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**Unraveling the roles of autophagy in proteome dynamics and energy
metabolism in plants under abiotic stress**

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Doctor Scientiae

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Thesis submitted to the Plant Physiology
Graduate Program of the Universidade
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the requirements for the degree of *Doctor
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Co-adviser: Jessica Aline Sousa Barros

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ABSTRACT

NOGUEIRA, Bianca Bueno, D.Sc., Universidade Federal de Viçosa, February, 2026.
Unraveling the roles of autophagy in proteome dynamics and energy metabolism in plants under abiotic stress. Adviser: Wagner Luiz Araujo. Co-adviser: Jessica Aline Sousa Barros.

Abiotic stresses impose major constraints on plant growth and productivity by disrupting cellular homeostasis, energy metabolism, and resource allocation. These challenges are further intensified by climate change, which increases the frequency, duration, and severity of environmental stresses. Because stress adaptation requires extensive metabolic reprogramming, mechanisms that maintain energetic balance and cellular integrity are critical for plant survival. Autophagy, a conserved intracellular recycling pathway, contributes to the degradation and remobilization of cellular constituents, providing substrates for energy production while maintaining organelle and proteome quality. Despite increasing recognition of its importance, the contribution of autophagy to energetic metabolism under prolonged abiotic stress remains incompletely understood. This thesis investigated the role of autophagy in maintaining plant energy homeostasis under two major abiotic constraints, high temperature and phosphate (Pi) starvation, using *Arabidopsis thaliana* as a model system. In Chapter 1, a comprehensive review synthesized current knowledge on the role of autophagy in plant heat-stress responses, highlighting its functions in thermotolerance, thermomemory, recovery, proteostasis, organelle quality control, and reproductive resilience. In Chapter 2, experimental analyses subsequently demonstrated that autophagy is essential for acclimation to prolonged warming, sustaining thermomorphogenic growth, photosynthetic performance, water conservation, and metabolic coordination. Proteomic and metabolic evidence revealed that autophagy preserves organelle integrity, promotes proteome stability, and prevents maladaptive activation of alternative catabolic pathways during chronic heat exposure. The role of autophagy in nutrient stress was investigated through mitochondrial and whole-plant responses to Pi starvation. In chapter 3, under Pi limitation, autophagy-deficient mutants exhibited accelerated depletion of intracellular Pi pools, impaired respiratory acclimation, and extensive remodeling of the mitochondrial proteome, indicating a critical role for autophagy in maintaining mitochondrial energetic competence. In chapter 4, at the whole-plant level, autophagy-deficient mutants showed disrupted root-to-shoot Pi redistribution, promoted Pi retention in roots and depletion in shoots, altered biomass allocation, and induced tissue-specific metabolic

reprogramming. Proteomic analyses further revealed that autophagy regulates the transition from plastidial carbon assimilation to glycolytic and respiratory energy production during prolonged Pi deprivation. Collectively, the results presented in this thesis establish autophagy as a regulator of energetic homeostasis under abiotic stress. Rather than functioning solely as a degradative recycling mechanism, autophagy emerges as a multi-scale homeostatic system that integrates nutrient recycling, organelle maintenance, proteome remodeling, and metabolic flexibility to sustain plant acclimation under prolonged environmental stress. These findings advance our understanding of the physiological and molecular mechanisms underlying stress resilience and identify autophagy as a promising target for the development of crops better adapted to future climatic and nutritional challenges.

Keywords: autophagy; energetic metabolism; proteomics; phosphate limitation; prolonged warming; *Arabidopsis thaliana*

RESUMO

NOGUEIRA, Bianca Bueno, D.Sc., Universidade Federal de Viçosa, fevereiro de 2026. **Desvendando os papéis da autofagia na dinâmica do proteoma e no metabolismo energético em plantas sob estresse abiótico.** Orientador: Wagner Luiz Araujo. Coorientadora: Jessica Aline Sousa Barros.

Os estresses abióticos impõem grandes restrições ao crescimento e à produtividade das plantas ao comprometer a homeostase celular, o metabolismo energético e a alocação de recursos. Esses desafios são ainda mais intensificados pelas mudanças climáticas, que aumentam a frequência, a duração e a severidade dos estresses ambientais. Como a adaptação ao estresse requer ampla reprogramação metabólica, mecanismos capazes de manter o equilíbrio energético e a integridade celular são essenciais para a sobrevivência das plantas. A autofagia, uma via conservada de reciclagem intracelular, contribui para a degradação e remobilização de constituintes celulares, fornecendo substratos para a produção de energia e, ao mesmo tempo, preservando a qualidade das organelas e do proteoma. Apesar do crescente reconhecimento de sua importância, a contribuição da autofagia para o metabolismo energético sob estresses abióticos prolongados ainda não é completamente compreendida. Esta tese investigou o papel da autofagia na manutenção da homeostase energética vegetal sob duas importantes restrições abióticas, altas temperaturas e deficiência de fosfato inorgânico (Pi), utilizando *Arabidopsis thaliana* como sistema modelo. No Capítulo 1, uma revisão abrangente sintetizou o conhecimento atual sobre o papel da autofagia nas respostas das plantas ao estresse térmico, destacando suas funções na termotolerância, memória térmica, recuperação pós-estresse, proteostase, controle de qualidade de organelas e resiliência reprodutiva. No Capítulo 2, análises experimentais demonstraram que a autofagia é essencial para a aclimação ao aquecimento prolongado, sustentando o crescimento termomorfogênico, o desempenho fotossintético, o controle da perda de água, e a coordenação metabólica. Evidências proteômicas e metabólicas revelaram que a autofagia preserva a integridade das organelas, promove a estabilidade do proteoma e previne a ativação inadequada de vias catabólicas alternativas durante a exposição crônica ao calor. O papel da autofagia sob estresse nutricional foi investigado por meio de respostas mitocondriais e da planta inteira à privação de Pi. No Capítulo 3, sob limitação de Pi, mutantes deficientes em autofagia apresentaram depleção acelerada dos estoques intracelulares de Pi, comprometimento da aclimação respiratória e extensiva remodelação do proteoma mitocondrial, indicando um papel crítico da autofagia na

manutenção da competência energética mitocondrial. No Capítulo 4, em escala de planta inteira, mutantes deficientes em autofagia apresentaram redistribuição comprometida de Pi entre raízes e parte aérea, promovendo retenção de Pi nas raízes e depleção de Pi na parte aérea, além de alterações na alocação de biomassa e reprogramação metabólica específica de tecido. As análises proteômicas revelaram ainda que a autofagia regula a transição da assimilação plastidial de carbono para a produção de energia via glicólise e respiração durante a privação prolongada de Pi. Coletivamente, os resultados apresentados nesta tese estabelecem a autofagia como uma reguladora da homeostase energética sob estresses abióticos. Em vez de atuar apenas como um mecanismo degradativo de reciclagem, a autofagia emerge como um sistema homeostático em múltiplas escalas, integrando reciclagem de nutrientes, manutenção de organelas, remodelação do proteoma e flexibilidade metabólica para sustentar a aclimação das plantas sob estresses ambientais prolongados. Esses resultados ampliam nossa compreensão dos mecanismos fisiológicos e moleculares que sustentam a resiliência ao estresse e identificam a autofagia como um alvo promissor para o desenvolvimento de culturas mais adaptadas aos futuros desafios climáticos e nutricionais.

Palavras-chave: autofagia; metabolismo energético; proteômica; limitação de fosfato; calor prolongado; *Arabidopsis thaliana*

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GENERAL INTRODUCTION

Plants, as sessile organisms, are continuously exposed to fluctuating environmental conditions that threaten growth, development, and survival. These challenges include a range of abiotic stresses, such as drought, heat, cold, hypoxia, oxidative stress, and nutrient limitation, as well as biotic threats from pathogens (Cheng et al. 2022). In response, plants activate complex and highly coordinated signaling networks that trigger extensive morphological, physiological, and metabolic reprogramming at both cellular and systemic levels. A fundamental component of this reprogramming is the maintenance of cellular energy balance, which becomes particularly critical under stress due to impaired enzymatic activity, limited substrate availability, and the increased energetic costs associated with repair, detoxification, and defense processes (Obata and Fernie 2012).

Disruptions to primary metabolism caused by stress pose a major threat to cellular energy homeostasis. Preserving energy balance is essential for sustaining defense responses and maintaining cellular viability. Accordingly, identifying the cellular mechanisms that mitigate metabolic collapse is a prerequisite for understanding plant stress tolerance. Among these protective processes, intracellular recycling pathways, particularly autophagy, play a crucial role in minimizing cellular damage and preserving metabolic stability under low-energy or nutrient-restricted conditions (Marshall and Vierstra 2018; Signorelli et al. 2019b; Barros et al. 2020).

Autophagy is an evolutionarily conserved catabolic process through which eukaryotic cells degrade and recycle dysfunctional or unnecessary cellular components. Under energy-limited conditions, autophagy reallocates internal resources by mobilizing macromolecules and organelles to sustain essential cellular functions (Araújo et al. 2011a). Basal autophagy contributes to quality control by removing damaged proteins and organelles, thereby preventing the accumulation of toxic cellular material. During stress or specific developmental transitions, including germination, flowering, and senescence, autophagy is strongly induced and facilitates nutrient remobilization. The resulting metabolites, including amino acids, fatty acids, and sugars, directly fuel energy metabolism and promote plant survival (Signorelli et al. 2019a; Sharma et al. 2022a).

In plants, three major forms of autophagy have been described: microautophagy, macroautophagy, and mega-autophagy (Marshall and Vierstra 2018; Magen et al. 2022). Microautophagy, which involves the direct engulfment of cytosolic material by tonoplast invagination or protrusion, is well characterized in yeast but remains poorly understood in plants, with its molecular machinery and regulatory mechanism still largely unresolved (Sieńko

et al. 2020; Yagyu and Yoshimoto 2024). Mega-autophagy represents a drastic, large-scale degradative response associated with programmed cell death (PCD) (Hatsugai et al. 2004; Van Doorn and Papini 2013). During mega-autophagy, the vacuolar membrane ruptures and releases hydrolytic enzymes into the cytoplasm, promoting extensive cellular degradation, particularly during hypersensitive responses and terminal PCD events (Hatsugai et al. 2004; Van Doorn and Papini 2013; Nabipour et al. 2023). By contrast, macroautophagy (hereafter referred to as autophagy) is the canonical and best-characterized pathway. It relies on the formation of double membrane autophagosomes that sequester cytosolic cargo and subsequently fuse with the vacuole. Upon delivery, autophagic bodies are degraded by vacuolar hydrolases, and the resulting metabolites are exported back to the cytosol to support metabolic homeostasis (Chen et al. 2019; Gross and Graef 2020; Yagyu and Yoshimoto 2024).

The autophagic machinery operates through the coordinated activity of a conserved set of autophagy-related (ATG) proteins. In plants, more than 40 ATG genes have been identified as components of the core autophagy pathway (Marshall and Vierstra 2018; Petersen et al. 2024a). These proteins can be grouped into five functional modules: (i) the ATG1-ATG13 kinase complex that initiates phagophore formation; (ii) ATG9-containing vesicles that supply membrane material and support phagophore expansion; (iii) the ATG2-ATG18 complex that mediates lipid transfer; (iv) the phosphatidylinositol-3-kinase (PI3K) complex required for vesicle nucleation; and (v) the ubiquitin-like ATG8 and ATG12 conjugation systems that drive autophagosome biogenesis and maturation (Chung 2019; Chiu et al. 2023a; Zhou et al. 2023b).

Among these components, ATG5, ATG7 and ATG2 are particularly crucial for sustaining autophagic flux. ATG5 and ATG7 play central roles in ATG8 lipidation, a key step in autophagosome formation (Cheng et al. 2022). ATG5 forms a complex with ATG12 to catalyze ATG8 conjugation to phosphatidylethanolamine (PE), thereby anchoring ATG8 to the autophagosomal membrane (Le Bars et al. 2014; Marshall and Vierstra 2018). ATG7 functions as an E1-like activating enzyme that initiates both the ATG12-ATG5 and ATG8-PE conjugation cascades (Phillips et al. 2008; Yamaguchi et al. 2018). ATG2 contributes to autophagosome expansion and closure by facilitating lipid transfer and coordinating the trafficking of ATG18a and ATG9 vesicles (Kang et al. 2018; Luo et al. 2023). Consequently, *atg* mutants fail to form functional autophagosomes, and frequently exhibit premature senescence under nitrogen, carbon, or phosphate (Pi) limitation (Barros et al. 2017b; Huang et al. 2019; Yue et al. 2021a; Li et al. 2022a).

Integrative studies combining autophagy mutants with metabolic profiling have revealed a central role for autophagy in sustaining nutrient homeostasis and ensuring adequate

metabolic resources for growth and survival (Wada et al. 2015; Cui et al. 2018; Sera et al. 2019; McLoughlin et al. 2020; Alseekh et al. 2022). The pronounced sensitivity of *atg* mutants to energy- and nutrient-limiting conditions (e.g., nitrogen, carbon, and Pi limitation) has been attributed to impaired respiration, altered lipid and starch metabolism, and defects in amino acid and organelle turnover (Guiboileau et al. 2012; Avin-Wittenberg et al. 2015; Masclaux-Daubresse 2016; Yoshitake et al. 2021, 2022). These metabolic perturbations are closely linked to dysfunction in endoplasmic reticulum (ER) homeostasis, defective peroxisomal β -oxidation, and impaired degradation of damaged proteins, lipid droplets, and starch granules (Chen et al. 2019; Havé et al. 2019; Barros et al. 2021; Gomez et al. 2021; Li et al. 2022a; Yoshitake et al. 2022).

In recent years, substantial advances have been made in understanding how autophagy is regulated under abiotic stress (Avin-Wittenberg 2019; Signorelli et al. 2019b; Gross and Graef 2020; Su et al. 2020; Chen et al. 2021). Among these stressors, heat stress (HS) and Pi-starvation represent two fundamentally distinct yet highly relevant challenges to plant energy metabolism, and therefore form the primary focus of this thesis.

HS destabilizes proteins, perturbs membrane integrity, and compromises mitochondrial function, leading to ATP depletion and excessive accumulation of reactive oxygen species (ROS) (Huang et al. 2012; Wang et al. 2018; Khan and Shahwar 2020; Scafaro et al. 2021). Autophagy contributes to thermotolerance by removing denatured proteins and damaged organelles, thereby preserving proteostasis, and supporting metabolic recovery (Zhou et al. 2014a; Zhang et al. 2022). By contrast, Pi limitation restricts ATP synthesis and disrupts essential biosynthetic and redox processes, including nucleotide metabolism, respiration, and carbon assimilation (Mikulska et al. 1998; Morcuende et al. 2007; Dissanayaka et al. 2021a). Pi deficiency alters carbon metabolism and can constrain mitochondrial energy metabolism through reduced Pi availability (Huang et al. 2008). In this context, autophagy likely serves as a key adaptive mechanism by enabling Pi recycling through the degradation of macromolecules and organelles, thereby sustaining mitochondrial respiration and energy production during prolonged nutrient deficiency (Yoshitake et al. 2022; Yoshitake and Yoshimoto 2023).

Despite substantial progress, key aspects of how autophagy contribute to stress adaptation remain unresolved. Although autophagy is recognized as essential for maintaining energy balance and stress survival, its specific roles in supporting mitochondrial function, regulating respiration, and preserving energetic homeostasis under Pi limitation and high temperatures require deeper investigation. Under Pi-starvation, the mechanisms by which autophagy shapes temporal and tissue-specific metabolic responses, particularly the differential

acclimation of roots and shoots from early to late stress phases, remain poorly understood. Similarly, although the role of autophagy during acute heat shock has been extensively characterized, its function under moderate, sustained moderate temperature increases (prolonged warming) remain largely unexplored. Whether autophagy performs distinct roles under these contrasting thermal regimes represents a key open question. Elucidating these mechanisms, including associated metabolic and proteomic reprogramming, is essential for understanding how plants preserve energetic homeostasis and resilience under diverse environmental constraints.

Layout and aims of the chapters

This thesis aims to elucidate how autophagy contributes to the maintenance of energy metabolism in plants exposed to abiotic stresses. Specifically, it investigates the physiological, biochemical, and molecular roles of autophagy under abiotic stress, particularly heat stress and Pi-starvation. The overarching goal is to advance our understanding of the functional relevance of autophagy in sustaining mitochondrial respiration, preserving metabolic homeostasis, and promoting stress resilience in *Arabidopsis thaliana*. To achieve these goals, complementary experimental approaches were employed, including phenotypic, metabolic, proteomic, and mitochondrial analyses across distinct stress conditions, tissues, and time points. Accordingly, the thesis is organized into four independent yet interconnected chapters, each comprising an introduction, results, discussion, and experimental methods, except for chapter 1, which represents a comprehensive review. The thesis concludes with a section synthesizing the main findings and outlining future perspectives on the role of autophagy in plant adaptation to energy stress.

Chapter 1: Beyond cellular recycling: autophagy as a central regulator of plant heat adaptation

Current knowledge of autophagy in plant heat responses has expanded considerably in recent years, yet its functions across different thermal scenarios remain fragmented. Although autophagy activation under elevated temperatures is well documented, the mechanisms through which it contributes to basal thermotolerance, acquired thermotolerance, thermomemory, and post-stress recovery are often considered independently. In this chapter, we synthesize current evidence demonstrating that autophagy functions far beyond the clearance of heat-damaged proteins and organelles. We highlight its emerging role as an integrative regulator of cellular homeostasis, coordinating proteostasis, organelle quality control, energy metabolism, hormonal

signaling, and stress-memory mechanisms during heat adaptation. By integrating findings across diverse heat-stress regimes, we propose that autophagy represents a central component of plant thermal resilience and discuss key knowledge gaps that must be addressed to improve our understanding of plant adaptation to a warming climate.

