependent on the N rate (Farjad et al. 2018). Measurements of bacterial cell numbers of the necrotroph *Erwinia amylovora* in infected *Arabidopsis thaliana* (*Arabidopsis*) revealed lower numbers when grown on low NO_3^- (0.5 mM) compared to high NO_3^- (5 mM). This was associated with transcriptional reprograming of defense genes, e.g., *PATHOGENESIS-RELATED GENE*2 and 5 (*PR2* and *PR5*) or salicylic acid (SA)-related genes (Farjad et al. 2018). Addition of NH_4^+, though, led to increased cases of elevated plant susceptibility, when infected with a necrotroph (7 out of 11 cases) (Table 1). Overall, we found that a plant's ability to withstand biotrophic attacks tends to be more successful when NH_4^+ is accessible, the opposite of what was shown for necrotrophs. The overall N addition rate might serve as a proxy for plant N status, which influences susceptibility additionally.

As shown above, plant N sources play critical roles for plant resistance. This observation motivates a further analysis of N transporters during pathogen attack. Camanes et al. (2012) investigated the response of NO_3^- transporters AtNRT2.1 and AtNRT2.2 to infection by the hemibiotrophic bacteria *P. syringae*. The *nrt2* mutant exhibited an increased immune response along with a reduced susceptibility and significant alterations in the transcriptome. The expression

Table 1 Effects of nitrate (NO_3^-) and ammonium (NH_4^+) availability on plant pathogen resistance

Type	Nutrition strategy	Positive effect of NO_3^- on plant resistance	Negative effect of NO_3^- on plant resistance	Positive effect of NH_4^+ on plant resistance	Negative effect of NH_4^+ on plant resistance
Bacteria	Biotroph	1	3	1	0
Fungi	Biotroph	1	4	2	1
Nematode/Protist	Biotroph	1	3	3	1
Virus	Biotroph	0	1	4	1
Oomycota	Biotroph	1	0	0	1
Bacteria	Hemibiotroph	1	2	2	1
Fungi	Hemibiotroph	5	3	3	5
Oomycota	Hemibiotroph	1	1	1	1
Bacteria	Necrotroph	0	2	0	0
Fungi	Necrotroph	9	4	4	7
Total	**Biotroph**	**4**	**11**	**10**	**4**
	Hemibiotroph	**7**	**6**	**6**	**7**
	Necrotroph	**9**	**6**	**4**	**7**

Results of a survey of different studies are summarized, comparing different pathogen types, separated by their nutrition strategy. The impact of different inorganic N sources on the plant's immune response during respective pathogen attacks were denoted. Effects are expressed through increased resistance and elevated susceptibility, respectively. Respective numbers express the count of experiments found, displaying a similar response. A summary of the counts is presented in bold, with no differentiation between different pathogen types, but grouped according to nutrition strategy. Respective references to the included studies can be found in Supplementary Tables 1–3

of SA marker genes was strongly increased compared to the wild type, and it was suggested that members of the AtNRT2 family might be important for the plant-pathogen interaction (Camanes et al. 2012). More recently it was shown that the *nrt2.5* mutant displayed similar responses (du Toit et al. 2020). Similarly, also NH_4^+ transporters such as AtAMT1.1 seem to play an important role for plant resistance (Pastor et al. 2014). *amt1.1* plants infected with *P. syringae* and *Plectosphaerella cucumerina*, a hemibiotrophic and a necrotrophic organism respectively, exhibited increased resistance, an effect that was enhanced by N depletion (Pastor et al. 2014). These findings lead to the hypothesis, that N transporters play a role in plant immune responses, by acting as regulators in N supply. We therefore ask the question whether other transporters that are involved in N uptake and N translocation and in particular the AATs could potentially also play a role in plant resistance.

A dual utilization of amino acids

It is well established that pathogens can feed on plant N reserves, mainly AAs, which makes them crucial players in the plant-pathogen interaction (Struck et al. 2004; Zeier 2013; Sonawala et al. 2018; Yang et al. 2020; Sharma 2020). It is energetically more beneficial for pathogens to directly acquire and metabolize plant AAs which is why a range of pathogens can directly target the induction of genes needed for AAT (Sonawala et al. 2018; Li et al. 2020). Having control over a plant's AA uptake and transport system can, therefore, be decisive for the survival of either the plant or the pathogen.