Chapter 2: Autophagy safeguards energy metabolism and proteome homeostasis during acclimation to prolonged warming in *Arabidopsis thaliana*

Rising global temperatures increasingly expose plants to prolonged periods of moderate warming that require physiological acclimation rather than short-term stress survival. Although autophagy is well recognized for its protective role during acute heat stress, its function during prolonged warming remains poorly understood. In this chapter, we demonstrate that autophagy is essential for the physiological, metabolic, and proteomic adjustments that enable *Arabidopsis thaliana* to acclimate to sustained elevated temperatures. Autophagy-deficient mutants exhibited impaired thermomorphogenic growth, reduced photosynthetic performance, elevated respiratory and transpiratory costs, and extensive metabolic reprogramming. Integrated metabolic and proteomic analyses revealed that autophagy supports organelle proteostasis, maintains carbon-nitrogen balance, preserves metabolic flexibility, and promotes efficient resource utilization during warming acclimation. We propose that autophagy acts as a central regulator of long-term thermal acclimation by coordinating organelle quality control, energy metabolism, and water-use balance, thereby sustaining growth and physiological homeostasis under prolonged warming.

Chapter 3: Autophagy sustains mitochondrial respiration during phosphate starvation by remodeling the plant mitochondrial proteome

Phosphate limitation imposes severe constraints on plant energy metabolism by restricting ATP synthesis and disrupting essential biosynthetic processes. In this chapter, we investigate how autophagy supports mitochondrial performance and metabolic stability under Pi-starvation. Using autophagy-deficient mutants, we show that autophagy is required to maintain respiratory capacity and mitochondrial proteome homeostasis when Pi availability is limited. Comparative mitochondrial proteomics indicate that autophagy promotes the selective turnover of metabolic and stress-related proteins, thereby supporting energy production and Pi recycling. We propose that autophagy functions as an adaptive mechanism that enables mitochondria to sustain efficient respiration under Pi limitation, ultimately preserving cellular energy balance and stress resilience.

Chapter 4: Exploring tissue- and time-dependent roles of autophagy in phosphate starvation responses of *Arabidopsis thaliana*

Phosphate starvation imposes major constraints on plant energy metabolism and requires coordinated regulation of nutrient allocation between roots and shoots. Although autophagy contributes to intracellular nutrient recycling, its role in systemic phosphate homeostasis and tissue-specific metabolic acclimation during Pi limitation remains poorly understood. We hypothesized that autophagy is required to maintain Pi and metabolic homeostasis across organs during progressive Pi-starvation. To test this, we investigated the role of autophagy in root-shoot Pi partitioning and organ-specific proteome remodeling during early and prolonged Pi limitation in *Arabidopsis thaliana*. Collectively, our findings demonstrate that autophagy is an integrator of Pi-starvation acclimation, coordinating Pi homeostasis, and proteome remodeling across tissues. By maintaining Pi allocation and metabolic flexibility during nutrient limitation, autophagy may support whole-plant resilience to prolonged Pi stress.

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CHAPTER 1 – Beyond cellular recycling: autophagy as a central regulator of plant heat adaptation

Beyond cellular recycling: autophagy as a central regulator of plant heat adaptation

ABSTRACT

Climate change is increasing the frequency, intensity, and duration of heat stress events, posing a major threat to plant productivity and global food security. To cope with elevated temperatures, plants must coordinate proteostasis, metabolic adjustment, stress signaling, and recovery processes across multiple temporal scales. Beyond its canonical role in the removal of damaged proteins and organelles, autophagy is increasingly recognized as an integrative mechanism that links thermotolerance, thermomemory, proteome remodeling, organelle quality control, and reproductive resilience. In this review, we synthesize recent advances in understanding how autophagy contributes to heat adaptation from acute heat shock through recovery and stress-memory phases. We highlight emerging mechanisms of selective autophagy, including specialized receptors, biomolecular condensates, and organelle-specific quality-control pathways, and discuss how autophagy interfaces with molecular chaperones, the ubiquitin-proteasome system, the unfolded protein response, and hormonal signaling networks. Despite rapid progress, most mechanistic knowledge derives from acute heat-shock studies, whereas the role of autophagy during other heat scenarios, such as prolonged warming, and under complex field-relevant stress combinations remain comparatively unexplored. Addressing these gaps will be essential for developing a unified understanding of heat adaptation and for harnessing autophagy to improve crop resilience in a warming climate.

Keywords: Heat stress; autophagy; basal thermotolerance; acquired thermotolerance; climate change.

INTRODUCTION

Climate change poses an escalating threat to global agriculture, as rising temperatures and increasingly frequent extreme heat events and heatwaves severely compromise plant productivity and crop stability. Current projections indicate that global temperatures may increase by 2.6 to 4.6 °C by the end of the century, with major consequences for productivity in key agricultural crops, including wheat, rice, and maize (Zhao et al. 2017; IPCC 2023). As sessile organisms, plants must withstand fluctuations in temperature and therefore rely on sophisticated mechanisms to perceive and respond to heat stress (HS).

Plant responses to high temperature vary according to stress intensity, duration, and recurrence. Moderate increases in ambient temperatures induce thermomorphogenesis, a suite of morphological adaptations that includes the acceleration of growth and flowering (Park et al.

2017; Casal and Balasubramanian 2019; Sun et al. 2024). When temperatures exceed species-specific thresholds, plants activate the heat stress response (HSR), which counteracts the detrimental effects of protein misfolding, membrane destabilization, and metabolic disruption (Sung et al. 2003; Jagadish et al. 2021; Samtani et al. 2022). Heat regimes are diverse, ranging from acute heat shock to recurrent heat waves and prolonged warming, and each imposes distinct physiological and cellular challenges (Khan and Shahwar 2020; Wang et al. 2020; Jagadish et al. 2021; Chaudhry and Sidhu 2022).

Plant survival under HS relies on both basal thermotolerance, defined as the intrinsic ability to withstand acute heat stress without prior acclimation, and the capacity to acquire thermotolerance following a non-lethal pre-exposure (Yeh et al. 2012; Li et al. 2018). Importantly, the protective state induced by mild stress can be maintained for days to weeks even after temperatures return to normal, reflecting the establishment of heat stress memory (HSM) or thermomemory (Lämke et al. 2016; Sedaghatmehr et al. 2019). Thermomemory enhances resilience to recurrent stress events and depends on coordinated transcriptional, chromatin-based, and metabolic adjustments (Sharma et al. 2019; Nishad and Nandi 2021; Balazadeh 2022; Li et al. 2025b).

The HSR integrates multiple protective systems, including heat shock proteins (HSPs), cytosolic and organellar chaperones, reactive oxygen species (ROS) signaling networks, and epigenetic reprogramming (Vacca et al. 2004; Wang et al. 2013; Distéfano et al. 2017; Borovik and Grabelnych 2018; Haider et al. 2021). In this context, autophagy has emerged as a major regulator of cellular homeostasis under HS. This evolutionarily conserved degradation pathway recycles damaged proteins, protein aggregates, membranes, and organelles, and supports survival under a variety of abiotic stresses (Klionsky and Emr 2000; Yoshimoto et al. 2004). In plants, autophagy contributes to both the establishment and resetting of thermomemory through its roles in proteostasis maintenance, metabolic homeostasis, organelle quality control, and post-stress recovery (Zhou et al. 2013; Sedaghatmehr et al. 2019; Yue et al. 2021b; Wan et al. 2023). Specialized cargo receptors and organelle-specific quality-control pathways further demonstrate that autophagy functions as a highly selective regulatory process rather than a purely degradative recycling mechanism, expanding its influence over proteostasis, signaling, and stress adaptation (Thirumalaikumar et al. 2021a; Yan et al. 2024; Hernández et al. 2026).

Despite rapid progress, major gaps remain in understanding how autophagy operates under realistic and multifactorial heat scenarios, including prolonged warming, recurrent heat events, and combined environmental stresses. How autophagy interfaces with other core

adaptive modules, including the chaperone network, the unfolded protein response (UPR), and multiple hormone-responsive signaling pathways, also remains insufficiently resolved.

In this review, we integrate recent advances in the role of autophagy across the major temporal phases of the heat response, encompassing acute heat shock, acclimation and acquired thermotolerance, heat stress memory, and post-stress recovery. These phases are distinguished by their temporal scale and dominant protective mechanisms, ranging from rapid proteostasis control during acute stress to longer-term transcriptional, epigenetic, and metabolic reprogramming during acclimation and memory. We then examine mechanistic insights into how autophagy coordinates these functions, focusing on selective cargo degradation, regulatory interplay with the ubiquitin-proteasome system (UPS) and the UPR, and modulation by hormonal and transcriptional networks. Here, autophagy is positioned as a multifaceted regulator that coordinates proteostasis, signaling, and energy balance to support plant adaptation to elevated temperatures, while highlighting critical knowledge gaps that must be addressed to guide future crop improvement.

MECHANISMS OF THERMOTOLERANCE: FROM PERCEPTION TO MEMORY

Heat stress (HS) disrupts cellular homeostasis at multiple levels, impairing protein stability, membrane integrity, organelle function, and metabolic coordination. One of the earliest events in heat perception is the increase in plasma membrane fluidity, which rapidly activates calcium ions (Ca^{2+})-permeable channels and induces transient cytosolic Ca^{2+} influx (Saidi et al. 2010; Wang and Huang 2017; Cano-Ramirez et al. 2021). Calcium signaling is subsequently decoded by calmodulins, calcineurin B-like proteins, calcium-dependent protein kinases (CDPKs), and calmodulin-binding transcription activators (CAMTAs), which initiate downstream heat-responsive transcriptional responses (Yuan and Poovaiah 2022; Kang et al. 2024). In parallel, plants also rely on protein thermosensors such as phytochrome B (PhyB), EARLY FLOWERING 3 (ELF3), and the intrinsically disordered protein THERMO-WITH ABA-RESPONSE 1 (TWA1), which directly integrate temperature signals into developmental and stress-responsive pathways (Legris et al. 2016; Press et al. 2016; Arico et al. 2019; Jung et al. 2020b; Bohn et al. 2024).

Heat-induced membrane destabilization rapidly propagates into oxidative and proteotoxic stress. HS perturbs chloroplast and mitochondrial electron transport chains, leading to excessive accumulation of reactive oxygen species (ROS) (Vacca et al. 2004; Lin et al. 2014; Zandalinas and Mittler 2021). Although uncontrolled ROS accumulation damages proteins, lipids, and nucleic acids, ROS also act as central signaling molecules that activate acclimatory

responses and stress signaling pathways. At the same time, elevated temperatures promote protein unfolding and aggregation, generating severe proteotoxic stress that threatens cellular viability (Wallace et al. 2015). In the endoplasmic reticulum (ER), accumulation of misfolded proteins activates the unfolded protein response (UPR), which enhances the expression of genes involved in protein folding, quality control, and degradation to restore ER proteostasis (Deng et al. 2011; Howell 2013; Malini et al. 2020). The cytoskeleton is also highly heat sensitive; both actin filaments and microtubules undergo destabilization under HS, impairing vesicle trafficking, intracellular transport, and cellular organization (Smertenko et al. 1997; Fan et al. 2016; Parrotta et al. 2016).

To counteract this disorder, plants activate interconnected protein quality control (PQC) systems that collectively preserve proteostasis during HS. Central among these are heat shock proteins (HSPs), the ubiquitin-proteasome system (UPS), and autophagy (Jacob et al. 2017; Pohl and Dikic 2019; Sedaghatmehr et al. 2019; Xie et al. 2024). HSPs function as molecular chaperones that stabilize unfolded proteins, prevent irreversible aggregation, and facilitate protein refolding or degradation (Ul Haq et al. 2019). The UPS primarily targets soluble and short-lived misfolded proteins, whereas autophagy preferentially removes protein aggregates, long-lived proteins, and damaged organelles through vacuolar degradation. Together with the UPR, these pathways form a highly interconnected proteostasis network in which the balance between protein refolding, degradation, and organelle quality control ultimately determines whether cells successfully acclimate or transition toward irreversible damage and cell death under severe stress (Simoni et al. 2022). Increasing evidence further suggests that the coordination among these pathways is dynamic and context dependent, varying according to stress intensity, duration and cellular compartmentalization (Box 1).

HS also affects cellular energy metabolism, forcing plants to reprogram metabolic pathways to maintain redox and energetic homeostasis (Wang et al. 2020; Scafaro et al. 2021; Zahra et al. 2023). Photosynthesis is particularly vulnerable to elevated temperatures, exhibiting reduced efficiency above species-specific thermal optima (Berry and Bjorkman 1980). One major limitation is the thermal instability of Rubisco activase, which compromises Rubisco activity and carbon fixation (Salvucci and Crafts-Brandner 2004a, 2004b; Perdomo et al. 2017). Consequently, chloroplasts undergo profound metabolic remodeling involving chlorophyll degradation, altered carbon metabolism, ROS accumulation, and activation of antioxidant defenses (Wang et al. 2018; Hu et al. 2020). In contrast, mitochondrial respiration is often enhanced during HS to sustain ATP production required for repair and stress adaptation (O'Leary et al. 2019). Increased activity of the alternative oxidase (AOX) pathway helps

maintain electron flow while limiting mitochondrial ROS generation (Huang et al. 2012; Scafaro et al. 2021; Sharma et al. 2022b). Because autophagy contributes both to protein quality control and to the recycling of carbon, nitrogen, and energy-rich metabolites, it is uniquely positioned to coordinate these proteostatic and metabolic dimensions of heat adaptation.

Once HS is perceived, plants activate HSR, which constitutes the core molecular basis supporting basal thermotolerance. Central to this response are Heat Shock Factors (HSFs), particularly HSFA1 isoforms, which are rapidly activated after heat-induced dissociation of HSP70-HSP90 complexes from inactive HSFA1 proteins (Hahn et al. 2011; Ohama et al. 2017). Activated HSFs induce the expression of protective genes, including HSPs, ROS-scavenging enzymes, and components of the PQC machinery (Wang et al. 2020; Xie et al. 2022; Löchli et al. 2023). Beyond immediate cytoprotection, the HSR also initiates longer-term acclimatory processes through activation of secondary regulators associated with heat stress memory (HSM), particularly HSFA2 and HSFA3 (Olas et al. 2021).

Exposure to a mild priming heat stimulus can establish thermomemory, a transient but actively maintained state that enhances tolerance to subsequent stress episodes (Staacke et al. 2025). HSFA2 and HSFA3 play central roles in this process by sustaining the expression of memory-associated genes such as *HSP18.1-CI* and *HSP25.3-P* after the initial stress subsides (Li et al. 2017; Liu et al. 2018). Maintenance of thermomemory is further reinforced through epigenetic regulation, particularly enrichment of the activating histone mark H3K4me3 at memory loci, which preserves transcriptional competence during recovery (Lämke et al. 2016; Liu et al. 2018). Persistent accumulation of protective proteins, including HSP21 and HSP101, additionally contributes to prolonged disaggregation capacity and stress preparation during recovery (Hong and Vierling 2000; Sedaghatmehr et al. 2016). Other regulatory layers, such as microRNA156 (miR156)-mediated repression of SQUAMOSA PROMOTER BINDING-LIKE (SPL) transcription factors, help stabilize heat-responsive transcriptional programs during extended recovery periods (Stief et al. 2014).

Thermomemory is also closely linked to metabolic and hormonal reprogramming. Several metabolites, including raffinose family oligosaccharides, branched-chain amino acids, tocopherols, and glucose, exhibit memory-associated accumulation patterns and contribute to osmoprotection, antioxidant defense, and energetic balance (Serrano et al. 2019). Glucose signaling through the TOR-E2Fa-HLP1 pathway promotes maintenance of H3K4me3 marks and sustains HSFA2-dependent transcription during memory establishment (Sharma et al. 2019; Olas et al. 2021). Hormonal pathways further modulate thermomemory, particularly Brassinosteroid (BS)-mediated activation of BES1 and ABA-dependent signaling pathways

that reinforce heat-responsive transcriptional programs (Albertos et al. 2022; Yao et al. 2022). More recently, biomolecular condensates typically formed by liquid-liquid phase separation have emerged as additional regulators of heat adaptation (Staacke et al. 2025). Stress granules (SGs) transiently sequester mRNAs and regulatory proteins, including HSFA2, HSFA7b, and AGO1, thereby modulating transcript stability and translational reactivation during stress and recovery (Tong et al. 2022; Blagojevic et al. 2024).

Although thermomemory enhances survival under recurring stress, maintaining a prolonged defense state is metabolically costly and may compromise growth and development. Plants therefore require mechanisms that not only establish protection but also actively reset stress-induced programs once temperatures normalize. This transition depends on selective degradation pathways capable of removing persistent protein aggregates, stress-associated proteins, and damaged organelles while restoring cellular homeostasis. In this context, autophagy emerges as a central regulator linking proteostasis, metabolic adaptation, recovery, and thermomemory resetting. The following sections examine how autophagy contributes across the distinct temporal phases of the heat stress response.

PART I: AUTOPHAGY ACROSS THE TEMPORAL STAGES OF HEAT STRESS

Autophagy in basal thermotolerance and acute heat shock

Autophagy is rapidly activated during acute heat shock and has emerged as an evolutionarily conserved component of basal thermotolerance across diverse plant species. Heat-induced autophagic responses have been documented in *Arabidopsis* (Zhou et al. 2013), pepper (Zhai et al. 2016, 2017), tomato (Zhou et al. 2014a; Cheng et al. 2016), apple (Wang et al. 2014), potato (Song et al. 2022), maize (Li et al. 2020), sweet orange (Fu et al. 2020) and *Brassica rapa* (Yue et al. 2021b). Under heat shock, upregulation of core ATG genes (e.g. *ATG5*, *ATG6*, *ATG12A*, and *ATG18A*) and accumulation of autophagosomes have been consistently observed across species (Zhou et al. 2014a; Cheng et al. 2016; Zhai et al. 2016). In apple, for instance, *MdATG18a* increases 10- to 15-fold within 4-8 h at 42 °C (Wang et al. 2014), while in sweet orange multiple ATG genes (*CsATG2*, *CsATG18e*, *CsATG18g*, *CsVTI12b*) are strongly heat induced (Fu et al. 2020). Mutants lacking *ATG5* or *ATG7* in *Arabidopsis* and tomato display increased heat sensitivity, characterized by accelerated protein aggregation, membrane damage, and reduced viability under heat shock (Zhou et al. 2013, 2014a; Cheng et al. 2016). These findings establish autophagy as a core component of basal thermotolerance.

Gain-of-function studies further reinforce the protective role of autophagy during acute HS. In apple, overexpression of MdATG18a enhances basal thermotolerance by limiting ROS accumulation, elevating SOD, POD, and CAT activity, and preserving photosynthetic performance under HS (Huo et al. 2020). MdATG18a-overexpressing plants maintain thylakoid structure and chloroplast envelope integrity, whereas wild-type (WT) plants display severe heat-induced damage (Huo et al. 2020). This protection correlates with enhanced autophagic flux, including increased autophagosome accumulation and upregulation of MdATG genes, supporting a direct role for autophagy in chloroplast preservation during heat shock.