Li et al. found substantial reprogramming of N and C metabolic pathways in kiwifruit tissues upon infection with *P. syringae*, i.e., an accumulation of specific AAs (Li et al. 2020). While the accumulation of some AAs can be beneficial for the pathogen, others can play important roles in plant resistance. Tryptophan and methionine, for instance, are known precursors for the synthesis of secondary metabolites with antimicrobial effects (Ahuja et al. 2012). Depending on the microbe, these metabolites accumulate in individual root cell layers and can contribute to increased resistance (Froschel et al. 2021). A similar response of citrus plants was described upon infection with the phloem-feeding biotroph *Candidatus liberibacter*, as the phloem sap of tolerant plants exhibited high amounts of tryptophan, tyrosine or phenylalanine; well-studied precursors for secondary metabolites and phenolics (Killiny and Hijaz 2016). Proline, a known radical scavenger, contributes to the regulation of cellular redox homeostasis (Smirnoff and Cumbes 1989). Gupta et al. (2020) recently corroborated the positive properties of proline during infection and analyzed upstream components. They identified miRNA involved in the regulation of proline biosynthesis, which is not only important for the plant immune response but is also involved in regulation of abiotic stresses (Gupta et al. 2020).

The above suggests that it is crucial to understand the molecular regulation of AA transport and accumulation because AAs can be used as N sources for the pathogen but also as protective agents for the plant. This leads to the question whether AATs are differently expressed during plant-pathogen interaction and if so, who the driver of this regulation is. Having control over the expression can, thus, decide over the fate of both, plants or pathogens (Hammes et al. 2006; Liu et al. 2010; Elashry et al. 2013; Pariyar et al. 2018; Sonawala et al. 2018; Froschel et al. 2021).

Responses of plant amino acid transporters to pathogen infection

The products of about 100 genes are known to facilitate AA transport in *Arabidopsis* and similar AATs have additionally been identified in many crop and tree species (Tegeder and Ward 2012; Pratelli and Pilot 2014; Yang et al. 2020). ATF (amino acid transporter family), APC (amino acid-polyamine-choline transporter family) and UMAMIT (usually multiple acids move in and out transporter family) represent the three main AAT families (Rentsch et al. 2007; Pratelli and Pilot 2014; Dinkeloo et al. 2018; Yang et al. 2020). ATFs can be divided into several subfamilies such as, e.g., AAPs (amino acid permeases) or LHTs (lysine histidine transporters) (Rentsch et al. 2007). CATs (cationic amino acid transporters) represent a subfamily within the APCs (Tegeder and Rentsch 2010).

Amino acid permeases (AAPs)

AAPs, a group of one-directional transporters, are involved in root AA uptake, phloem loading, xylem-phloem transfer, and seed loading (Fischer et al. 1995; Okumoto et al. 2002, 2004; Lee et al. 2007; Svennerstam et al. 2008; Zhang et al. 2010; Santiago and Tegeder 2016). It is well known that AAPs are highly conserved between various species (Benedito et al. 2010; Zhao et al. 2012, 2017; Limpens et al. 2013; Garneau et al. 2018; Duan et al. 2020; Llebrés et al. 2021; Omari Alzahrani 2021).

Several members of the AAP family were found to be differentially regulated upon biotrophic interactions. AtAAPs demonstrated enhanced gene expression after plant-parasitic nematode infection and increased resistance in respective knockout mutants (Hammes et al. 2005; Elashry et al. 2013; Marella et al. 2013). Analysis of *aap1*, *aap2* and *aap6* knockout mutants displayed decreased reproduction of cyst nematodes (Elashry et al. 2013). Similarly, *aap3* and *aap6* exhibited reduced reproduction of root-knot nematodes

(Marella et al. 2013). Recently, the role of CsAAP2A in cucumber became evident as knockout plants displayed resistance to downy mildew (Berg et al. 2021). A functional analysis of AAPs in tomato plants, when challenged with the hemibiotrophic *Phytophthora infestans* (*P. infestans*), displayed that mutations in the tomato homologues *SlAAP5A* and *SlAAP5B* led to similar effects (Berg et al. 2021). It is reasonable that an infection causes a differential regulation of local AATs in specific cell types. It would also be conceivable that a transporter is being regulated in opposing directions upon infection of the same pathogen, however, in different cells. A recent study zoomed in on these questions and compared expression patterns in four specific root cell layers (rhizodermis, cortex, endodermis, and stele), when *Arabidopsis* was challenged with, in their nutrition strategy varying, microbes (Froschel et al. 2021). When looking at the cell layer-specific transcript abundance after hemibiotrophic *P. parasitica* infection, it was found that *AtAAP3*, *AtAAP5* and *AtAAP6* were induced in the stele, however, *AtAAP6* was additionally upregulated in the cortex (Froschel et al. 2021). Responses to hemibiotrophic, vascular *Verticillium longisporum* (*V. longisporum*) varied within the AtAAP family: *AtAAP4* was the only representative that was upregulated and only in the cortex. *AtAAP1*, in the cortex, and *AtAAP2*, in the rhizodermis, were found to be downregulated after infection (Froschel et al. 2021).

Based on the above publications, it can be suggested that AAPs are negative regulators in plant defense against (hemi-) biotrophic pathogens. An increase in AAT transcript abundance might reduce plant defense reactions which would be beneficial for the pathogen. Alternatively, these transporters might be exploited by pathogens to steer plant AA transport, elevating the amount of accessible AAs in infected leaves and creating an artificial sink that pathogens can feed on (Berg et al. 2021).

Cationic amino acid transporters (CATs)

Some AATs affect the plant immune system in a positive way, like AtCAT1 (Yang et al. 2014). The infection with hemibiotrophic *P. syringae* caused elevated transcript levels of *AtCAT1* and increased resistance. Overexpression of *AtCAT1* led to the constitutive expression of SA related and *PR1* genes, as well as an increase in SA levels. Since *AtCAT1* expression responded quickly to the infection it seems that it is involved in the systemic resistance of the plant (Yang et al. 2014).

Usually multiple acids move in and out transporter family (UMAMITs)

Most AATs operate as one-directional symporter, transporting AAs along a proton gradient (Bush 1993; Frommer et al. 1993; Hsu et al. 1993), however, UMAMITs are an exception. Driven by an electrochemical gradient, UMAMITs transport AAs in both directions (Ladwig et al. 2012; Muller et al. 2015). Due to their bi-directional activity, AtUMAMITs are involved in multiple physiological roles ranging from phloem loading/unloading, over xylem-phloem transport, to transport to sink tissues (Ladwig et al. 2012; Muller et al. 2015; Besnard et al. 2016). When looking at the cell layer-specific transcript abundance, all differentially regulated AtUMAMIT genes found upon presence of the hemibiotroph *P. parasitica* were downregulated: *AtUMAMIT11/38/41* were differentially regulated in the rhizodermis and the cortex. Besides, *AtUMAMIT11* was additionally downregulated in the stele. *AtUMAMIT33* was regulated in the cortex and *AtUMAMIT5* in the rhizodermis as well as the stele (Froschel et al. 2021). *AtUMAMIT18* expression in the rhizodermis and stele, *AtUMAMIT5* in the stele, and *AtUMAMIT34* expression in the cortex were downregulated upon hemibiotrophic *V. longisporum* infection. The opposite effect, an increase in transcripts, was seen for *AtUMAMIT5/31* (cortex), *AtUMAMIT38* (endodermis) and *AtUMAMIT14* (stele) (Froschel et al. 2021). Based on the analysis of transgenic *Arabidopsis* lines, Besnard et al. (2021) suggested that AtUMAMIT14 is a positive regulator in plant pathogen resistance. When challenged with the biotrophic oomycota *Hyaloperonospora arabidopsidis*, *AtUMAMIT14* overexpression lines displayed enhanced expression of SA marker genes as well as SA levels, leading to increased resistance (Besnard et al. 2021). The example of UMAMITs visualizes a diverse set of responses, where individual genes can be regulated opposingly depending on the cell type, and genes within the transporter family are regulated inconsistently. It might be that their bi-directional transport ability causes different responses, which is why the individual role of each transporter during plant-pathogen interaction needs to be carefully evaluated.

Lysine histidine transporters (LHTs)

In *Arabidopsis*, 10 AtLHT paralogs (Rentsch et al. 2007) exist with different specificity and cellular location. AtLHT1, the first identified transporter of this family (Chen and Bush 1997; Hirner et al. 2006; Svennerstam et al. 2007) is involved in leaf mesophyll import as well as root uptake of acidic and neutral AAs, both at naturally occurring concentrations (Svennerstam et al. 2011), and from agricultural soil (Ganeteg et al. 2017). AtLHT1 also transports non-proteinogenic AAs, like 1-aminocyclopropane-1-carboxylic acid (ACC), just as its paralog AtLHT2 (Shin et al. 2015; Choi et al. 2019). ACC serves as a precursor of the phytohormone ethylene (ET) and as a signaling molecule on its own (Van de Poel and Van Der Straeten 2014; Vanderstraeten et al. 2019). AtLHT1 can be exploited to shuttle

novel AA-coupled pesticides inside a plant (Jiang et al. 2018; Chen et al. 2018). Homologs of AtLHT1 were also identified and studied in, e.g., rice, poplar, lotus, tea and ginseng (Guether et al. 2011; Zhang et al. 2013; Wang et al. 2019; Guo et al. 2020; Gratz et al. 2021; Li et al. 2021). The *Arabidopsis* knockout mutant *lht1-1* displayed an early senescence phenotype (Hirner et al. 2006; Svennerstam et al. 2007).