Despite this generally pro-survival role, autophagy does not increase linearly with heat intensity. Instead, it operates within narrow physiological limits. Severe or prolonged exposure can suppress autophagic flux; in *Arabidopsis* seedlings, autophagy becomes nearly blocked after 12 h at 37 °C or 2 h at 45 °C (Jung et al. 2020). This collapse reflects heat-induced disruption of membrane trafficking, denaturation of autophagy enzymes, or exhaustion of the proteostasis network. These threshold effects highlight the importance of precise autophagy modulation, since insufficient activity leads to proteostasis collapse, whereas excessive activity may promote detrimental degradation of essential cellular components.

When HS exceeds the buffering capacity of autophagy, plant cells appear to activate alternative, autophagy-independent death pathways. Distéfano et al. (2017) identified a ferroptosis-like cell death pathway in *Arabidopsis* root hairs exposed to extreme heat (55 °C), characterized by ROS accumulation, lipid peroxidation, and mitochondrial shrinkage. Notably, this process occurred independently of ATG5 and ATG7, indicating that canonical autophagy is neither required nor protective at such lethal thresholds. These findings raise a fundamental question: how do plant cells sense the tipping point at which autophagy-mediated cytoprotection is abandoned in favor of terminal cell death pathways? Defining the regulatory threshold at which autophagy-mediated cytoprotection gives way to irreversible cell death will be essential for understanding the upper limits of plant heat resilience.

Proper activity of autophagy during acute HS additionally requires transcriptional regulators and selective cargo receptors. The heat-responsive transcription factor WRKY33 is required for induction of autophagy under high temperature, and its silencing suppresses ATG5, ATG7, and NBR1 expression, reduces autophagosome accumulation, and heightens heat sensitivity in *Arabidopsis* and tomato (Zhou et al. 2014a, 2015). The selective autophagy receptor NBR1 likewise contributes to basal thermotolerance: *nbr1* mutants accumulate insoluble proteins and exhibit reduced survival under heat shock (Zhou et al. 2014a, 2014b).

These observations show that effective basal thermotolerance requires not only the core ATG machinery but also proper transcriptional activation and selective cargo recognition.

Within this tightly balanced system, members of the ATG8 gene family function as key modulators of thermotolerance, and accumulating evidence indicates that individual ATG8 isoforms contribute differently across distinct heat regimes and exposure durations. In pepper, *CaATG8e* and *CaATG8c* exhibit contrasting roles depending on the duration of heat exposure (Zhai et al. 2017; Liang et al. 2023). Under acute heat shock (45 °C for 6 h), silencing of *CaATG8e* enhances thermotolerance, reducing leaf wilting, chlorophyll loss, and electrolyte leakage while increasing expression of heat-responsive genes such as *CaHsfA2*, *CaHSP16.4*, and *CaHSP70.1* (Liang et al. 2023). Under prolonged HS (45 °C for 16 h), however, silencing *CaATG8c* impairs survival, resulting in severe wilting, reduced chlorophyll content, and lower SOD activity (Zhai et al. 2017).

The contrasting phenotypes observed after *CaATG8e* and *CaATG8c* silencing indicate that, although autophagy universally depends on ATG8 conjugation, individual isoforms contribute differently to autophagic capacity across heat regimes. During acute stress, restricting autophagy via *CaATG8e* silencing may prevent excessive turnover of essential components (Liang et al. 2023), whereas during prolonged exposure, sustained autophagic flux mediated by isoforms such as *CaATG8c* becomes indispensable for clearing persistently damaged proteins and organelles (Zhai et al. 2017). Thus, the functional divergence between *CaATG8e* and *CaATG8c* likely reflects a combination of intrinsic biochemical specialization and differential engagement of autophagy pathways as HS progresses. These observations suggest that *CaATG8e* participates in early, highly sensitive regulatory steps of the heat response, whereas *CaATG8c* appears more closely associated with sustained proteostasis and organelle quality control during prolonged exposure.

Notably, overexpression of both *CaATG8e* and *CaATG8c* in Arabidopsis led to reduced thermotolerance, though through distinct mechanisms (Zhai et al. 2017; Liang et al. 2023). Overexpression of *CaATG8e* in Arabidopsis reduces thermotolerance, causing excessive autophagosome buildup, impaired germination, and increased bleaching during HS (Liang et al. 2023). Similarly, overexpression of *CaATG8c* also diminishes survival, but through a different mechanism, suppressing key stress-response genes such as *AtRD29A*, *AtAPX2*, and *AtDREB2A* (Zhai et al. 2017). Both insufficient and excessive ATG8 activity therefore disrupt the finely tuned autophagic balance required for basal thermotolerance.

Recent isoform-resolved analyses in Arabidopsis further support the non-redundant nature of ATG8 family members. Chiaro et al. (2025) demonstrated that ATG8 isoforms differ

markedly in lipidation efficiency, partner interactions, and cargo selectivity, forming distinct functional modules with specialized roles in organelle turnover and membrane dynamics. Although not heat-specific, these findings provide a mechanistic explanation for the contrasting phenotypes of CaATG8e and CaATG8c and underscore why ATG8 isoforms cannot be considered interchangeable during heat adaptation.

Similar patterns of ATG8 specialization have also been reported in other plant species. Genome-wide analyses in chrysanthemum (*Chrysanthemum morifolium*) identified an expanded ATG8 family (e.g., 17 CmATG8 genes) whose members display distinct temporal expression patterns during prolonged HS (40 °C). Some isoforms, such as *CmATG8g*, peak within the first hours, whereas others (e.g., *CmATG8c*, *CmATG8d*, *CmATG8o*) reach maximal induction only after 48-72 h (Luo et al. 2025). These expression dynamics suggest that plants maintain a diversified set of ATG8 paralogs, each potentially tuned to distinct phases of the heat response, supporting proteostasis across shifting stress regimes.

Collectively, evidence from loss- and gain-of-function studies indicates that basal thermotolerance depends not only on robust induction of autophagy but also on isoform-specific, dosage-sensitive, and time-dependent regulation of ATG8 activity. These observations also point toward a broader quantitative principle: heat resilience depends on maintaining autophagic flux within an optimal range. Both insufficient and excessive autophagic activity destabilize proteostasis, highlighting a major unresolved question regarding how plants calibrate autophagy according to heat intensity and duration.

Autophagy in acquired thermotolerance and thermomemory

Evidence accumulated over the last years indicates that autophagy participates not only in early heat responses but also in thermomemory establishment and its subsequent resetting. Zhang et al. (2021b) highlighted the role of autophagy in the initial response to HS, where the *atg5* mutant showed reduced early induction of stress-responsive genes, including those involved in signaling and protein quality control. Although differences between WT and *atg5* decreased as stress progressed, this early transcriptional impairment indicates that autophagy helps establish the priming layer of thermotolerance, providing a molecular foundation for thermomemory. Consistent with this, Wang et al. (2024a) reported that heat priming in tall fescue not only enhanced whole-plant thermotolerance but also preserved PSII photochemical efficiency during a subsequent triggering stress. Using TMT-based proteomics across three phases, pre-priming (S0), primed/recovery (R4), and triggering (S5), they identified 526 heat stress memory proteins (HSMPs). During the memory phase (R4), proteins associated with

autophagy and proteostasis were significantly enriched, and several autophagy-linked HSMPs, including the translation initiation regulator EIF2A, were selectively retained (Wang et al. 2024a). This enrichment indicates that autophagy remains active after heat priming, likely contributing to thermomemory maintenance by regulating the turnover and persistence of memory-associated effectors and sustaining translational capacity.

Sedaghatmehr et al. (2019) provided mechanistic evidence that autophagy negatively regulates thermomemory persistence by promoting HSP degradation during recovery. In autophagy-deficient mutants (*atg2-1*, *atg5-1*, *atg12ab*, *atg18a-2*), HSP proteins remained elevated after stress subsided despite similar transcript levels, and these mutants exhibited enhanced thermomemory, reflected in higher survival and biomass after a second heat episode. These findings demonstrate that autophagy acts as a post-translational regulator of thermomemory duration by promoting the clearance of persistent memory-associated HSPs during the post-stress phase. A complementary layer of regulation was identified by Thirumalaikumar et al. (2021a), who showed that NBR1, a selective autophagy receptor, targets thermomemory-associated proteins, including HSP90.1 and ROF1, for degradation during recovery. In *atg5-1* and *atg18a-2* mutants, NBR1 accumulated excessively, and loss-of-function *nbr1-2* plants displayed enhanced thermomemory but reduced basal thermotolerance, confirming that NBR1 fine-tunes memory duration while maintaining proteome integrity. Importantly, autophagy cooperates with the plastid metalloprotease FtsH6 in HSP21 turnover, indicating that organelle-specific proteases and autophagy jointly form a plastid proteolytic module that helps control the persistence of plastidial thermomemory effectors after heat exposure (Sedaghatmehr et al. 2021).

Thermoprimering also triggers strong autophagic activity in the shoot apical meristem (SAM), a region critical for developmental plasticity and stress responsiveness (Sedeh and Bazgir 2021). Sedeh and Bazgir (2021) reported transcriptional upregulation of multiple ATG genes, including ATG8 isoforms, during both priming and the subsequent recovery phase. Sustained expression of ATG5 and ATG8B following the initial heat stimulus, together with induction of selective autophagy markers such as ATI1, ATI2, NBR1, and TSPO, indicates a prolonged and spatially distinct autophagic response within the SAM. Confocal imaging of GFP-ATG8A further confirmed persistent autophagosome formation in meristematic tissues, supporting the idea that autophagy contributes to thermomemory-associated protection of meristem function.

Li et al. (2024) further showed that stress granules (SGs) act as dynamic regulatory platforms controlling the timing of autophagy activation. Under HS, SGs sequester core

autophagy components (including the ATG1/ATG13 kinase complex and ATG5), delaying bulk autophagy activation (Li et al. 2024). As the stress subsides, SG disassembly releases these ATG proteins back into the cytoplasm, facilitating the rapid, synchronous activation of autophagy required to clear heat-induced ubiquitinated aggregates during the recovery phase (Li et al., 2024). This SG-mediated mechanism ensures that autophagy is activated only after the initial stress phase, thereby coordinating the timing of thermomemory resetting and the clearance of persistent stress-associated proteins.

Chloroplast-derived signaling pathways also contribute to autophagy-dependent regulation of thermomemory. Pollastri et al. (2021) demonstrated that *cas* mutants, deficient in chloroplast calcium-sensing receptors, displayed prolonged thermomemory and elevated HSP17.6 levels during recovery. Although ATG transcript levels were unchanged, autophagic activity was inferred to be lower: free amino acid levels failed to increase in *cas* mutants 24 h after priming, and HSP17.6 protein persisted for days despite stable transcript levels. These findings suggest that chloroplast calcium signaling modulates autophagy-dependent turnover of memory-associated proteins, linking organelle-derived cues to the regulation of thermomemory persistence. In a related context, Pan et al. (2023) showed that overexpression of the *Haloxylon ammodendron* 14-3-3 protein HaFT-1 enhanced thermotolerance in *Arabidopsis* through coordinated activation of proline metabolism, ROS scavenging, and autophagy. Increased expression of ATG8a in transgenic lines, together with improved ROS clearance, suggests that HaFT-1 primes multiple protective systems, including autophagy, to reinforce both basal tolerance and thermomemory. For a concise synthesis of the role of autophagy in proteome resetting and memory control, see Sedaghatmehr and Balazadeh (2024).

Current evidence supports a multifaceted role for autophagy in thermomemory, functioning as a dynamic regulator of memory duration rather than merely a degradative stress-response pathway. Through selective turnover of HSPs (Sedaghatmehr et al. 2019; Thirumalaikumar et al. 2021a), SG-mediated temporal control of autophagy activation (Li et al. 2024), chloroplast-derived signaling pathways (Pollastri et al. 2021), and protection of meristematic tissues (Sedeh and Bazgir 2021), autophagy modulates both the persistence and the timely erasure of HS memory effectors. This dual role enables plants to maintain protective stress memory long enough to withstand recurrent heat episodes while preventing excessive persistence of energetically costly defense programs.

Autophagy in post-stress recovery

In contrast to the extensive literature on acute HS, the contribution of autophagy during post-stress recovery remains comparatively underexplored. Emerging evidence indicates that autophagy contributes to the restoration of cellular homeostasis by coordinating redox balance, hormonal reprogramming, and organellar turnover after heat exposure.

Early evidence linking autophagy to post-stress recovery emerged from studies on HSP101, a central chaperone required for thermotolerance acquisition (Charng et al. 2006). While HSP101 accumulates during HS, Wu et al. (2013) showed that its abundance declines rapidly during recovery, and this turnover is partially autophagy-dependent. Pharmacological inhibition of autophagy with E-64d delayed HSP101 degradation, demonstrating that autophagy contributes to the timely clearance of stress-induced chaperones. This controlled turnover likely facilitates restoration of basal proteostasis by removing stress-induced chaperones once their protective functions are no longer required.

Transcriptomic profiling of *Brassica rapa* identified ATG8A and ATG8E as central regulatory hubs within stress-responsive co-expression networks (Yue et al. 2021b). Notably, their induction followed genotype-specific trajectories. The heat-tolerant line activated ATG8 expression during the stress phase, whereas the sensitive genotype showed maximal induction only during recovery (Yue et al. 2021b). These contrasting temporal patterns suggest that autophagy is integrated differently into tolerant versus sensitive programs, potentially reflecting distinct strategies for damage limitation versus damage repair. ATG8 expression co-occurred with genes associated with oxidative stress mitigation (e.g., DJ1A), ABA-related transcriptional regulators (CPL1, VAL1), and cytokinin-related signaling (AAE3), indicating that autophagy participates in a broader rewiring of stress-resolution pathways (Yue et al. 2021b).

Li et al. (2025) further demonstrated that autophagy can even impair recovery under specific genetic backgrounds. In *Arabidopsis*, *fes1a* mutants, which lack a key HSP70 co-chaperone and are highly thermosensitive, blocking constitutive autophagy through mutations in core ATG genes (e.g., *atg7*) rescued both survival and chlorophyll retention during post-heat-stress recovery. Mechanistically, this rescue did not result from increased heat-shock protein accumulation but from the stabilization of peroxisomes. Confocal imaging and proteomic analyses showed that *atg7 fes1a* mutants retained higher peroxisome numbers, resulting in enhanced catalase activity, more efficient scavenging of H₂O₂ and O₂⁻, and increased auxin (IAA) biosynthesis via peroxisomal β -oxidation. Importantly, disrupting peroxisome function, either chemically with the catalase inhibitor 3-AT or genetically by overexpressing the peroxisomal transporter ABCD1, re-sensitized the *atg7 fes1a* mutants to

heat. These findings indicate that, in a chaperone-deficient background, autophagy-dependent peroxisome turnover can become detrimental by removing organelles required for ROS detoxification and hormone-mediated recovery responses. This contrasts with the findings of Yue et al. (2021), where ATG8 induction was associated with recovery in a stress-sensitive line, highlighting that autophagy may exert opposing effects depending on the cellular context and genetic background.

These apparently contrasting effects indicate that the contribution of autophagy to post-stress recovery is highly context dependent. Recovery efficiency likely depends not simply on the magnitude of autophagic activity, but rather on the nature of the substrates being targeted. Selective removal of damaged proteins and dysfunctional organelles can accelerate restoration of cellular homeostasis, whereas excessive degradation of protective or metabolically important organelles may compromise redox balance, hormone biosynthesis, and growth resumption. Thus, the physiological outcome of autophagy during recovery appears to depend on how degradation pathways are coordinated with the cellular demands of the post-stress state.

The transition from stress exposure to recovery is accompanied by extensive reprogramming of autophagic activity. Recovery-specific shifts, such as delayed ATG8 activation in sensitive *Brassica* genotypes (Yue et al. 2021b), indicate that autophagy is not simply maintained after stress cessation, but instead redeployed according to the physiological demands of recovery. In some contexts, autophagy facilitates recovery by removing obsolete chaperones, oxidized proteins, and dysfunctional organelles, thereby supporting restoration of redox, metabolic, and hormonal homeostasis. In others, particularly when protective organelles become limiting (e.g. peroxisomes), excessive autophagic degradation may impair recovery capacity. These findings establish post-stress recovery as a distinct regulatory phase in which autophagy must transition from a predominantly cytoprotective degradation program to a selective remodeling mechanism that preserves organelles and signaling components required for growth resumption. A central unresolved question is therefore how plants discriminate between structures that remain beneficial for acclimation and those become liabilities during recovery.

PART II: FUNCTIONAL INTERSECTIONS AND REGULATION

Synergistic proteostasis: coordination between autophagy, molecular chaperones, the UPS, and the UPR during heat stress

Heat stress imposes a challenge to cellular proteostasis by causing protein unfolding, aggregation, organellar dysfunction, and trafficking defects. Plants counteract this proteotoxic

burden through the coordinated activity of multiple protein quality control (PQC) systems, including molecular chaperones, the ubiquitin-proteasome system (UPS), the unfolded protein response (UPR), and autophagy. Rather than acting independently, these pathways form an integrated and highly dynamic proteostasis network whose balance appears to determine whether cells successfully acclimate or undergo irreversible damage under HS (Table 1).

Current evidence supports a functional division of labor between degradation systems. The UPS primarily removes soluble, short-lived, or moderately misfolded proteins, whereas autophagy preferentially targets protein aggregates, long-lived proteins, and damaged organelles that exceed proteasomal capacity (Pohl and Dikic 2019; Raffeiner et al. 2023; Sharma et al. 2024). This distinction becomes particularly important during severe HS, when aggregate formation rapidly increases and proteasomal buffering alone becomes insufficient. Consistent with this idea, proteasome inhibition enhances NBR1 accumulation and stimulates compensatory autophagic responses, suggesting that plants dynamically rebalance degradation routes according to proteotoxic load (Jung et al. 2020a).