The role of AtLHT1 during pathogen infection has been investigated in several studies: *AtLHT1* transcript levels were elevated when the host was infected with the biotrophic powdery mildew fungus *Erysiphe cichoracearum* (*E. cichoracearum*) (Liu et al. 2010) or the biotrophic nematode *Heterodera schachtii* (Elashry et al. 2013). Also, upon infection with the hemibiotrophic bacteria *P. syringae*, the fungi *Colletotrichum higginsianum* (*C. higginsianum*) (Liu et al. 2010) and *V. longisporum* (Froschel et al. 2021) as well as the oomycete *P. parasitica* (Froschel et al. 2021), *AtLHT1* was upregulated. Most biotrophs feed on the apoplast or apoplast-like compartments and assimilate nutrients directly from their living host (Szabo and Bushnell 2001; Fatima and Senthil-Kumar 2015; Wang et al. 2020). It has been shown, that pathogens can reprogram plant transport proteins for their benefit, in order to, e.g., gain nutrients (Delmotte et al. 2009; Spanu and Panstruga 2017). This opens for the possibility that the pathogen, rather than the host plant, may steer the expression of *AtLHT1*.

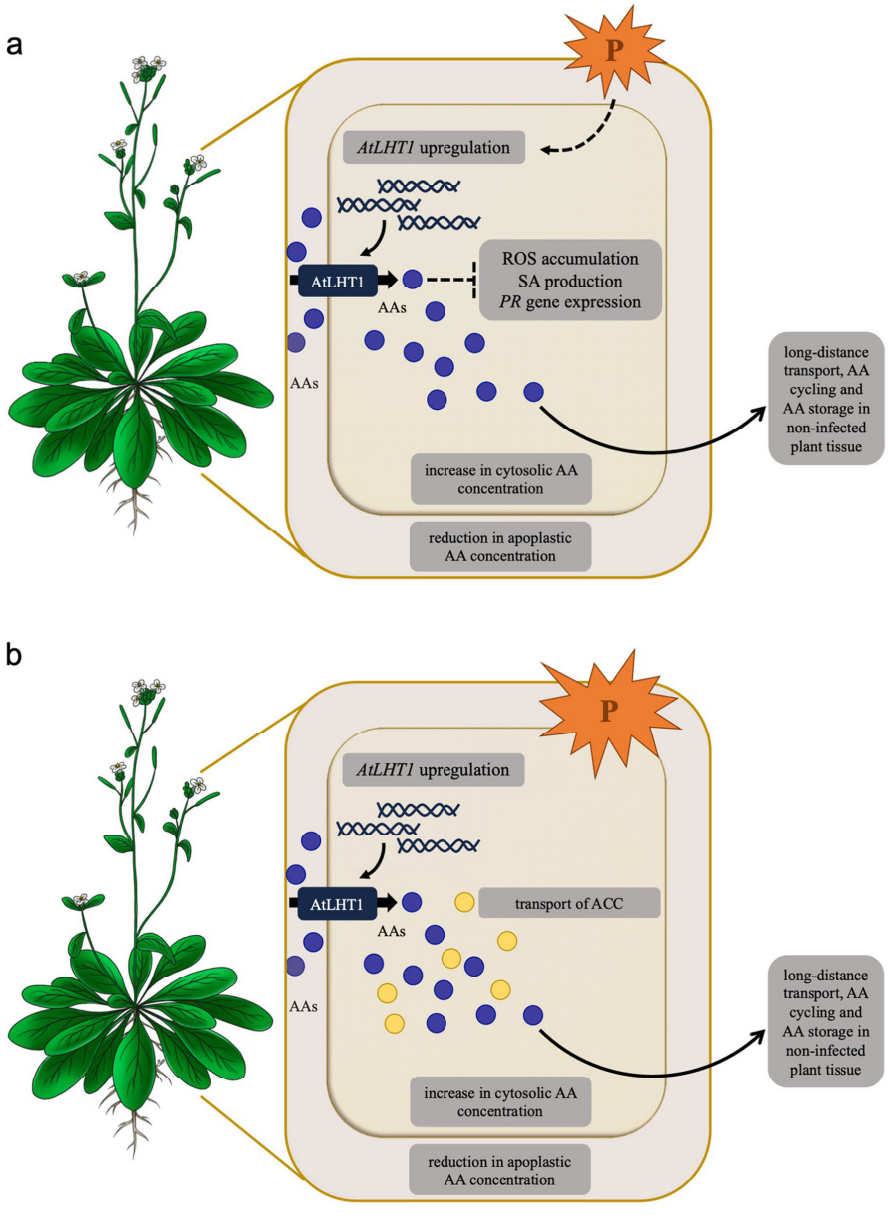
From a plant's perspective, it would be beneficial to increase the uptake of AAs from the apoplast to lower AAs accessibility for biotrophic pathogens and to secure its AA resources away from the infected area. This means an increased remobilization of AAs would require increased expression of AATs as part of a slash-and-burn defense strategy (Masclaux-Daubresse et al. 2010) (Fig. 1a). The increased expression of *AtLHT1* could be seen as a defense strategy caused by the plant to drain a maximum of AAs out of the apoplast in order to starve the pathogen.

However, and in contrast to the predictions from this hypothesis, *lht1-1* knockout mutants displayed increased resistance to *P. syringae, C. higginsianum* and *E. cichoracearum*, highlighting that AtLHT1 is a negative regulator in plant defenses (Liu et al. 2010). Disruption of *AtLHT1* displayed different defense responses such as increased callose deposition, hypersensitive cell death and the constitutive expression of genes belonging to the SA defense pathway such as *PR1* (Liu et al. 2010). The response is very similar to what was described for mutants of different AAPs (Elashry et al. 2013; Marella et al. 2013; Berg et al. 2021; Froschel et al. 2021). Liu et al. (2010) hypothesized that AtLHT1's role in plant resistance was linked to its ability to transport glutamine. The absence of AtLHT1 causes a lack of glutamine within the cell, which leads to an altered redox status and enhanced immunity due to an accumulation of reactive oxygen species (ROS) and induced programmed cell death (PCD) (Liu et al. 2010). This suggests that the increased expression of *AtLHT1* observed during the infection may be caused by the biotrophic pathogens, in order to inhibit the activation of the SA defense and, hence, an increase in plant resistance (Fig. 1a).

On the contrary, necrotrophic pathogens break plasma membranes and induce PCD in the host prior to nutrient uptake. While the SA pathway plays little role, the ethylene/jasmonic acid (ET/JA)-mediated response contributes to defense against necrotrophic pathogens (Glazebrook 2005; Pieterse et al. 2012; Huang et al. 2020). Furthermore, it has been shown that plants react in an analogous way to nematodes as to necrotrophic pathogens by activating the ET/JA pathway (Przybylska and Obrępalska-Stęplowska 2020). Similar to what has been observed for biotrophic pathogens, increased *LHT1* transcript levels were also found upon interaction with necrotrophic pathogens *Botrytis cinerea* (Xiong et al. 2018) and *Erwinia amylovora* (Farjad et al. 2018). Farjad et al. confirmed the involvement of *AtLHT1* during pathogen attack: *AtLHT1* resembled the expression profile of other defense associated genes by being induced during infection, behaving opposing to other N metabolism related genes. Potentially this serves an increased transport of ACC, supporting ET-based plant defense, as AtLHT1 and AtLHT2 were found to transport the ET precursor (Shin et al. 2015; Choi et al. 2019). This hypothesis is in line with the finding, that *lht1-1* mutants displayed no increased resistance to necrotrophic pathogen infection such as *Sclerotinia sclerotiorum* (Liu et al. 2010) or the nematode *H. schachtii* (Elashry et al. 2013). Necrotrophic pathogens would not benefit from increasing the transcript abundance of *AtLHT1*, which therefore might display a plant response in order to transport ACC as defense mechanism as well as to transport AAs away from the invaded tissue (Fig. 1b).