Selective autophagy receptors appear to function as central coordinators of this proteostasis network. NBR1 not only mediates aggregate clearance during acute HS but also regulates thermomemory resetting through selective degradation of HSP90.1-ROF1 complexes during recovery (Thirumalaikumar et al. 2021a). Similarly, the E3 ubiquitin ligase CHIP cooperates with NBR1 to limit aggregate accumulation under heat stress, and loss of either pathway compromises thermotolerance (Zhou et al. 2014b; Zhang et al. 2021a). These findings suggest that ubiquitination may serve as a shared molecular language linking UPS-mediated recognition with autophagy-dependent clearance. However, recent evidence that *Arabidopsis* NBR1 can also target non-ubiquitinated proteins through liquid-liquid phase separation (LLPS) (Yan et al. 2024) indicates that selective autophagy likely recognizes a larger spectrum of proteotoxic signals than previously appreciated.

Heat-induced proteostasis also depends heavily on molecular chaperones. HSPs buffer protein misfolding, stabilize metastable intermediates, and facilitate refolding, thereby limiting aggregate formation during the early phases of stress (Ul Haq et al. 2019). Yet increasing evidence indicates that chaperones and autophagy are mechanistically interdependent rather than sequentially activated systems. HSP101 turnover during recovery requires autophagy, and blocking vacuolar degradation leads to abnormal HSP101 persistence after stress (Wu et al. 2013). Similarly, CaATG6 associates with HSP90 family members in pepper (Zhai et al. 2016), while overexpression of autophagy (ATG)-related genes such as MdATG18a, MdATG8i, and SlATG8f enhances HSP accumulation and improves thermotolerance across species (Huo et al.

2020, 2021; Song et al. 2024). Conversely, excessive persistence of HSP-associated complexes can interfere with thermomemory resetting, requiring selective autophagic clearance mechanisms mediated by NBR1 (Thirumalaikumar et al. 2021a). Together, these observations support a model in which chaperones and autophagy function cooperatively to balance immediate cytoprotection with long-term proteome reorganization.

The unfolded protein response (UPR) provides an additional regulatory layer connecting ER stress with autophagy-dependent proteostasis. HS disrupts ER protein folding capacity, activating canonical UPR signaling pathways such as IRE1b and bZIP60 (Malini et al. 2020). In *Arabidopsis*, IRE1b signaling promotes selective removal of misfolded ER proteins via autophagy (Yang et al. 2016), while in maize heat-induced splicing of bZIP60 enhances ATG8 lipidation and thermotolerance (Li et al. 2020). Importantly, this coordination also depends on chaperone-associated proteins such as the J-protein AtDjC5 (HSP40-family), which is required for autophagy induction during ER stress (Shen et al. 2022). Rather than functioning solely as a stress-sensing pathway, the UPR may act as a regulatory interface that links ER proteostasis status with autophagic capacity, thereby coordinating protein refolding, degradation, and recovery during HS.

Transcriptional regulators further integrate these proteostasis systems into unified heat-responsive pathways. The master heat shock transcription factor HsfA1a directly activates both chaperone genes and autophagy regulators such as ATG10 and BAG5b, thereby promoting autophagosome formation and clearance of ubiquitinated protein aggregates (Xie et al. 2022; He et al. 2025). HsfA1a also induces expression of the UPR regulator bZIP60, linking cytosolic heat shock signaling with ER stress responses (Löchli et al. 2023). Similarly, WRKY33 coordinates the induction of ATG genes and NBR1 during HS, enabling efficient autophagic responses and aggregate clearance (Zhou et al. 2014a, 2015). Together, these regulators appear to orchestrate a multilayered PQC network capable of dynamically adjusting degradation, refolding, and signaling capacities according to heat severity and duration.

Despite increasing evidence for extensive crosstalk between PQC pathways, major mechanistic questions remain unresolved. Although substantial progress has been made in defining individual points of interaction between autophagy, molecular chaperones, the UPS, and the UPR, how these systems collectively coordinate proteostasis decisions during HS remains largely unknown. A central unresolved question is how cells allocate damaged proteins among competing quality-control routes. How is it determined whether a damaged protein should be refolded by chaperones, degraded by the proteasome, sequestered into biomolecular condensates, or eliminated through autophagy? Equally unclear is how these pathways are

quantitatively coordinated during the transition from acute stress to recovery. The observation that excessive autophagy can become detrimental under certain conditions further suggests that thermotolerance depends not on maximal activation of individual PQC systems, but on their dynamic and balanced deployment according to proteotoxic load. Resolving how these proteostasis decisions are integrated across space, time, and stress intensity remains a major challenge for understanding the limits of plant heat resilience.

Selective autophagy pathways in plant heat stress adaptation

The best-characterized selective autophagy pathway during HS is NBR1-mediated aggrephagy. NBR1 functions as a selective receptor by simultaneously binding ubiquitinated cargo and ATG8, thereby promoting autophagic clearance of protein aggregates. Although severe HS can constrain bulk autophagic flux, NBR1-dependent aggregate targeting remains strongly activated (Jung et al. 2020a; Zhang and Chen 2020). In *Arabidopsis*, *nbr1* mutants accumulate insoluble protein aggregates and exhibit reduced basal thermotolerance, particularly in combination with autophagy-deficient backgrounds such as *atg7* (Zhou et al. 2013, 2014b). Upstream regulation of this pathway involves WRKY33, which activates expression of ATG5, ATG7, and NBR1 and promotes heat-induced aggrephagy. Accordingly, silencing WRKY33 suppresses autophagosome formation and intensifies aggregate load (Zhou et al. 2014a, 2015).

Although NBR1 primarily recognizes ubiquitinated cargo, recent findings indicate that cargo recognition during plant aggrephagy is more complex than originally envisioned. In *Arabidopsis*, NBR1 undergoes liquid-liquid phase separation (LLPS) and targets non-ubiquitinated proteins such as ROF1 and EXO70E2 via its ZZ and FW domains, two conserved cargo-binding motifs (Yan et al. 2024). These NBR1 condensates may organize cargo prior to autophagosome formation, expanding the spectrum of substrates that can be incorporated into autophagic degradation pathways. Supporting a broader role for condensate-mediated selective autophagy, ERC1 (Enhancer of RCC1) was identified as a heat-responsive protein that forms cytoplasmic condensates and recruits both NBR1 and ATG8 during HS (Chung et al. 2025). Loss of ERC1 compromises autophagosome formation and thermotolerance, whereas enhanced ERC1 condensation promotes autophagic activity and heat resilience (Chung et al. 2025). Additional complexity emerged with the identification of CESAR as a conserved selective autophagy receptor required for proteostasis under heat stress (Hernández et al. 2026). CESAR associates with ubiquitinated protein aggregates and functionally cooperates with NBR1 during their autophagic clearance, indicating that aggregate recognition is mediated by a receptor

network rather than a single cargo adaptor (Hernández et al. 2026). Rather than operating through a single NBR1-centered pathway, heat-induced aggrephagy appears to involve a coordinated network of receptors and condensate-forming proteins that expand cargo recognition capacity under proteotoxic stress. The emerging challenge is therefore to understand how NBR1, CESAR, ERC1, and other components are coordinated to define cargo selectivity during heat-induced proteotoxic stress.

Beyond aggregate clearance, NBR1-dependent aggrephagy also contributes to thermomemory regulation. Thirumalaikumar et al. (2021a) demonstrated that NBR1 targets the heat shock protein HSP90.1 and its co-chaperone ROF1 (Rotamase FKBP1) for selective degradation. This complex regulates HSFA2, a key transcription factor that maintains heat-induced gene expression. By modulating the turnover of the HSP90.1-ROF1 complex, NBR1-mediated autophagy influences the extent and duration of thermomemory, thereby facilitating the transition from stress-associated transcriptional programs back to developmental growth. Additionally, during recovery from HS, autophagy contributes to metabolic remodeling, as autophagy-deficient and *nbr1* plants fail to accumulate proteogenic dipeptides and display altered lipid remodeling profiles (Thirumalaikumar et al. 2021b). NBR1-dependent aggrephagy is therefore positioned at the interface between proteostasis maintenance, thermomemory regulation, and metabolic remodeling during recovery from HS.

HS also disturbs ER redox balance, secretory flux, and protein folding capacity, thereby triggering UPR-associated pathways. In Arabidopsis, the ATI3-UBAC2 module has emerged as a selective ER-associated autophagy pathway involved in thermotolerance (Zhou et al. 2018). The ATI3 proteins interact with ATG8 through canonical AIM/LIR motifs and associate with the ER-localized adaptor UBAC2 (Zhou et al. 2018). Loss of ATI3 or UBAC2 compromises heat tolerance and increases sensitivity to tunicamycin-induced ER stress, while both proteins accumulate in ATG8-positive puncta that traffic to the vacuole in an ATG5-dependent manner during HS (Zhou et al. 2018). Additionally, UBAC2 overexpression stimulates autophagosome formation, suggesting roles in both cargo recruitment and pathway activation. The ATI3-UBAC2 module expands the ER proteostasis network beyond canonical ERQC and ERAD pathways, potentially providing an additional degradation route when ER folding and quality-control capacities become saturated during HS. However, the specific cargos targeted through this pathway remain unknown.

Chloroplasts are vulnerable to HS because of their central roles in photosynthesis and redox homeostasis. In *Chlamydomonas reinhardtii*, disruption of chloroplast proteostasis induces broad stress responses involving molecular chaperones, proteases, and autophagy-

related pathways, supporting chloroplast-to-nucleus signaling mechanisms associated with cellular proteostasis remodeling (Ramundo et al. 2014). In higher plants, the chloroplast-localized 2-Cys peroxiredoxins (2-CP1 and 2-CP2) rapidly accumulate under HS and are essential for maintaining redox homeostasis (Cheng et al. 2016). Silencing 2-CPs increases protein oxidation, the accumulation of insoluble proteins, and heat sensitivity, which in turn triggers an earlier and stronger induction of autophagy genes (ATG5 and ATG7) (Cheng et al. 2016). However, direct evidence that damaged chloroplast components are selectively targeted for degradation under these conditions is still lacking. Autophagy also contributes to chloroplast-associated protein quality control during HS and recovery. The plastid-localized heat shock protein HSP21 accumulates during heat priming and is cleared via autophagy during recovery (Sedaghatmehr et al. 2021). In *atg* mutants or upon pharmacological inhibition of vacuolar degradation, HSP21 turnover is delayed, whereas HSP21 co-localizes with ATG8a-positive autophagic structures, supporting direct autophagic targeting (Sedaghatmehr et al. 2021). Furthermore, AT11 expression is induced during recovery, and *at11* mutants exhibit prolonged accumulation of HSP21 together with enhanced heat stress memory (Sedaghatmehr et al. 2021), linking selective autophagy to the controlled removal of thermomemory-associated proteins after stress.

The chloroplast-associated degradation (CHLORAD) pathway, which mediates ubiquitin-dependent turnover of chloroplast outer membrane proteins, can engage NBR1-dependent autophagic mechanisms under stress conditions (Wan et al. 2023). Under HS, NBR1 associates with ubiquitinated TOC components at the chloroplast periphery and promotes their delivery to ATG8-positive autophagic structures (Wan et al. 2023). Consistent with this role, *atg7* and *nbr1* mutants exhibit enhanced heat sensitivity, whereas NBR1 overexpression improves thermotolerance (Wan et al. 2023). Whether NBR1-mediated turnover extends to entire chloroplast structures or remains restricted to ubiquitinated envelope-associated proteins remains unresolved.

Maintenance of the Golgi apparatus is crucial for protein trafficking and cellular homeostasis during HS. Zhou et al. (2023) identified a non-canonical ATG8-dependent pathway involved in Golgi recovery following acute HS. Heat stress induces the formation of ATG8-positive puncta associated with Golgi membranes that operate independently of canonical autophagy initiation complexes (Zhou et al. 2023b). In this pathway, ATG8 recruits the clathrin light chain protein (CLC2) through an AIM-LDS interface, facilitating Golgi remodeling and vacuolar trafficking of accumulated cargo (Zhou et al. 2023b). Impaired CLC2 recruitment in *atg5-1* mutants and delayed Golgi restoration in *clc2-1* plants demonstrate the

importance of ATG8-mediated membrane remodeling for post-stress cellular recovery. ATG8 therefore appears capable of coordinating membrane remodeling and organelle recovery independently of canonical autophagosome biogenesis, expanding its functional repertoire beyond degradative autophagy. Whether similar ATG8-dependent Golgi quality-control mechanisms operate during prolonged warming or in other plant species remains unknown.

Selective autophagy during HS emerges as a set of compartment-specific quality-control pathways rather than a single degradation process. Aggrephagy, ER-associated autophagy, chloroplast-associated turnover, and Golgi remodeling target distinct forms of proteotoxic and organellar damage generated during stress and recovery. Yet a central question remains unresolved: how do plant cells prioritize cargo among competing selective autophagy routes? Whether different pathways are activated according to cargo identity, organelle status, stress intensity, or recovery stage remains largely unknown. Defining this hierarchy of cargo selection and pathway activation represents a major challenge for understanding how selective autophagy contributes to thermotolerance.

Autophagy at the interface of hormone and stress signaling during heat stress

Heat-induced autophagy is regulated through interconnected signaling networks integrating phytohormonal, metabolic, and redox-associated pathways. Within this framework, autophagy functions not merely as a downstream stress response, but as an active component of cellular reprogramming, coordinating proteostasis, metabolic adjustment, and stress recovery. Rather than acting as simple linear inducers, these signaling pathways appear to modulate the magnitude, timing, and physiological consequences of autophagy across distinct phases of HS and recovery.

Potential links between phytohormone signaling and autophagy regulation during HS have emerged from promoter analyses and transcriptomic studies, although the upstream regulatory mechanisms remain poorly resolved. In potato, several *StATG8* promoters contain cis-elements associated with abscisic acid (ABA)- and salicylic acid (SA)-responsive transcription, while *StATG8-2.1* is strongly induced during HS (Song et al. 2022). However, the presence of hormone-responsive elements alone does not demonstrate direct hormonal control of ATG transcription, highlighting the need for functional studies connecting these promoter signatures with in vivo transcriptional regulation during HS. A more integrated relationship between ABA signaling and autophagy was reported in cucumber, where increased ABA accumulation in luffa-grafted plants was associated with enhanced thermotolerance, reduced *csa-miR159b* abundance, induction of the target transcription factors *CsGAMYB1* and

CsMYB29-like, and increased autophagosome accumulation under HS (Li et al. 2016). Importantly, the study did not establish a direct transcriptional connection between the ABA-miR159b module and the autophagic machinery. Rather, the coexistence of ABA signaling, altered miRNA regulation, enhanced autophagy, and improved heat tolerance suggests that these processes operate within a coordinated stress-response network. Consistent with this view, contrasting *Brassica rapa* genotypes display genotype- and time-dependent regulation of ABA-associated regulators (e.g., *SPK2E*, *ATHB-7*, *ABI2*) together with differential expression of *ATG8A* and *ATG8E* during HS and recovery (Yue et al. 2021b), supporting the hypothesis that ABA-related pathways may contribute to the temporal tuning of autophagic responses during heat acclimation and recovery.

Melatonin, polyamines, jasmonic acid (JA), and γ -aminobutyric acid (GABA) intersect with autophagy primarily through the regulation of proteotoxic stress, redox homeostasis, and metabolic buffering during HS. In tomato, endogenous melatonin levels peak at 40 °C, and exogenous melatonin application directly upregulates ATG genes and stimulates autophagosome formation to degrade aggregated ubiquitinated proteins under HS (Xu et al. 2016). Similarly, in tomato reproductive tissues, melatonin enhances autophagic clearance to preserve pollen viability and alleviate heat-induced oxidative stress (Qi et al. 2018). However, it is important to distinguish direct autophagic activation from indirect systemic adjustments. Although Jahan et al. (2021) showed that melatonin suppresses heat-induced senescence and represses excessive ABA biosynthesis in vegetative tissues, autophagic structures and flux were not directly assessed. Thus, an important unresolved question is whether melatonin directly regulates the recruitment of core ATG machinery during HS or instead establishes a protective metabolic state that indirectly supports cellular quality control.

Transcriptomic profiling of heat-stressed *atg5* mutants reveals a severe misregulation of JA-associated gene networks (Zhang et al. 2021b). Because JA contributes to defense signaling, growth restraint, and oxidative balance during HS, the disruption of JA-associated transcriptional networks in *atg5* mutants, together with increased ROS accumulation, positions autophagy as an important component of JA-dependent stress responses during heat exposure (Zhang et al. 2021b). Concurrently, metabolic reprogramming via GABA accumulation has been linked to autophagy-related responses during combined high light and heat stress (HL+HS) (Balfagón et al. 2022). Both GABA-deficient (*gad3*) and *atg* mutants exhibit hypersensitivity and photosynthetic impairment under combined stress (Balfagón et al. 2022), supporting close functional interactions between autophagy, metabolic signaling, and redox homeostasis. However, the mechanistic directionality of this relationship remains unresolved.

It is still unclear whether GABA functions upstream as a signaling metabolite capable of modulating autophagy or instead represents a downstream metabolic component whose homeostasis depends on functional autophagic recycling.

Brassinosteroid (BR) signaling provides one of the clearest examples of how heat-response pathways and autophagy may converge on common regulatory nodes. Under acute HS, BES1, a key BR transcription factor, is rapidly dephosphorylated and activated independently of canonical BR signaling, allowing it to bind directly to heat shock elements (HSEs) alongside HSFA1 to induce heat-responsive genes, including HSP70 and HSP90, thereby promoting thermotolerance (Albertos et al. 2022). During the thermomemory phase, BES1 accumulates and directly sustains the expression of memory-associated genes such as APX2 and HSFA3, highlighting its role in maintaining post-stress transcriptional programs (Yao et al. 2022). Independently, BES1 has been identified as a selective autophagy substrate targeted for NBR1-dependent degradation (Nolan et al. 2020). Although this mechanism has not yet been directly examined during HS or thermomemory, it raises an intriguing possibility that selective autophagy contributes to the temporal control of BES1 abundance after stress. Such a mechanism could provide a means to balance thermomemory maintenance with the eventual restoration of developmental growth programs once stress conditions subside.