Regulation of amino acid transporters through additional physiological processes

The dominant players in plant defense are the antagonistic phytohormones SA and ET/JA (Huang et al. 2020; Zhang et al. 2020). The involvement of other phytohormones and crosstalk among the different players is well studied (Pieterse et al. 2012; Huang et al. 2020; Zhang et al. 2020; Aerts et al. 2021). The SA-mediated defense seems to be more effective against biotrophs and hemibiotrophs whereat the ET/JA-mediated defense targets necrotrophic microbes (Glazebrook 2005; Huang et al. 2020; Zhang et al. 2020). The link between SA-mediated defense and AAT regulation has been studied (Liu et al. 2010; Yang et al. 2014; Besnard et al. 2021), whereas not much is known about ET/JA-regulated defense against necrotrophs in connection to



AAT regulation. Recently, much work has been done on understanding the molecular underpinnings of leaf senescence. Due to the fact that the *lht1-1* mutant displays an early senescence-like phenotype (Hirner et al. 2006; Svennerstam et al. 2007), we aimed to identify regulatory targets, that play a role in plant senescence and pathogen defense, and at the same time display a connection to the regulation of AATs (Fig. 2).

The transcription factor ORESARA1 (AtORE1) targets promoters of senescence-associated genes and directly

◂**Fig. 1** Response of the plant amino acid transporter AtLHT1 to pathogen attack. Upon attack by biotrophic pathogens (orange P), the transcript abundance of *AtLHT1* is increased (**a**). An increased gene expression leads to an increased AtLHT1 protein abundance at the plasma membrane, which causes an active import of AAs (purple dots) into the cytosol. As a consequence, a depletion of apoplastic- and an increase of cytosolic AA concentrations occurs. This might be a direct response by the plant to apoplastic-feeding pathogens, in order to empty the apoplast and shuttle AAs into the cytosol. From there, AAs can be exported to healthy plant tissues. Due to the fact that *lht1-1* mutants display increased pathogen resistence due to the accumulation of reactive oxygen species (ROS), salicylic acid (SA) production and pathogenesis-related (PR) gene expression, the upregulation of *AtLHT1* might by steered by the biotrophic pathogen itself (dotted arrow). This action might avoid SA defense responses and might increase chances for the pathogen to survive. Upon attack by a necrotrophic pathogen, *AtLHT1* is also elevated (**b**). This might, however, be an exclusive response by the plant. AtLHT1 transports the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) (yellow dots). Mostly ET/JA-mediated responses contribute to the defense against necrotrophic pathogens. Additionally, an upregulation of the transporter might contribute to the shuttling of AAs to healthy, more distal plant tissues. Hence, the observed upregulation of *AtLHT1* might be mostly a protective measure, steered by the plant

mediates PCD (Oh et al. 1997; Kim et al. 2009; Balazadeh et al. 2010; Farage-Barhom et al. 2011; Al-Daoud and Cameron 2011; Matallana-Ramirez et al. 2013; Qiu et al. 2015; Durian et al. 2020). AtORE1 itself is targeted for degradation by the RING-type E3 ubiquitin ligase NITROGEN LIMITATION ADAPTATION (AtNLA) (Park et al. 2018). Deubiquitination events, however, stabilize AtORE1 and promote leaf senescence (Park et al. 2019). ET is involved in a positive regulation of *AtORE1*. More specifically, AtEIN3, a transcription factor acting downstream of EIN2, represses *miR164*, a negative regulator of *AtORE1*, and can in parallel bind to the *AtORE1* promoter (Kim et al. 2009, 2014; Li et al. 2013). Together with AtEIN3, *AtORE1* then activates transcription of chlorophyll catabolic genes in an ET dependent manner (Qiu et al. 2015). AtORE1 additionally activates *ACC SYNTHASE2* (*AtACS2*) and *AtACS6* expression, leading to enhanced ET production, displaying a coherent feed-forward loop for ET dependent leaf senescence (Qiu et al. 2015; Zhang et al. 2021). Interestingly, the action of AtORE1 and AtNLA are tightly connected to plant defense responses (Zhang et al. 2021). *Arabidopsis* infection with the hemibiotroph *V. dahliae* caused premature leaf senescence. It was shown that a microbial elicitor interfered with the interaction between AtORE1 and AtNLA, which, in turn, stabilized AtORE1, enhanced ET production and, thus, promoted senescence (Zhang et al. 2021). Recently, it was shown that AtORE1 is activated through protein phosphorylation via the calcium (Ca^{2+}) kinase AtCPK1 (Durian et al. 2020). This kinase has previously been analyzed and it was shown that *AtCPK1* is upregulated upon pathogen infection and was found to be a positive regulator in plant resistance due to activation of SA biosynthesis (Coca and San Segundo 2010). Interestingly, also plants infected with necrotrophs displayed increased resistance, although no ET derived defense responses were found (Coca and San Segundo 2010). In a preprinted study, it was suggested that AtNLA displays a negative regulator in plant defense against necrotrophs (Val-Torregrosa et al. 2021-preprint). *nla* mutants displayed increased callose deposition as well as increased resistance. Upon pathogen attack, transcript levels of *AtNLA* were reduced (Val-Torregrosa et al. 2021-preprint).