Conversely, recent evidence indicates that the contribution of autophagy to thermotolerance is not universally beneficial, particularly in the context of selective organelle turnover. In *Arabidopsis fes1a* mutants, which lack a key HSP70 nucleotide exchange factor and are highly heat-sensitive, suppression of constitutive autophagy unexpectedly restores acquired thermotolerance (Li et al. 2025b). Mechanistically, this rescue is associated with the accumulation of peroxisomes that would otherwise be removed by pexophagy, thereby enhancing the conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA) and increasing endogenous auxin levels (Li et al. 2025b). The restoration of thermotolerance in *atg7 fes1a* plants indicates that constitutive pexophagy can become maladaptive under severe proteotoxic stress by prematurely removing metabolically beneficial organelles. More broadly, this study challenges the prevailing assumption that increased autophagic activity is invariably advantageous during HS and highlights the importance of distinguishing bulk autophagy from selective organelle-specific pathways when evaluating stress adaptation mechanisms.

Safeguarding reproduction under heat stress

Reproductive development is among the most heat-sensitive phases of the plant life cycle, with elevated temperatures impairing pollen development, fertilization, and seed set. In

tomato, Song et al. (2024) demonstrated that overexpression of *SLATG8f* enhances pollen viability under HS, despite increasing susceptibility in vegetative tissues. *SLATG8f*-overexpressing (OE) plants exhibited elevated autophagic flux, revealed by increased autophagosome formation and stronger expression of *ATG8* genes and HSPs in floral organs. Scanning electron microscopy showed less morphological defects in OE pollen after prolonged heat exposure, and pollen viability, initially lower in OE plants, ultimately surpassed that of WT plants. Consistent with these findings, Xie et al. (2022) identified a dedicated HSFA1a-ATG10 regulatory module in pollen that activates autophagy to prevent the accumulation of toxic protein aggregates, thereby sustaining pollen viability during HS. The sensitivity of gametophytic cells to proteotoxic stress may therefore impose a higher dependence on autophagic quality-control mechanisms than in vegetative tissues during HS. Interestingly, these same *SLATG8f*-OE lines exhibited elevated damage in leaves, including chloroplast and mitochondrial disruption, suggesting that the physiological consequences of elevated autophagic activity are highly tissue-dependent. Whereas sustained autophagy appears to preserve proteostasis in reproductive tissues, the increased organellar damage observed in vegetative tissues of the same *SLATG8f*-OE lines indicates that elevated autophagic activity is not always beneficial. These contrasting phenotypes suggest that reproductive and vegetative tissues operate under distinct autophagic thresholds, with the optimal level of autophagic activity depending on tissue-specific metabolic demands and stress sensitivities.

Similar patterns emerge across diverse reproductive systems. In *Brassica napus* and *Hordeum vulgare*, HS-induced microspore cell death was associated with activation of autophagy and proteolytic pathways, including increased *HvATG5* and *HvATG6* expression and autophagosome accumulation, which coincided with improved embryogenic potential (Pérez-Pérez et al. 2019). This autophagy-mediated cytoprotection involved the removal of ROS and damaged cellular components, supporting microspore survival and developmental reprogramming. Additionally, Mohammadi et al. (2025) evaluated the contribution of autophagy and antioxidant systems in maintaining reproductive success in heat-tolerant (SAFI5) and heat-sensitive (DH13) cultivars of *Brassica napus*. Under HS, SAFI5 maintained high pollen germination rates and viability, while DH13 showed significant reproductive failure. This resilience was linked to enhanced activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which reduced ROS accumulation in reproductive tissues. Notably, SAFI5 also exhibited elevated gene expression of autophagy markers (*BnATG8d*, *BnNBR1*) and greater autophagosome formation in tissues experiencing oxidative stress. Beyond gametophytic protection, autophagy may also contribute to later

reproductive processes. In tomato, *SlATG8f* overexpression improves fruit set, accelerates fruit maturation, and preserves fruit quality traits under high temperatures, accompanied by altered expression of ATG8 family member and HSP response genes during fruit development (Cheng et al. 2026). Although autophagic flux measurements were not performed, the findings nevertheless suggest that autophagy-associated pathways may contribute to reproductive performance beyond pollen viability, influencing developmental and physiological processes that ultimately determine yield and fruit quality under heat stress.

The emerging picture is that reproductive tissues may require substantially different autophagic regimes than vegetative organs during HS. While enhanced autophagic activity consistently correlates with improved pollen viability, microspore survival, and reproductive success, the same increase can be associated with organellar damage in vegetative tissues (Song et al. 2024). The mechanisms underlying this apparent trade-off remain unclear. Whether reproductive cells are intrinsically more dependent on autophagic recycling, or whether distinct selective autophagy pathways operate in reproductive and vegetative tissues, remains unknown. Resolving these differences will be essential for exploiting autophagy in crop improvement without compromising vegetative performance. Furthermore, identifying the cargos, receptors, and regulatory networks that operate specifically in reproductive tissues will be essential for understanding how autophagy preserves fertility and yield stability under future warming scenarios.

CONCLUDING REMARKS

Autophagy has emerged as a central component of plant heat adaptation. Beyond its established role in the removal of damaged proteins and organelles, autophagy is increasingly recognized as a systems-level regulator that integrates proteostasis, energy metabolism, redox homeostasis, thermomemory, hormone signaling, and reproductive resilience during heat stress. Recent advances have challenged the traditional view of autophagy as a uniformly beneficial degradative pathway, revealing context-dependent functions that vary according to tissue type, developmental stage, stress intensity, and recovery status. At the same time, the discovery of selective autophagy pathways has expanded the functional landscape of autophagy far beyond bulk cellular recycling. Here, autophagy emerges as a major integrative component of plant adaptation to heat stress.

Future perspectives

Recent discoveries are transforming our understanding of autophagy during heat stress from a broadly degradative process into a highly selective and dynamically regulated network. The identification of specialized receptors and regulators, including NBR1, ERC1, and CESAR, together with emerging roles for biomolecular condensates and liquid-liquid phase separation, suggests that selective autophagy operates through a far more sophisticated cargo-recognition system than previously appreciated (Yan et al. 2024; Chung et al. 2025; Hernández et al. 2026). A major challenge for future research will be to determine how these components interact to establish cargo specificity and how selective autophagy pathways are coordinated during different stages of stress and recovery.

The current literature remains heavily dominated by acute heat-shock studies, yet plants in natural environments are primarily exposed to other scenarios, such as prolonged warming (Wang et al. 2020), recurrent heat events, and fluctuating temperature regimes. Increasing evidence indicates that autophagy performs distinct functions depending on stress duration and intensity, developmental stage, tissue identity, and recovery status. Whether plants deploy distinct autophagic programs across different thermal regimes, tissues, and developmental stages remains unresolved, limiting our ability to develop a unified framework of heat adaptation.

Future studies must also move beyond single-stress paradigms. Heat stress rarely occurs in isolation and is frequently accompanied by drought, nutrient limitation, or high irradiance. Emerging evidence suggests that autophagy acts as an important integration point between multiple stress-response pathways and contributes to resilience under combined stress conditions (Balfagón et al. 2022; Hickey et al. 2024). However, the molecular mechanisms through which autophagy prioritizes and coordinates responses to simultaneous environmental constraints remain largely unknown. Resolving this gap will be essential for determining whether current mechanistic models derived from controlled environments remain valid under realistic field conditions.

Recent studies also challenge the long-standing assumption that enhanced thermotolerance can be achieved simply through increased autophagic activity. Functional specialization among ATG isoforms, together with examples in which excessive or misdirected autophagy becomes detrimental, indicates that successful heat adaptation depends on precise regulation rather than maximal autophagic flux (Liang et al. 2023; Chiaro et al. 2025; Ma et al. 2025). Future efforts should therefore focus on understanding autophagic selectivity, flux dynamics, and pathway specialization. Such knowledge will be essential for determining

whether specific autophagy components, receptors, or naturally occurring allelic variants can be harnessed to improve crop resilience under increasingly frequent and severe heat events.

Outstanding Questions

How is autophagic flux regulated across different thermal regimes, including acute heat shock, prolonged warming, and recurrent heat episodes?

Do plants activate distinct selective autophagy pathways in response to different forms of heat-induced cellular damage, and how are these pathways coordinated during stress and recovery?

How do biomolecular condensates contribute to selective autophagy during heat stress, and what determines their cargo composition, dynamics, and recruitment to the autophagic machinery?

What molecular mechanisms determine cargo recognition and prioritization during heat stress, and how are substrates allocated between refolding, proteasomal degradation, and selective autophagy?

How do individual ATG isoforms and selective autophagy receptors shape cargo selectivity, thermotolerance, and thermomemory?

How is autophagy coordinated with other proteostasis pathways, including molecular chaperones, the ubiquitin-proteasome system, and the unfolded protein response, during heat stress?

How do autophagic responses differ between vegetative and reproductive tissues, and what mechanisms underlie their distinct sensitivities to elevated temperature?

How is autophagy integrated with other environmental constraints that frequently accompany heat stress, such as drought, high-light, and nutrient limitation?

Can natural genetic variation in autophagy-related pathways be exploited to improve thermotolerance in crops without compromising growth and productivity under non-stress conditions?

Which autophagy-associated traits, regulators, or biomarkers are most suitable for crop breeding and biotechnological approaches aimed at improving heat resilience?

SUPPLEMENTAL DATA

Table 1. Major functional interactions between autophagy and other proteostasis pathways during heat stress.

Proteostasis pathway	Main role during heat stress	Functional interaction with autophagy	Key regulators/components	Proposed unresolved questions
Molecular chaperones	Protein refolding, stabilization, and prevention of irreversible aggregation	Cooperate with autophagy in aggregate clearance, proteostasis buffering, and thermomemory resetting	HSP101, HSP90.1, HSP21, ROF1, Hsa32	How is the transition from protein refolding to autophagic degradation determined? Do specific HSPs preferentially cooperate with selective autophagy pathways during different heat phases?
Ubiquitin-proteasome system (UPS)	Removal of soluble and short-lived ubiquitinated proteins	Compensates for or cooperates with autophagy depending on proteotoxic load and substrate properties	CHIP, NBR1, ubiquitin ligases	How are substrates dynamically partitioned between the UPS and autophagy? Which signals trigger the shift from proteasomal degradation to aggrephagy during severe or prolonged heat stress?
UPR/ER stress signaling	Restoration of ER folding capacity and secretory homeostasis	Activates selective ER-phagy and coordinates ER quality control under heat stress	IRE1b, bZIP60, ATI3, UBAC2, AtDjC5	Which ER cargos are selectively degraded during heat stress? Does ER-phagy contribute differently during acute heat shock, thermomemory, and prolonged warming?
Selective autophagy pathways	Removal of protein aggregates and damaged organelles	Integrates signals from multiple PQC systems to selectively remodel the stress-damaged proteome	ATG8, NBR1, ATI1/2, ATI3	How is cargo specificity regulated across distinct heat phases? Are additional heat-specific autophagy receptors or adaptors still unidentified in plants?
Transcriptional PQC regulation	Coordinates stress-responsive proteostasis programs	Synchronizes autophagy, chaperone expression, and ER stress responses	HsfA1a, WRKY33, bZIP60	How are transcriptional and post-translational regulatory layers integrated to fine-tune autophagic flux during heat stress and recovery?

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**CHAPTER 2 – Autophagy safeguards energy metabolism and proteome homeostasis
during acclimation to prolonged warming in *Arabidopsis thaliana***

Autophagy safeguards energy metabolism and proteome homeostasis during acclimation to prolonged warming in *Arabidopsis thaliana*

ABSTRACT

Global warming increasingly exposes plants to sustained, sublethal temperature elevations that require long-term acclimation rather than short-term survival. While autophagy is well-established as a protector against acute heat shock, its role in prolonged warming remains largely unknown. Here, we investigated the function of autophagy during acclimation to prolonged warming (28 °C for 7 days) in *Arabidopsis thaliana* using the autophagy-deficient mutants *atg5-1* and *atg7-2*, combined with physiological, metabolic, and quantitative proteomic analyses. Autophagy-deficient plants failed to sustain thermomorphogenic growth and exhibited impaired photosynthetic performance, elevated respiratory and transpiratory costs, and extensive metabolic reprogramming during warming acclimation. In particular, *atg7-2* displayed the simultaneous accumulation of carbon reserves, amino acids, and stress-associated metabolites despite increased respiration, indicating a loss of metabolic coordination rather than resource limitation. Proteomic analyses revealed widespread disruption of chloroplast function, protein synthesis, and organelle proteostasis, accompanied by activation of alternative catabolic pathways and stress-response processes. Our results demonstrate that autophagy is essential for prolonged warming acclimation by maintaining organelle integrity, metabolic flexibility, and resource-use efficiency. Our results establish autophagy as a key mechanism of thermal acclimation capacity and a promising target for improving crop resilience in a warming climate.

Keywords: Autophagy; prolonged warming; proteomic remodeling; energy metabolism.

INTRODUCTION

Global warming has become one of the foremost challenges to plant growth, development, and productivity, intensifying concerns over global food security (Dasgupta and Robinson 2022; Rosegrant et al. 2024). The IPCC (2023) estimates a greater than 50% likelihood that global mean temperature will reach or exceed 1.5 °C between 2021 and 2040, highlighting the urgency of understanding how plants cope with sustained warming. In plant physiology, heat stress (HS) includes diverse thermal scenarios that differ in intensity and duration (Wahid et al. 2007; Sedaghatmehr et al. 2019; Xie et al. 2022; Hickey et al. 2024). Because of this complexity, research on plant HS has progressed in multiple complementary directions with some studies focus on short, acute heat shock to dissect rapid cellular and molecular responses, whereas others investigate prolonged, moderate warming to explore

physiological acclimation and ecological outcomes (Wang et al. 2020; Jagadish et al. 2021). Although differing in scope and timescale, both approaches share the common goal of understanding how elevated temperatures reshape plant function. Greater integration and crosstalk between these perspectives are essential for a deep understanding of plant adaptation and vulnerability under progressive climate warming.

At the cellular level, elevated temperatures compromise membrane integrity, perturb photosynthesis and respiration, induces protein misfolding, enhances reactive oxygen species (ROS) accumulation, and disrupts the cytoskeleton, ultimately impairing cellular homeostasis and metabolism (Haider et al. 2021; Chaudhry and Sidhu 2022). Heat exposure triggers metabolic changes associated with impaired electron transport and ROS production, which in turn stimulate alternative respiratory pathways such as alternative oxidase (AOX) and membrane-bound NADPH oxidase (Miller et al. 2009; Wang et al. 2013; Borovik and Grabelnykh 2018). Photosynthesis is particularly heat-sensitive, with the oxygen-evolving complex of Photosystem II (PSII) and carbon assimilation being primary targets (Anzano et al. 2022). Stress-induced proteolysis and amino acid release can sustain ATP production under high energy demand, while heat shock proteins (HSPs) preserve proteome integrity (Araújo et al. 2011b; Hildebrandt 2018; Ul-Haq et al. 2019).

Autophagy, a conserved eukaryotic catabolic pathway, plays a pivotal role in recycling damaged proteins and organelles to maintain metabolic balance under stress (Signorelli et al. 2019b; Azmat et al. 2024). Among the core autophagy genes, ATG5 and ATG7 play central roles in the lipidation of ATG8, which is critical for autophagosome formation and autophagic flux (Cheng et al. 2022). ATG5 forms a complex with ATG12 to catalyze ATG8 conjugation to phosphatidylethanolamine (PE), anchoring it to the autophagosome membrane (Le Bars et al. 2014; Marshall and Vierstra 2018). ATG7, an E1-like activating enzyme, initiates the conjugation cascades by activating both ATG8 and ATG12 (Phillips et al. 2008) enabling their subsequent transfer and covalent linkage to their respective partners (Yamaguchi et al. 2018). Consequently, autophagy-defective mutants of *Arabidopsis thaliana* fail to form autophagosomes, exhibit general disruptions in autophagy-associated processes, and undergo premature senescence under nitrogen- or carbon-limiting conditions (Barros et al. 2017b; Huang et al. 2019; Yue et al. 2021a; Li et al. 2022a).

High temperature is a well-established inducer of autophagy, which plays multifaceted roles in heat stress (HS) responses as a cellular quality control mechanism to degrade misfolded proteins and oxidized components (Yang et al. 2016; Gomez et al. 2021). Acute heat shock exposes plants to near-lethal temperatures and triggers rapid autophagy that removes heat-

denatured or proteins aggregates when chaperone capacity is overwhelmed (Zhou et al. 2013, 2014a; Thirumalaikumar et al. 2021a). Thermomemory represents a different scenario in which a prior mild heat stimulus improves tolerance to a later, more severe event. In this case, autophagy acts during recovery to degrade excess HSPs and reset the cellular state, balancing stress readiness with growth demands. Furthermore, a non-canonical autophagy pathway is recruited during HS to restore Golgi function, revealing autophagy roles during HS recovery (Zhou et al, 2023).

While the critical role of autophagy in proteostasis and oxidative stress responses during heat shock is well established, it is unclear whether autophagy also supports long-term acclimation to moderate warming. This condition does not represent a transient shock to survive or a memory to be reset, but rather a new environmental baseline to which the organism must continuously adjust its entire physiology. Plants deploy distinct acclimatory strategies depending on the thermal regimes (Wang et al., 2020). Recent mechanistic work has linked warm-temperature growth signaling to autophagy by showing that PIF4 activates autophagy to promote thermomorphogenesis through selective degradation of the circadian repressor TOC1 (Li et al. 2025). Moreover, no studies have comprehensively examined how *atg* mutants respond physiologically and metabolically to prolonged warming.

To address this knowledge gap, we investigated autophagy function during acclimation to prolonged warming in *Arabidopsis thaliana*. Using *atg* mutants (*atg5-1* and *atg7-2*), four-week-old plants were exposed to prolonged warming (28 °C for 7 days), and multiple parameters were assessed, including thermomorphogenic growth, photosynthetic performance, respiratory and transpiratory responses, carbon and nitrogen metabolism, and proteomic reprogramming. Our results indicate that autophagy is required to sustain thermomorphogenic growth, constrain respiratory and hydraulic costs, maintain carbon-nitrogen balance, and support proteome homeostasis during prolonged warming. Together, our findings identify autophagy as a central regulator of long-term thermal acclimation, linking cellular quality control with metabolic flexibility, resource-use efficiency, and physiological homeostasis under sustained warming.

RESULTS

Autophagy supports coordinated growth and photosynthetic acclimation during prolonged warming

Plants respond to prolonged warming by adjusting physiological processes and energetic metabolism via reduced stomatal conductance, enhanced photosynthesis, and

alteration in the TCA cycle (Wang et al. 2020). To investigate the role of autophagy in the acclimation of plants under mild, sustained temperature elevation, *atg* mutants (*atg5-1* and *atg7-2*) were exposed to prolonged warming (28 °C for 7 days) (Fig. S2). The *atg5-1* and *atg7-2* lines used here are autophagy-deficient (*atg*) and impaired in ATG8 lipidation and autophagic flux (Thompson et al. 2005; Phillips et al. 2008; Minina et al. 2018). Both *atg* mutants were notably smaller than WT plants under control conditions, consistent with the established role of autophagy in promoting vegetative growth, as previously characterized (Barros et al. 2017b) (Fig. 1A).

To further characterize growth and morphological alterations in *atg* plants under elevated temperature conditions, shoot dry weight and leaf area were quantified as indicators of biomass accumulation and growth expansion (Fig. 1B-C). Petiole elongation was clearly observed in WT plants exposed to elevated temperature, representing a classic thermomorphogenic response associated with adaptive growth modulation (Quint et al. 2016) (Fig. 1A). Under control conditions, the *atg* mutants exhibited significantly lower biomass accumulation than WT, reduced by approximately 40-50% (Fig. 1B). This deficit persisted under warming, with no significant effect of temperature on biomass accumulation in any genotype. Analysis of leaf area dynamics revealed a more pronounced genotype-specific response (Fig. 1C). Under control conditions, both *atg5-1* and *atg7-2* already displayed significantly reduced leaf area compared to WT. WT plants exhibited a significant increase in leaf area under warming (+17%), reflecting typical thermomorphogenic growth enhancement. In contrast, both *atg5-1* and *atg7-2* maintained similar leaf area under control and warming conditions.

Given the alterations in growth parameters, we next assessed PSII photochemical efficiency (*Fv/Fm*) and pigment composition as indicators of chloroplast performance (Fig. 1D). *Fv/Fm* measurements revealed a modest but consistent reduction in *atg7-2* under warming compared to WT. WT maintained stable *Fv/Fm* values in response to warming, whereas *atg7-2* showed a small but significant decline compared to its control.

To complement these findings, pigment levels were quantified to further assess chloroplast function (Fig. 1E-H). Chlorophyll *a* content in WT plants remained stable under warming, whereas it was significantly reduced in *atg7-2* by approximately 24% (Fig. 1E). Under warming, both *atg5-1* and *atg7-2* exhibited significant reductions compared to WT (21% and 27%, respectively). Additionally, *atg5-1* showed a significant decrease relative to its control conditions. For chlorophyll *b*, a significant difference was detected only between WT and *atg5-1* under warming (*atg5-1* ~24% lower) (Fig. 1F). Total chlorophyll content in WT

plants remained stable across treatments, whereas it was significantly reduced in *atg7-2* compared with WT under control condition. Under warming, both *atg* mutants displayed significantly decreased total chlorophyll levels compared to WT (*atg5-1*: ~22%, and *atg7-2*: ~30% lower) (Fig. 1G). Carotenoid content did not differ significantly among genotypes under control conditions; however, in *atg5-1* it showed a modest yet significant reduction under warming compared to its control (~8% decrease) (Fig. 1H). The reduced pigment levels in *atg* mutants under warming are consistent with the decreased leaf greenness, most evident in *atg7-2* (Fig. 1A).

Lack of autophagy impairs respiratory balance and water-use efficiency under warming

To investigate whether autophagy contributes to the regulation of gas exchange responses during prolonged warming, we evaluated photosynthetic CO₂ assimilation (A_N), dark respiration (R_D), and transpiration rates during the day (E_{day}) and night (E_{night}) (Fig. 2). Photosynthetic rate in WT plants remained stable under warming (~6.3 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), whereas *atg5-1* and *atg7-2* showed significantly reduced A_N compared to WT under control conditions (~20% and ~34% lower, respectively) (Fig. 2A). Notably, these differences were no longer apparent under warming, which may reflect a mild heat-induced reduction in photosynthetic performance in WT plants, resulting in similar A_N values across genotypes under this condition.

R_D exhibited a distinct genotype-dependent response to warming (Fig. 2B). Under control conditions, R_D did not differ significantly among genotypes. However, under warming, both WT and *atg5-1* maintained comparable R_D values (1.15 and 1.41 $\mu\text{mol m}^{-2} \text{ s}^{-1}$, respectively), whereas *atg7-2* exhibited a pronounced and significant increase (2.66 $\mu\text{mol m}^{-2} \text{ s}^{-1}$), more than doubling relative to the WT.

The daytime transpiration rate (E_{day}) revealed a significant correlation between genotype and temperature ($P < 0.05$, factorial ANOVA) (Fig. 2C). Under control conditions, E_{day} did not differ among genotypes (~2.9 $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$). Interestingly, WT plants significantly reduced E_{day} under warming (~28% decrease) compared to control. In contrast, both *atg5-1* and *atg7-2* exhibited markedly higher E_{day} values compared to WT under warming, increasing by approximately 75% and 104%, respectively. Notably, *atg7-2* nearly doubled its E_{day} relative to WT under warming, indicating impaired stomatal regulation in *atg* plants during the day.

Night-time transpiration (E_{night}) followed a similar pattern (Fig. 2D). While no differences were observed among genotypes under control conditions, warming induced a

moderate increase in E_{night} in WT plants and a stronger increase in *atg* mutants. Under warming, *atg7-2* exhibited the highest E_{night} ($2.49 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), with an increase of nearly 100% compared to WT, while *atg5-1* showed intermediate values, with an increase of approximately 39% relative to WT.

Autophagy coordinates metabolic reprogramming to support acclimation to prolonged warming

Given the observed growth limitation and increased energy and water costs in *atg* plants under prolonged warming, we next examined whether autophagy contributes to the regulation of carbon reserve mobilization, metabolic homeostasis, and nitrogen metabolism. To this end, we quantified metabolites associated with carbon storage, central respiration, and nitrogen utilization in whole rosettes of WT and *atg* plants following prolonged warming.

Starch and soluble sugars exhibited genotype-dependent adjustments to prolonged warming (Fig. 3A; Fig. S3). Under control conditions, starch and glucose levels were similar across genotypes, whereas fructose and sucrose were modestly elevated in both *atg* mutants compared to WT. In WT, warming showed stable starch levels, reduced glucose, and increased fructose. In contrast, while *atg5-1* largely resembled the WT in maintaining stable starch levels, *atg7-2* accumulated substantial amounts of starch and sucrose under warming. Moreover, although fructose increased in *atg7-2*, glucose remained unchanged. The simultaneous accumulation of storage carbohydrates (starch, sucrose) and hexoses suggests altered carbon utilization in *atg7-2*.

Under control conditions, total soluble protein content did not differ significantly among genotypes, whereas *atg7-2* displayed slightly lower free amino acids than the WT (Fig. 3B-C). Under warming, soluble protein content decreased, particularly in WT, whereas total free amino acids showed a slight increase. In contrast, *atg5-1* showed no clear alteration in either parameter between control or warming conditions. Under warming, both *atg* mutants maintained higher soluble protein levels than WT, consistent with reduced protein turnover in the absence of functional autophagy.

To gain deeper insight into the metabolic reprogramming associated with autophagy deficiency under prolonged warming, we performed metabolic profiling of soluble sugars, polyols, organic acids, and secondary metabolites using GC-MS (Fig. 4). Among sugars and polyols (Fig. 4A), raffinose and trehalose accumulated in *atg7-2* under warming compared to both WT and *atg5-1*. WT plants tended to reduce raffinose under warming, whereas *atg7-2* increased. Trehalose levels were already elevated in *atg7-2* under control conditions and

increased further under warming. Ribose increased in WT and *atg5-1* under warming but decreased in *atg7-2*. In addition, myo-inositol accumulated specifically in *atg7-2* under warming, while remained unchanged in WT and *atg5-1*. Similarly, erythritol and glycerol increased in all genotypes under warming, but more strongly in *atg7-2*.

Organic acids and secondary metabolites also exhibited contrasting patterns between WT and *atg7-2* (Fig. 4B). Glycolic and glyceric acids showed modest genotype differences under control conditions; under warming, glyceric acid decreased in WT but accumulated in both mutants, particularly *atg7-2*. Threonic and galactonic acids were consistently higher in *atg7-2* than in WT under both conditions and increased further with warming. Gluconic and erythronic acids also tended to increase in *atg7-2* under warming, while levels in WT declined or remained stable. Citric and succinic acids, key TCA cycle intermediates, decreased under warming in WT but increased in *atg7-2* (Fig. 4B). Similarly, shikimic acid and the phenolic precursor sinapinic acid remained stable or declined in WT but accumulated in *atg7-2*.

To further explore how autophagy influences nitrogen metabolism during prolonged warming, we quantified selected proteinogenic and non-proteinogenic amino acids and related metabolites, by GC-MS (Fig. 5). Among proteinogenic amino acids (Fig. 5A), alanine decreased in WT under warming, whereas *atg7-2* showed the opposite pattern, with a strong accumulation compared to WT. Glycine accumulated in *atg7-2* compared to WT and *atg5-1* under warming. Valine decreased in WT under warming but showed a tendency to increase in *atg7-2*. Phenylalanine and glutamate also increased in *atg7-2* under warming, whereas they remained stable in WT and *atg5-1*. Threonine remained unchanged across all genotypes.

Non-proteinogenic amino acids and related metabolites (Fig. 5B) further reflected altered nitrogen metabolism in *atg7-2*. GABA, β -alanine, pyroglutamate, and ethanolamine accumulated in *atg7-2* under warming, in contrast to stable or declining levels in WT and *atg5-1*. Notably, GABA and β -alanine decreased in WT but increased in *atg7-2*. Similarly, pyroglutamate and ethanolamine remained stable or showed only minor changes in WT and *atg5-1* but accumulated in *atg7-2* under warming.

Proteomic remodeling in autophagy-deficient plants under prolonged warming

To assess how autophagy contributes to plant acclimation to prolonged warming, we performed Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS) in rosettes of WT and the *atg7-2* mutant under control and prolonged warming conditions. Given that *atg7-2* exhibited pronounced physiological and metabolic alterations under prolonged warming, it became a suitable model for characterizing autophagy-dependent

proteome homeostasis. Consistent with previous studies, loss of ATG7 produced a stronger physiological phenotype than loss of other ATGs, making *atg7-2* the most informative genotype for detailed proteomic characterization (Barros et al. 2017; Kang et al. 2018; Wan et al. 2023).

Principal component analysis (PCA) revealed a clear separation between genotypes along PC1 (20.2% variance), whereas the prolonged warming response was captured by PC2 (8.3% variance) (Fig. S4). Among the 6,124 identified proteins, differentially abundant proteins (DAPs; FDR < 0.05) were extracted to define both warming-induced and autophagy-dependent alterations. A total of 368 proteins were differentially abundant in WT plants following prolonged warming relative to control (204 increased and 164 decreased), whereas 1,071 proteins were differentially abundant in *atg7-2* (543 increased and 528 decreased) (Fig. 6A, B).

Prolonged warming induced the accumulation of several aquaporins in both genotypes, including PIP1-1, PIP1-2, PIP2-1, PIP2-2, PIP2-3, PIP2-6, and TIP1-2 (Fig. 6B; Fig. S5). Additional members of the MIP family accumulated specifically in *atg7-2*, including PIP1-5, PIP2-7, TIP2-1, and TIP2-2, whereas PIP1-3 increased only in WT. Several molecular chaperones associated with heat acclimation, including HSP101, HSP60, HSP70-4, HSP89.1, and HSP90-1, also increased in abundance in both genotypes, while HSP60-3A and HSP70T-2 accumulated specifically in *atg7-2*.

In WT plants, warming predominantly induced the accumulation of major intrinsic protein (MIP) family proteins, including several plasma membrane intrinsic proteins (PIP aquaporins) (Fig. S5). WT plants showed selective enrichment of proteins associated with cellular organization and structural remodeling, including expansins, pectin-related proteins, hydroxyproline-rich glycoproteins, and microfilament- and microtubule-associated proteins, whereas these categories were not enriched in *atg7-2*. No functional categories were significantly downregulated in WT.

In *atg7-2*, the accumulation of MIP proteins was also identified, together with the accumulation of proteins associated with amino acid and lipid catabolism, aminoacyl-tRNA synthetase activity, and peroxisomal metabolism (Fig. S5). Proteins involved in branched-chain amino acid (BCAA), cysteine degradation, and fatty acid degradation were among the proteins that accumulated in *atg7-2*. In addition, proteins associated with cellular redox metabolism accumulated in *atg7-2* under prolonged warming, including catalase isoforms (CAT1 and CAT3), superoxide dismutase 3 (CSD3), L-ascorbate peroxidase 3 (APX3), monodehydroascorbate reductases (MDAR1/MDAR4), peroxiredoxin-2B (PRX2B), NADPH-dependent thioredoxin reductase 3 (NTRC), nucleoredoxin 1 (NRX1), and oxoprolinase 1 (OXP1) (Fig. S7D). Conversely, proteins associated with coenzyme and RNA metabolism

decreased in abundance in *atg7-2* under warming (Fig. S6). Proteins associated with tetrapyrrole metabolism showed reduced abundance in *atg7-2* under warming. These included proteins involved in 5-aminolevulinic acid formation, chlorophyll metabolism, heme biosynthesis and modification, and protoporphyrin IX metabolism (Fig. S6). Additionally, proteins associated with ribosomal structure, organellar RNA processing, RNA modification, and RNA splicing were reduced in abundance. In parallel, members of the FTSH protease family (FTSH4, FTSH5, FTSH11, and FTSH12) showed reduced abundance under warming (Fig. S7D).

To further characterize the enrichment of amino acid degradation pathways, differentially abundant proteins were integrated with metabolite data (Fig. 7). Multiple enzymes associated with valine, leucine, and isoleucine accumulated in *atg7-2* under warming, including proteins participating in transamination, acyl-CoA oxidation, hydration, and thiolysis reactions (Fig. 7A). These coordinated changes involved enzymes associated with transamination, acyl-CoA oxidation, hydration, and thiolysis reactions within the BCAA degradation pathways. Similarly, proteins associated with cysteine degradation accumulated in *atg7-2* (Fig. 7B). Proteins involved in glutamate-associated pathways also showed coordinated changes together with alterations in glutamate-derived metabolites, including the accumulation of glutamate, alanine and GABA (Fig. 7C). In addition, multiple proteins associated with fatty acid degradation, glycerolipid metabolism, phospholipase activity, and peroxisomal β -oxidation accumulated in *atg7-2* under warming (Fig. S5).

DISCUSSION

Climate warming imposes sustained pressure on plant energy metabolism, challenging the balance between growth, productivity, and stress resilience (Hatfield and Prueger 2015). Prolonged warming elicits coordinated acclimation responses involving thermomorphogenesis, chloroplast and stomatal adjustments, respiratory shifts, and metabolic reprogramming to optimize carbon and nitrogen use (Wang et al. 2020). While autophagy is well established as a key regulator of proteostasis and organelle quality control during acute HS (Zhou et al. 2013, 2014a, 2023b; Jiang et al. 2020; Wan et al. 2023), its contribution to long-term acclimation under mild but sustained warming remains unclear. A recent mechanistic study directly connects thermomorphogenesis signaling to autophagy, showing that the transcription factor PIF4 activates autophagy to promote growth through selective degradation of the circadian clock repressor TOC1 (Li et al. 2025). Our study now extends this model from short-term growth signaling to long-term thermal acclimation. While Li et al. (2025) characterized

autophagy function in response to 12 hours of warming (28 °C), we demonstrate that under prolonged warming (28 °C for 7 days), autophagy transitions from an early regulatory trigger to a housekeeping system. Our findings indicate that functional autophagy is beneficial for successful acclimation of *Arabidopsis thaliana* under prolonged warming. The physiological, metabolic, and proteomic impairments observed in *atg7-2* redefine autophagy from a transient stress response into a continuous, proactive system supporting cellular maintenance, resource management, and metabolic stability. Together, our findings indicate that autophagy can contribute to carbon and nitrogen homeostasis, proteome stability, and long-term acclimation under sustained warming.

Autophagy supports thermomorphogenic growth and chloroplast resilience during prolonged warming

Thermomorphogenesis involves increased leaf expansion and elongation growth driven by warm-temperature signaling and hormone-dependent reprogramming (Quint et al. 2016; Bellstaedt et al. 2019). The PHYB-PIF4 module initiates this response by promoting auxin biosynthesis and transport (Bellstaedt et al. 2019; Lee et al. 2021; Meng et al. 2025). While this signaling cascade is sufficient to trigger hypocotyl cell elongation (Zhou et al. 2023a), sustained growth under prolonged warming requires additional mechanisms to preserve metabolic and cellular homeostasis.

Under prolonged warming, WT plants exhibited enhanced leaf expansion without a proportional increase in biomass, suggesting a shift toward surface expansion rather than structural carbon investment. In contrast, *atg* mutants showed reduced biomass under both control and warming conditions and failed to sustain leaf expansion. This phenotype is consistent with previously reported growth limitations associated with impaired nutrient recycling and increased metabolic demand (Guiboileau et al. 2012; Avin-Wittenberg et al. 2015; Barros et al. 2017b; Chiu et al. 2023), and indicates that autophagy is required to support the metabolic demands underlying thermomorphogenic growth.

Recently, a mechanistic link between thermomorphogenesis signaling and autophagy has been established, showing that the transcription factor PIF4 directly activates *atg* genes during early responses to warming (Li et al. 2025a). While this study demonstrates that autophagy can be transcriptionally induced as part of warm-temperature signaling, our results indicate that autophagy remains important during prolonged warming, contributing to the maintenance of metabolic and physiological homeostasis throughout the acclimation period. Although the autophagic flux was not assessed, the metabolic reprogramming observed in *atg7-*

2, including the induction of amino acid and lipid catabolism, suggests that autophagy normally contributes to resource recycling and metabolic buffering during warming. In WT plants, this function may reduce the need for activation of compensatory degradation pathways, thereby supporting sustained growth and thermomorphogenic development under elevated temperature.