It was recently shown that AtORE1 and AtNLA additionally play a role in the regulation of AtLHT1 (Fig. 2). The ubiquitin ligase AtNLA targets pathways connected to organic N remobilization by targeting AATs during N deficiency (Liao et al. 2020). Transcript abundance of several AATs was found upregulated in the *nla* mutant and *AtLHT1* displayed the highest regulation. A proteomic analysis confirmed the regulation of AtLHT1 by AtNLA (Liao et al. 2020), however, it remains to be tested whether this regulation is due to a direct interaction between AtLHT1 and the ligase. The authors additionally speculated whether AtORE1 is controlling transcription of *AtLHT1* (Liao et al. 2020), however, an upregulation of *AtLHT1* in *AtORE1* overexpression lines has not been observed (Matallana-Ramirez et al. 2013). It remains unclear whether AtORE1 serves as TF regulating *AtLHT1*.

Given this complex regulatory crosstalk between different physiological processes, it can be speculated whether AtLHT1 is subject to additional molecular regulation. Due to the fact that miR164 is an important player at the interface between ET signaling and senescence (Kim et al. 2009, 2014; Li et al. 2013), and miRNAs in general play important roles in plant immunity (Val-Torregrosa et al. 2021), future studies should evaluate whether *AtLHT1* may also be regulated through the action of miRNAs. As mentioned above, the signaling compound and ET precursor ACC is transported by members of the AtLHT family (Van de Poel and Van Der Straeten 2014; Shin et al. 2015; Choi et al. 2019; Vanderstraeten et al. 2019), which provides a direct link between the ET signaling- and AA uptake pathways. In addition, Chen et al 2012 found the ER-localized ETHYLENE RESPONSE SENSOR2 (ERS2) (Hua et al. 1998), to interact with AtLHT1 in yeast (Chen et al. 2012). Novel findings about the poplar homolog PtrLHT1.2 being not exclusively localized at the PM but also at the ER (Gratz et al. 2021), raise the question about a potential functional importance of this potential interaction, that remains to be tested *in planta*. Given the fact that ERS2 is a receptor kinase (Moussatche and Klee 2004) whose activity is not needed for ET signaling, it raises the question whether the kinase targets substrates outside the ET pathway and, thus, could be involved in additional responses (Chen et al. 2009; Lacey and Binder 2014). This opens up for the hypothesis that AtLHT1 could be post-translationally modified in an