WT plants maintained PSII photochemical efficiency and chlorophyll content under prolonged warming. Chloroplasts act not only as photosynthetic organelles but also as central metabolic and signaling hubs in temperature responses (Zahra et al. 2023; Amaral et al. 2024). Under prolonged warming, *atg* plants, particularly *atg7-2*, displayed consistent reductions in PSII photochemical efficiency, while chlorophyll and carotenoid content generally maintained. These results indicate compromised PSII functional stability rather than acute pigment loss or severe photoinhibition. Moderate increases in temperature actively reprogram chloroplast metabolism, triggering starch degradation and sucrose production, which in turn stabilizes PIF4 and promotes thermomorphogenic growth (Fan et al. 2025).

Autophagy-deficient mutants exhibit extensive alterations in chloroplast ultrastructure, including the disorganization of thylakoid membranes and impaired chloroplast remodeling under carbon-limiting conditions (Barros et al. 2021, 2023). Under heat shock, selective autophagy pathways dynamically regulate chloroplast protein import by targeting the TOC apparatus, a mechanism critical for maintaining organelle quality control and plant survival during heat (Wan et al. 2023), whereas autophagy deficiency accelerates chlorophyll loss, growth inhibition, and premature senescence under prolonged warming (Jiang et al. 2020). The reduced abundance of proteins associated with tetrapyrrole metabolism, particularly those involved in chlorophyll biosynthesis, in *atg7-2* suggests impaired chloroplast function. In addition, FTSH proteases involved in organelle proteostasis, including FTSH4, FTSH5, FTSH11, and FTSH12, were less abundant in *atg7-2* under prolonged warming. FTSH4, a mitochondrial isoform, contributes to protection against oxidative protein damage (Opalińska et al. 2017) and to mitochondrial protein quality control under prolonged warming (Maziak et al. 2021). Likewise, the chloroplast-envelope protease FTSH11 has been implicated in thermotolerance and maintenance of photosynthetic performance under elevated temperatures (Chen et al. 2006, 2018; Adam et al. 2019), whereas FTSH12 has been associated with chloroplast biogenesis, protein import, and maintenance of chloroplast proteome homeostasis (Mielke et al. 2021; Li et al. 2022b). Although direct regulatory links between autophagy and these FTSH isoforms remain largely unexplored, FTSH6 has been shown to cooperate with autophagy in the turnover of the chloroplast small heat-shock protein HSP21 during recovery from heat stress, revealing functional coordination between organelle-localized proteolytic

systems with autophagic degradation pathways (Sedaghatmehr et al. 2016, 2021). The simultaneous reduction of multiple FTSH proteases in *atg7-2* suggests that autophagy deficiency compromises organelle proteostasis at multiple levels, which may cause the accumulation of dysfunctional chloroplast and mitochondrial components during prolonged warming. Consistent with this interpretation, the reduction of these proteases coincided with decreased PSII efficiency and reduced abundance of chloroplast-associated proteins, supporting the idea that disruption of organelle quality-control systems contributes directly to the photosynthetic and metabolic defects underlying the warming-sensitive phenotype of *atg7-2*.

Autophagy constrains respiratory and transpiratory costs to maintain energy and water balance

High temperatures increase mitochondrial respiration rates and ATP demand (Atkin and Tjoelker 2003; Scafaro et al. 2021; Sharma et al. 2022b). In acclimating plants, respiration is adjusted to maintain energy balance while minimizing unnecessary carbon loss (Dusenge et al. 2019; O’Leary et al. 2019). Under prolonged warming, respiration remained unchanged in WT plants, consistent with respiratory acclimation, a process that returns respiratory rates toward baseline levels during sustained exposure to elevated temperatures while maintaining cellular energy supply (Atkin and Tjoelker 2003; O’Leary et al. 2019; Posch et al. 2019; Scafaro et al. 2021). In contrast, *atg7-2* exhibited a marked increase in respiration together with elevated daytime and nighttime transpiration, indicating a failure to achieve the physiological adjustments observed in WT plants.

This phenotype was accompanied by extensive proteome remodeling, including enrichment of amino acid degradation, lipid degradation, and peroxisomal pathways. Notably, these changes occurred despite the accumulation of starch and soluble sugars, indicating that increased respiratory demand was not driven by depletion of carbon reserves. Instead, the results point to impaired metabolic coordination during warming acclimation. The proteomic remodeling observed in *atg7-2* suggests that, in the absence of autophagy, anabolic, catabolic, and stress-related processes become simultaneously activated, increasing maintenance costs and reducing the metabolic flexibility required for efficient acclimation to elevated temperature.

One possible contributor to this phenotype is altered mitochondrial quality control. Autophagy participates in the removal of dysfunctional mitochondria, and defects in this process can impair respiratory efficiency and increase ROS production (Kurihara et al. 2012; Li et al. 2014; Broda et al. 2018; Nakamura et al. 2021). Although mitochondrial function was not directly assessed here, the accumulation of citrate and succinate together with induction of

amino acid degradation pathways and reduced abundance of the mitochondrial quality-control protease (FTSH4) suggest reorganization of mitochondrial metabolism in *atg7-2* during warming. Multiple enzymes involved in branched-chain amino acid degradation accumulated in the mutant, indicating enhanced conversion of amino acids into respiratory intermediates. Similarly, the enrichment of lipid degradation pathways was associated with the accumulation of proteins involved in peroxisomal β -oxidation. The induction of these alternative catabolic routes despite the accumulation of soluble sugars and starch suggests that their activation is unlikely to reflect carbon starvation. Rather, these responses may represent compensatory metabolic adjustments associated with increased energetic demand and impaired metabolic reprogramming during warming acclimation.

The accumulation of peroxisomal proteins provides an additional link to the constitutive ROS accumulation previously reported in autophagy-deficient guard cells, which compromises stomatal regulation and water-use efficiency (Fig S5) (Yamauchi et al. 2019). Multiple antioxidant proteins also accumulated specifically in *atg7-2* under warming (e.g. CAT1/CAT3, CSD3, MDAR1/MDAR3, APX3). The coordinated induction of components of the ascorbate-glutathione cycle, thioredoxin system, and peroxide-detoxification pathways suggests increased demand for ROS-scavenging capacity, which may be consistent with elevated oxidative pressure during warming acclimation. Consistent with this interpretation, *atg7-2* also accumulated shikimate and sinapinic acid under warming, indicating increased investment in secondary metabolic pathways commonly associated with stress responses.

Excessive transpiration during both day and night in *atg* mutants, particularly in *atg7-2*, points to impaired stomatal regulation. The accumulation of trehalose, raffinose, myo-inositol, erythritol, and glycerol indicates substantial reconfiguration of carbon metabolism toward metabolites commonly associated with osmotic and oxidative stress responses. Whether this accumulation contributes directly to protection or instead reflects persistent stress signaling remains unclear. Nevertheless, the elevated transpiration rates together with the accumulation of these metabolites are consistent with a disruption of water balance in autophagy-deficient plants under warming. Autophagy has been implicated in drought responses and stomatal regulation through modulation of ROS and ABA signaling in guard cells (Liu et al., 2009; Yamauchi et al., 2019). Our findings extend this connection to prolonged warming and suggest that autophagy contributes to the coordination of energy metabolism and water use during thermal acclimation.

Because aquaporin abundance increased in both genotypes, the elevated transpiration observed in *atg7-2* cannot be attributed to impaired induction of water transport proteins.

Instead, the accumulation of additional aquaporins in the mutant (PIP1-5, PIP2-7, TIP2-1, and TIP2-2) may reflect a compensatory response to altered water relations. The persistence of elevated transpiration despite this response suggests that hydraulic adjustments alone were insufficient to restore water-use control, pointing instead to defects in stomatal regulation and cellular homeostasis. Together with the evidence for altered redox metabolism and ROS-associated responses, these findings support a role for autophagy in coordinating water relations, oxidative balance, and metabolic acclimation during prolonged warming.

Autophagy maintains carbon-nitrogen balance and metabolic flexibility during prolonged warming

Autophagy has emerged as a central regulator of carbon-nitrogen integration by coordinating the recycling of proteins, lipids, and organelles while maintaining cellular energy balance under abiotic stress (Masclaux-Daubresse 2016; Marshall and Vierstra 2018; Signorelli et al. 2019a; Barros et al. 2021). Prolonged warming requires coordinated adjustments in carbon allocation, nitrogen remobilization, and energy metabolism to sustain growth, while limiting excessive respiratory costs (Vasseur et al. 2011; Wang et al. 2020). In WT plants, starch levels remained stable and soluble sugars showed only moderate adjustments under warming, including decreased glucose and increased fructose levels. Although biomass accumulation did not increase, WT plants displayed enhanced leaf expansion, indicating that carbon metabolism remained sufficiently flexible to support thermomorphogenic growth and acclimation. Such responses are consistent with previous studies showing that warm-acclimated plants reprogram carbohydrate metabolism to maintain growth and energetic balance under sustained elevated temperatures (Dusenge et al. 2019; Scafaro et al. 2021)

In contrast, *atg7-2* accumulated starch, sucrose, amino acids and respiratory intermediates despite exhibiting higher respiratory activity. This combination indicates that neither carbon nor nitrogen availability is limited during prolonged warming. Instead, the simultaneous accumulation of storage compounds, free metabolites, and catabolic enzymes suggests a failure to efficiently channel available resources into growth and cellular maintenance.

WT plants showed relatively small changes in soluble sugars, polyols, and organic acids, under warming, consistent with efficient metabolic reprogramming during acclimation. By contrast, carbon-rich metabolites, including raffinose, trehalose, myo-inositol, erythritol, and glycerol accumulated in *atg7-2*. These metabolites are commonly associated with osmotic, oxidative, and abiotic stress responses (O'Hara et al. 2013; Elsayed et al. 2014) and their

persistent accumulation suggests chronic metabolic stress rather than successful acclimation. Similar metabolic signatures have been associated with impaired carbon utilization and disrupted stress responses, as observed in glycerol-accumulating *gli1* mutants (Eastmond 2004). These results suggest that carbon metabolism becomes progressively disconnected from metabolic demand in the absence of autophagy.

Organic acid metabolism further supported the existence of impaired metabolic coordination in *atg7-2*. Whereas WT plants showed reductions in citrate, succinate, glyceric acid, and sugar acids, consistent with active carbon turnover during acclimation, citrate and succinate accumulated in *atg7-2* despite elevated respiration. Accumulation of TCA-cycle intermediates together with increased respiratory rates suggests that carbon oxidation remains active but becomes inefficient, potentially reflecting downstream metabolic bottlenecks or altered mitochondrial homeostasis (Sweetlove et al. 2010; Araújo et al. 2011a). This interpretation is further supported by the simultaneous induction of amino acid degradation, fatty acid β -oxidation, and peroxisomal pathways revealed by the proteomic analysis.

The accumulation of threonic and galactonic acids suggest enhanced ascorbate turnover, a response often associated with oxidative stress. Although ROS accumulation was not directly measured, multiple lines of evidence suggest increased oxidative pressure in *atg7-2* during prolonged warming, including reduced PSII efficiency, elevated respiration, accumulation of peroxisomal proteins, induction of fatty acid β -oxidation pathways, and the increased abundance of antioxidant enzymes involved in ROS detoxification. The accumulation of shikimic and sinapinic acids in *atg7-2* also suggests increased allocation of carbon toward phenylpropanoid metabolism, a pathway frequently induced during abiotic stress and involved in the production of protective secondary metabolites (Masclaux-Daubresse 2016). Because phenylpropanoid biosynthesis draws carbon from primary metabolism through the shikimate pathway (Dong and Lin 2021), this response may reflect increased investment in antioxidant and stress-defense pathways at the expense of growth.

Autophagy is a major contributor to nitrogen recycling through controlled protein turnover, particularly under conditions that increase metabolic demand (Li et al. 2015; Masclaux-Daubresse et al. 2017). Elevated temperatures accelerate protein turnover and increase the requirement for metabolic reorganization (Hildebrandt 2018; Santos Júnior et al. 2025). Both WT and *atg7-2* exhibited reduced soluble protein content and slightly increased total free amino acid levels under warming. However, the composition of the amino acid pool differed substantially between genotypes, indicating that autophagy influences not only nitrogen release but also its effective reutilization.

The accumulation of alanine and GABA in *atg7-2* occurred alongside increased abundance of amino acid degradation enzymes, elevated respiration, and reduced abundance of ribosome-associated proteins, suggesting that nitrogen released through proteolysis was not efficiently redirected toward anabolic processes. Alanine formation can divert pyruvate away from constrained mitochondrial metabolism (Rocha et al. 2010), whereas the GABA shunt provides an alternative route connected to the TCA cycle that contributes to redox regulation (Bouché and Fromm 2004; Carillo 2018). Similar responses have been reported during heat stress (Balfagón et al. 2022) and are consistent with the elevated respiratory demand observed in *atg7-2*. They may also reflect increased mitochondrial burden resulting from impaired mitophagy and reduced organelle quality control (Broda et al. 2018). In WT, autophagy may buffer these pathways by maintaining the turnover of damaged mitochondria (mitophagy) and by recycling amino acids in a controlled manner, thus avoiding the accumulation of intermediates that signal metabolic imbalance (Broda et al. 2018).

Additional accumulation of β -alanine, pyroglutamate, and ethanolamine in *atg7-2* further supports disruption of amino acid and membrane-associated metabolism. β -alanine has been associated with altered nitrogen recycling and polyamine metabolism under stress conditions (Parthasarathy et al. 2019). Pyroglutamate accumulation indicates elevated γ -glutamyl cycle activity and glutathione turnover, consistent with oxidative pressure (Noctor et al. 2015). This interpretation is further supported by the increased abundance of γ -glutamyl transferases GGT1 and GGT3 together with OXP1, enzymes involved in glutathione recycling and γ -glutamyl metabolism. The coordinated accumulation of these proteins and pyroglutamate suggests enhanced glutathione turnover in *atg7-2*, reinforcing the evidence for persistent redox stress during prolonged warming. Ethanolamine accumulation suggests intensified phospholipid degradation and altered phosphatidylethanolamine metabolism (Lynch and Dunn 2004). Since PE is the lipid anchor for ATG8 during autophagosome formation, a process central to membrane remodeling, autophagy impairment can directly disrupt PE homeostasis and phospholipid-nitrogen metabolism (Zou et al. 2025).

The accumulation of amino acids metabolites and proteins in *atg7-2* under prolonged warming suggests that the primary limitation is not amino acid production itself, but rather their effective reintegration into anabolic and respiratory metabolism. This interpretation is supported by proteomic evidence showing a reduced abundance of proteins associated with ribosomal function, plastid translation, RNA metabolism, indicating a diminished capacity for protein synthesis. Impaired translational capacity would limit the efficient utilization of amino acids released through proteolysis, thereby contributing to their accumulation despite the

activation of amino acid degradation pathways (Hildebrandt et al. 2015; Hildebrandt 2018; Hirota et al. 2018). Consistent with this interpretation, proteins involved in aminoacyl-tRNA synthesis accumulated in *atg7-2*, suggesting continued investment in translational machinery despite reduced abundance of ribosomal components. Collectively, these changes indicate a metabolically incoherent state in which protein degradation, amino acid accumulation, and alternative respiratory pathways are activated simultaneously, while the capacity to channel these resources into productive growth and biosynthesis is diminished.

CONCLUSION

Our findings demonstrate that autophagy is essential for successful acclimation to prolonged warming in *Arabidopsis thaliana*. While WT plants maintained photosynthetic performance, respiratory acclimation, metabolic flexibility, and thermomorphogenic growth, autophagy-deficient mutants, particularly *atg7-2*, exhibited impaired leaf expansion, increased respiratory and transpiratory costs, disruption of carbon and nitrogen metabolism, and extensive proteome remodeling. The accumulation of storage compounds, amino acids, stress-associated metabolites, antioxidant proteins, and alternative catabolic pathways indicates that autophagy contributes not only to nutrient recycling but also to the coordination of organelle maintenance, redox homeostasis, and energy metabolism. Together, these results identify autophagy as a central integrator of proteostasis and metabolic acclimation during prolonged warming, enabling plants to balance growth, resource utilization, and stress resilience in a warming climate.

METHODS

Plant material, heat treatments and growth

All *Arabidopsis thaliana* plants used in this study were of the Columbia-0 ecotype. The T-DNA loss-of-function mutant lines *atg5-1* (SAIL_129_B07) and *atg7-2* (GK-655B06), previously characterized by Barros et al. (2017a), and the corresponding wild type (Col-0) were used in this study. Seeds were surface sterilized and imbibed for 4 days at 4 °C in the dark on 0.7% (w/v) agar plates containing one-half-strength Murashige and Skoog medium (Sigma-Aldrich; pH 5.7). Seeds were subsequently germinated and grown at 22 °C under short-day conditions (8 h of light/16 h of dark), 60% relative humidity, and 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

For heat treatment, 3-week-old plants were transferred to control and treatment conditions. Adapted from Wang et al. (2020), the warming temperature (W) was set at 6 °C above control, 28 °C, during 7 days. After warming treatment, whole rosettes from at least six

independent samples per genotype were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

Growth parameters were evaluated in 4-week-old plants by measuring leaf area and dry weight. At this stage, the plant rosette growth is nearly complete and leaves are fully expanded. Leaf area was measured at 3 and 7 days by importing photographs and processing the resulting images on ImageJ/Fiji software (Schindelin et al. 2012; Schneider et al. 2012).

Measurements of gas exchange and chlorophyll fluorescence

Gas exchange parameters were measured by using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR Inc., Lincoln, NE) with a portable photosynthesis system. Light was supplied from a series of light-emitting diodes located above the cuvette, providing an irradiance of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, consistent with our growth conditions. The reference CO_2 concentration was set at 400 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air. All measurements were performed at the respective temperature regime, and vapor pressure deficit was maintained at 2 ± 0.2 kPa, while the amount of blue light was maintained at 10% of the photon flux density to optimize stomatal aperture. The determination of photosynthetic parameters, including photosynthesis rate (A_N), dark respiration (R_d), and day (E_{day}) and night transpiration (E_{night}) was performed in 4-week-old plants. R_d and E_{night} were measured after 2 hours in the dark period using the same gas exchange system described above.

The maximum quantum yield of PSII (F_v/F_m), which corresponds to the potential quantum yield of the photochemical reactions of Photosystem II (PSII) and represents a measure of photochemical efficiency, was measured using a MINI-PAM fluorometer (Heinz Walz, Effeltrich, Germany). F_v/F_m was determined in leaves dark-acclimated for at least 30 min (Maxwell and Johnson 2000).