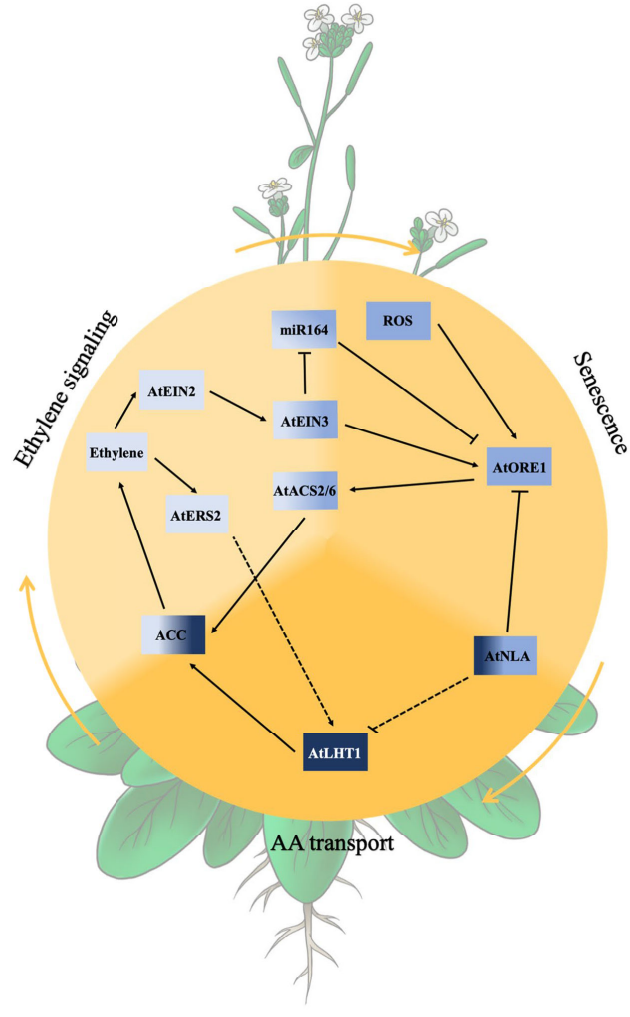


Fig. 2 The molecular regulation of amino acid transporters is influenced by diverse regulatory pathways. Using the example of AtLHT1, the influence of individual key players important for ethylene (ET) signaling and senescence in the context of pathogen defense is depicted. AtLHT1 transports the signaling molecule and ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The ET receptor kinase ETHYLENE RESPONSE SENSOR2 (AtERS2) might interact with AtLHT1 and thus depicts a potential feedback loop in dependence of ET. ET presence in parallel represses the activity of miRNA164, through the action of the transcription factor (TF) EIN3. miRNA164 itself is a negative regulator of the TF ORESARA1 (AtORE1), a key player in plant senescence. AtEIN3 activates *AtORE1* transcription directly whereas AtORE1 then activates the expression of *ACC SYNTHASE2* (*AtACS2*), displaying a feed-forward loop. AtORE1 itself is directly regulated by the ubiquitin ligase NITROGEN LIMITATION ADAPTATION (AtNLA), which also regulates AtLHT1 through either direct or indirect action. Dashed lines indicate potential regulatory connections that remains to be tested

ET-dependent way; a speculation that remains to be tested. The strong connection between AtLHT1 and ET leads to the question if unknown defense responses against necrotrophs exist, that involve the action of AtLHT1. Pathogen attack triggers Ca^{2+} influx into the cell (Nishad et al. 2020), which can then lead to phosphorylation and activation of AtORE1 (Coca and San Segundo 2010; Durian et al. 2020). Overexpression of *AtCPK1* leads to increased resistance of plants upon necrotrophic attack, the molecular regulation for this is, however, so far unknown (Coca and San Segundo 2010). The suggested downregulation of *AtNLA* upon necrotrophic interaction (Val-Torregrosa et al. 2021-preprint) would lead to a potential reduction in AtORE1 degradation. Overall, this would increase AtORE1 activity and PCD as well as senescence (Oh et al. 1997; Kim et al. 2009; Balazadeh et al. 2010; Farage-Barhom et al. 2011; Al-Daoud and Cameron 2011; Matallana-Ramirez et al. 2013; Qiu et al. 2015; Durian et al. 2020). This, a beneficial outcome for necrotrophs, would stand in contrast to the fact that a high accumulation of AtORE1 would increase ACC production via ACS2/6, and thus, ET accumulation (Qiu et al. 2015; Zhang et al. 2021). Reduced transcript accumulation of *AtNLA* would additionally lead to an increase in *AtLHT1* (Liao et al. 2020). AtLHT1 could then contribute to the

production of ET by transport of ACC (Shin et al. 2015; Choi et al. 2019) and, potentially, ET triggered resistance to necrotrophic microbes. It becomes evident that many common players in the regulation of pathogen resistance, leaf senescence and AAT regulation have overlapping functions. In future experiments, it has to be carefully determined, in which way the crosstalk between those players has an influence on plant microbes and plant resistance.

The complex network behind plant pathogen defense depends on several factors such as soil N availability and composition of the soil N pool which would affect both the internal N status of the plant and its energy status. Both plants and pathogens possess toolboxes, containing different signaling molecules such as ROS or hormones, but also transcription factors to concur the respective other. These responses are deeply interwoven with a machinery of cell-type specific regulation of AATs and, hence, the accumulation or depletion of specific AAs. The unique response signatures that are being formed upon association of a pathogen then contributes to the susceptibility of the plant.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-022-01244-1.

Acknowledgements We thank the Kempe Foundations and the Knut and Alice Wallenberg Foundation for financial support. In addition, we are grateful to Dr. Rumen Ivanov for his valuable comments on this manuscript. Sincere apologies to all colleagues whose scientific contributions could not be included in this review due to space limitations.

Author contributions Conceptualization: RG; Literature search: LT, JC, RG; Writing—original draft preparation: LT, RG; Writing—review and editing: LT, JC, TN, RG; Funding acquisition: TN, RG; Supervision: TN, RG.

Funding Open access funding provided by Swedish University of Agricultural Sciences. This review was supported by the Kempe Foundations (Grant Numbers JCK-2015.1 and JCK-2122 awarded to Torgny Näsholm and Regina Gratz) and the Knut and Alice Wallenberg Foundation (Grant Number 2016.0352 awarded to Torgny Näsholm).

Data availability Not applicable.

Code availability Not applicable.

Material availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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