Determination of metabolite levels

Approximately 5 mg of powdered leaf material was used for metabolite extraction. Metabolite extraction was performed by rapid grinding of the tissue in liquid nitrogen followed by the immediate addition of methanol and ribitol, as described by Lisec et al. (2006). In the soluble fraction, the contents of chlorophyll a, chlorophyll b, and carotenoids were determined according to Wellburn (1994), whereas glucose, fructose, sucrose, and starch were determined according to Fernie et al. (2001). Total amino acids and soluble proteins were determined as described by Yemm et al. (1955) and Bradford (1976), respectively. Proline was quantified as

described by Carillo and Gibon (2011). The organic acids malate and fumarate were determined according to Nunes-Nesi et al. (2007).

Extraction and derivatization of polar metabolites were carried out using the protocol described by Lisec et al. (2006). The metabolites were analyzed by gas chromatography coupled to mass spectrometry (GC-MS; QP-PLUS 2010, Shimadzu, Japan). Mass spectra were analyzed using Xcalibur® 2.1 (Thermo Fisher Scientific, Waltham, MA, USA). Metabolites were identified using the Golm Metabolome Database (<http://gmd.mpimp-golm.mpg.de/>) (Kopka et al. 2005). Metabolite levels were normalized to the amount of ribitol and the dry weight used for extraction.

Protein extraction, digestion, and sample preparation for proteome analysis via mass spectrometry

The procedure is based on the single-pot solid-phase-enhanced sample preparations (SP4) protocol for proteomics experiments of Johnston et al. (2022). In brief, proteins were solubilized with sodium dodecyl sulfate (SDS) from lyophilized plant powder. Disulfide bridges were reduced with dithiothreitol (DTT) and subsequently alkylated with iodoacetamide (IAM). Glass beads were used to bind the denatured proteins. The beads were washed multiple times with 80 % ethanol to get rid of interfering components for the digestion. Finally, the proteins were digested overnight with trypsin (Mass Spectrometry Grade, Promega). The peptide-containing supernatants were collected and desalted on 50 mg Sep-Pak tC18 columns (Waters). The purified peptides were quantified with a quantitative Peptide Assay Kit (Thermo Scientific) and adjusted to 400 ng · µl⁻¹ in 0.1 % formic acid.

Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS)

400 ng of peptides were injected via a nanoElute 2 UHPLC (Bruker Daltonik, Bremen, Germany) and separated on an analytical reversed-phase C18 column (Aurora Ultimate 25 cm x 75 µm, 1.6 µm, 120 Å; IonOpticks). Using MS grade water and a multi-staged gradient acetonitrile containing 0.1 % formic acid (0 min, 2 %; 54 min, 25 %; 60 min, 37 %; 62 min, 95 %; 70 min, 95 %), peptides were eluted and ionized by electrospray ionization with a CaptiveSpray 2 ion source and a flow rate of 300 nl · min⁻¹. The ionized peptides were separated, fragmented and analyzed with a standard data-dependent acquisition parallel accumulation-serial fragmentation application method (DDA-PASEF) of the system with the following settings: Ion mobility window of 0.7-1.5 V s cm⁻², 4 PASEF ramps, target intensity

14,500 (threshold 1,200), and a cycle time of ~0.53 s. The analysis was performed on a timsTOF-HT mass spectrometer (Bruker Daltonik, Bremen, Germany).

Data processing and functional annotation

The ion mobility spectrometry (IMS-MS/MS) spectra from both experiments were analyzed with MaxQuant software (Cox and Mann 2008) using default search parameters and TAIR10 (Arabidopsis.org) as database for protein identification. The calculation of label-free quantification (LFQ) values and intensity-based absolute quantification (iBAQ) values were both enabled. Data evaluation was performed using Perseus software (Tyanova et al. 2016). Proteins were excluded from further analysis if they were not detected in at least $n-1$ biological replicates in at least one of the sample groups. Subsequently, missing values were replaced with randomly chosen low values from a normal distribution. Significant changes were determined from the LFQ values using Student's *t*-tests followed by FDR correction ($\text{FDR} < 0.05$).

Statistical data analyses

The experiments were conducted using a completely randomized design with a minimum of three biological replicates per treatment. Phenotypic experiments were independently repeated at least twice to confirm reproducibility. Data were first tested for normality prior to further analysis. Depending on the experimental design, differences among means were evaluated using either two-way factorial ANOVA, one-way ANOVA, or Student's *t*-test, with significance accepted at $P < 0.05$. For factors showing significant main or interaction effects in ANOVA, means were compared using Tukey's Honestly Significant Difference (HSD) post hoc test ($P < 0.05$). Statistical analyses were performed using R statistical software (www.r-project.org) with the ExpDes package. Graphs were generated in GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and RStudio (RStudio, Boston, MA, USA) using the ggplot2 package.

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FIGURES

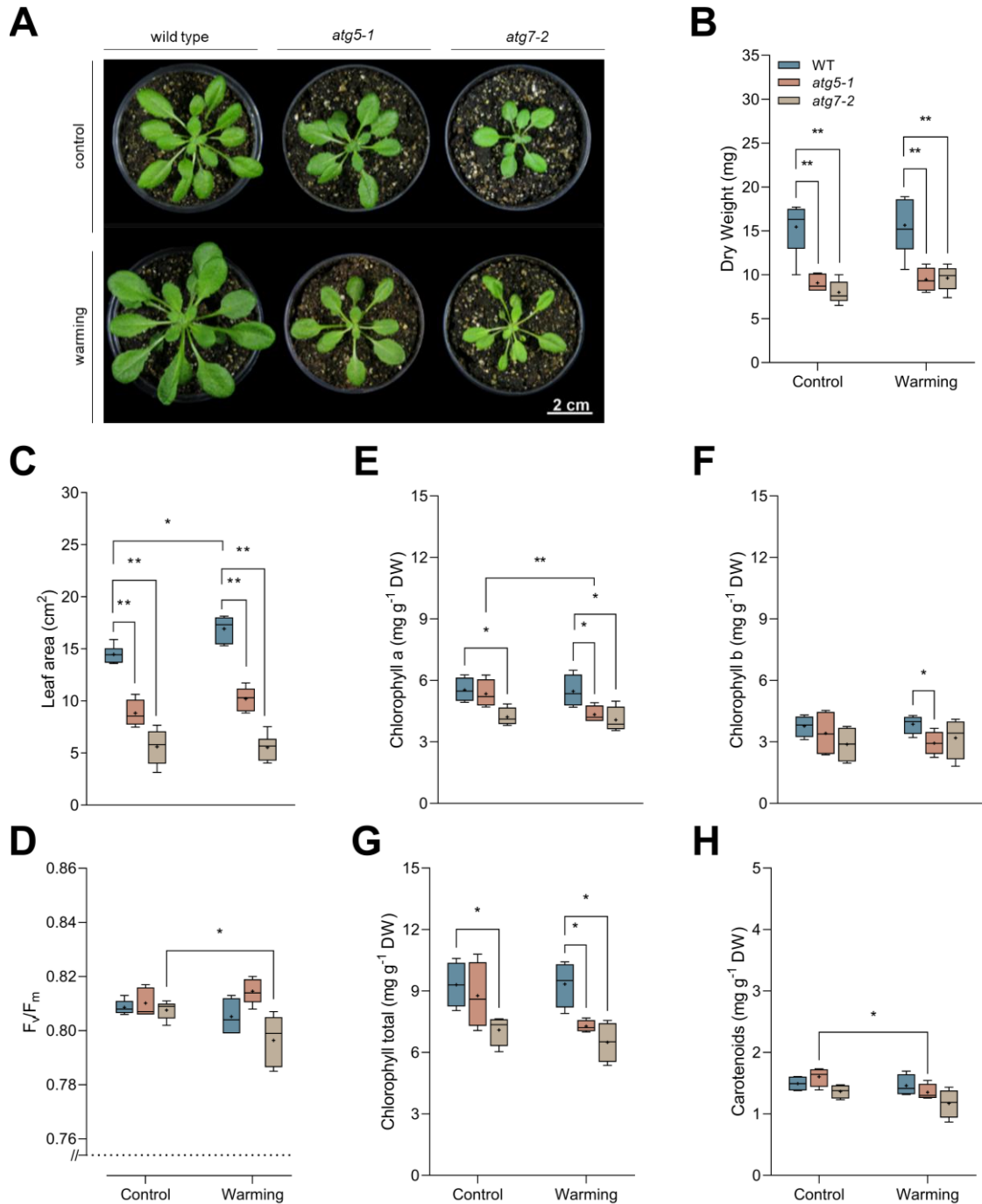


Figure 1. Autophagy deficiency impairs growth, and photosynthetic performance under control and prolonged warming conditions in *Arabidopsis thaliana*. (A) Representative phenotypes, (B) dry weight, (C) leaf area, (D) maximum quantum efficiency of PSII (F_v/F_m), (E) chlorophyll a, (F) chlorophyll b, (G) total chlorophyll, and (H) carotenoids of wild-type (WT), *atg5-1*, and *atg7-2* rosettes. Plants were grown under short-day conditions for 4-week-old short-day-and subsequently exposed to control (22 °C) or prolonged warming (28 °C) for 7 days. Data are presented as means $\pm$ SE (n = 4-6). Asterisks indicate significant difference between comparisons based on a two-tailed Student's *t*-test (* P < 0.05; ** P < 0.01; *** P < 0.001). Scale bars = 2 cm.

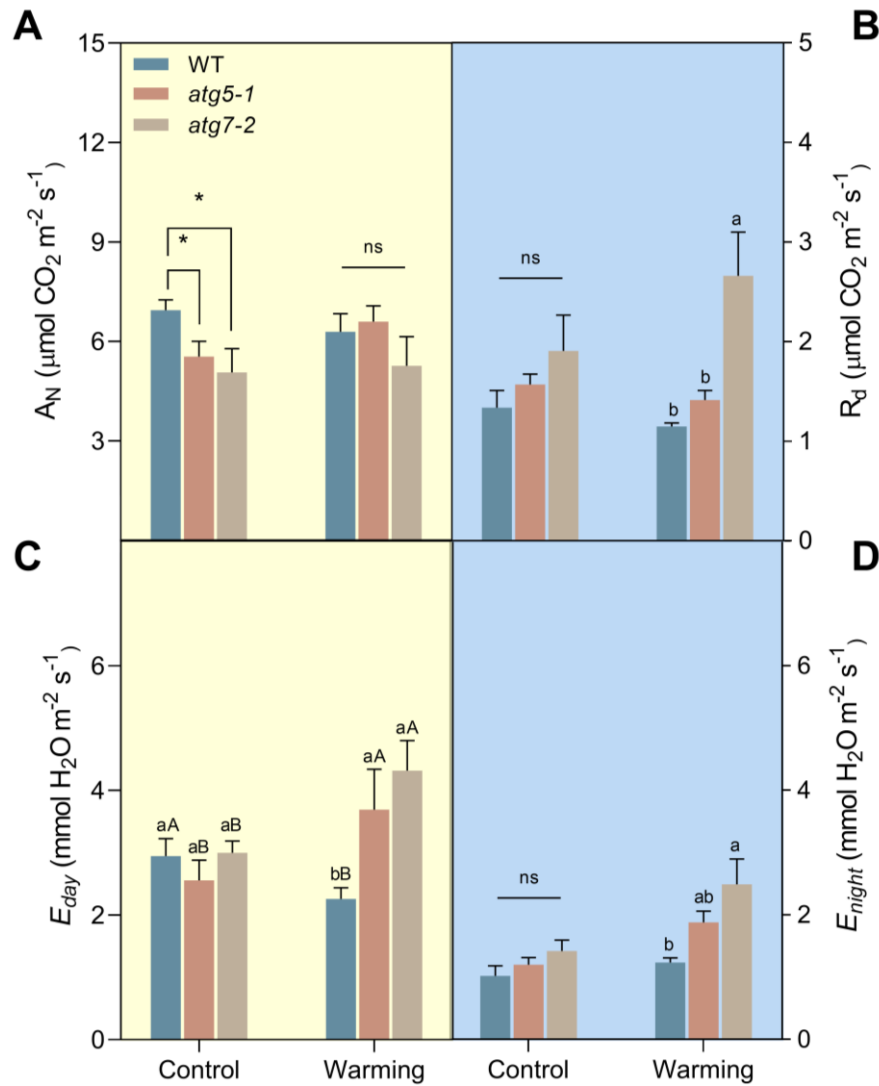


Figure 2. Autophagy balance dark respiration and transpiration responses under prolonged warming in *Arabidopsis thaliana*. (A) Net CO₂ assimilation rate (A_N), (B) dark respiration rate (R_d), (C) daytime transpiration rate (E_{day}), and (D) nighttime transpiration rate (E_{night}) of wild-type (WT), *atg5-1* and *atg7-2* plants. Four-week-old plants grown under short-day conditions were exposed to control (22 °C) or prolonged warming (28 °C) for 7 days prior to measurements. Gas-exchange parameters were measured using an open-flow infrared gas-exchange analyzer system equipped with a whole-plant cuvette. Data are presented as mean $\pm$ SE ($n = 5$). For E_{day} , statistical analysis was performed using two-way ANOVA with genotype and treatment as fixed factors ($P < 0.05$), followed by Tukey's post-hoc test for pairwise comparisons among genotype vs temperature combinations ($P < 0.05$). Different lowercase and uppercase letters indicate significant differences among genotypes within a given temperature and between temperatures within a genotype, respectively. For the other parameters, asterisks indicate significant differences based on a two-tailed Student's t -test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). "ns" indicates non-significant effects.

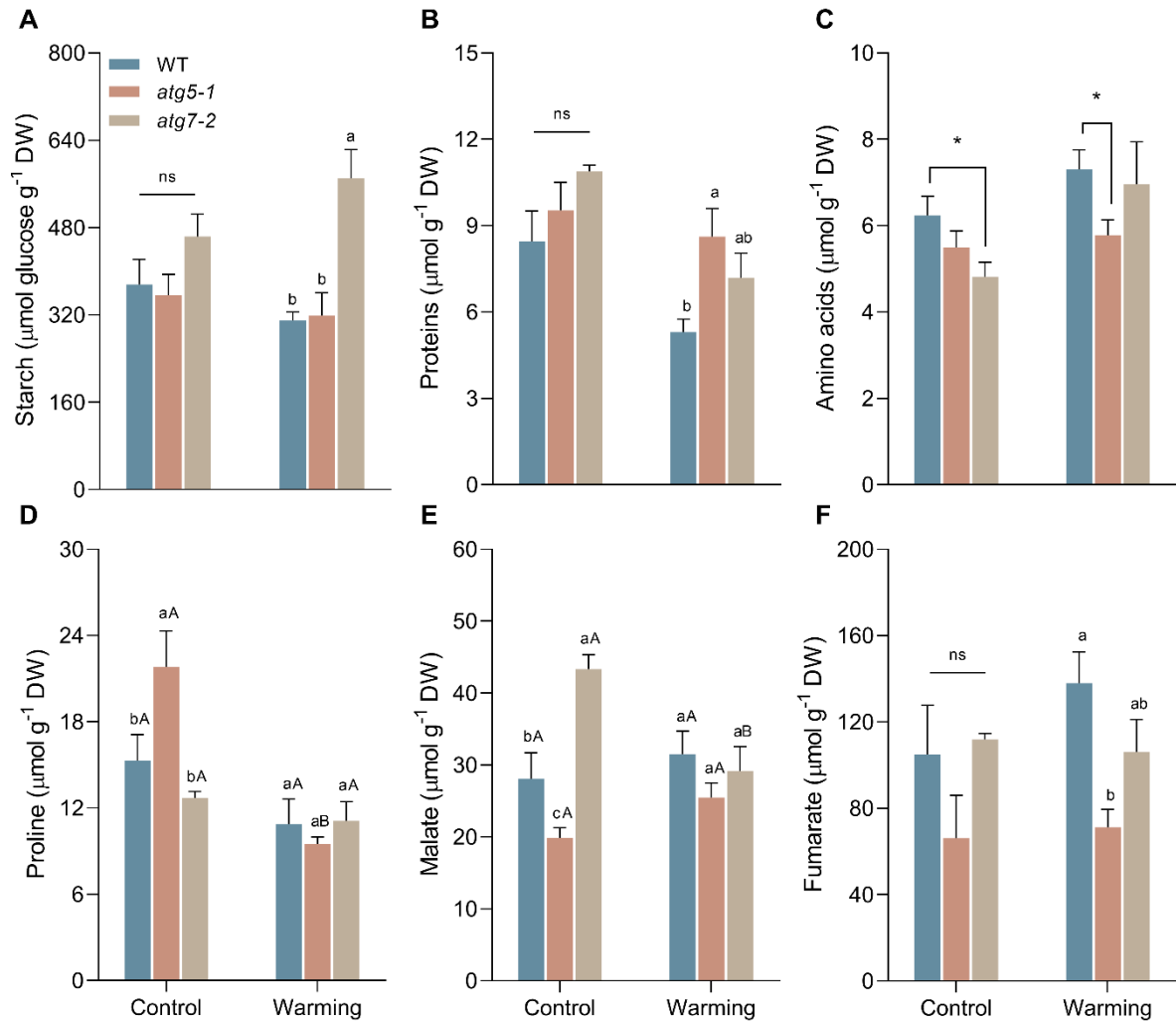


Figure 3. Autophagy deficiency affects nitrogen and carbon metabolic pools in *Arabidopsis thaliana* following prolonged warming. (A) Starch, (B) protein, (C) amino acids, (D) malate, and (E) fumarate were measured using whole rosettes of 4-week-old short-day-grown *Arabidopsis* plants after 7 d following control (22°C) and prolonged warming (28°C). Data presented as mean $\pm$ SE of $n = 4$ biological replicates. Statistical analyses were performed using two-way ANOVA with genotype and treatment as fixed factors. For metabolites with a significant genotype vs. treatment interaction ($P < 0.05$), pairwise comparisons among all genotype vs. treatment combinations were conducted using Tukey's test ($P < 0.05$). Different lowercase and uppercase letters indicate significant differences among genotypes within a treatment and between treatments within a genotype, respectively. For metabolites showing only a significant main effect of genotype or treatment, one-way ANOVA followed by Tukey's test was applied separately for the respective factor, and different lowercase letters indicate significant differences ($P < 0.05$). "ns" indicates no significant effects.

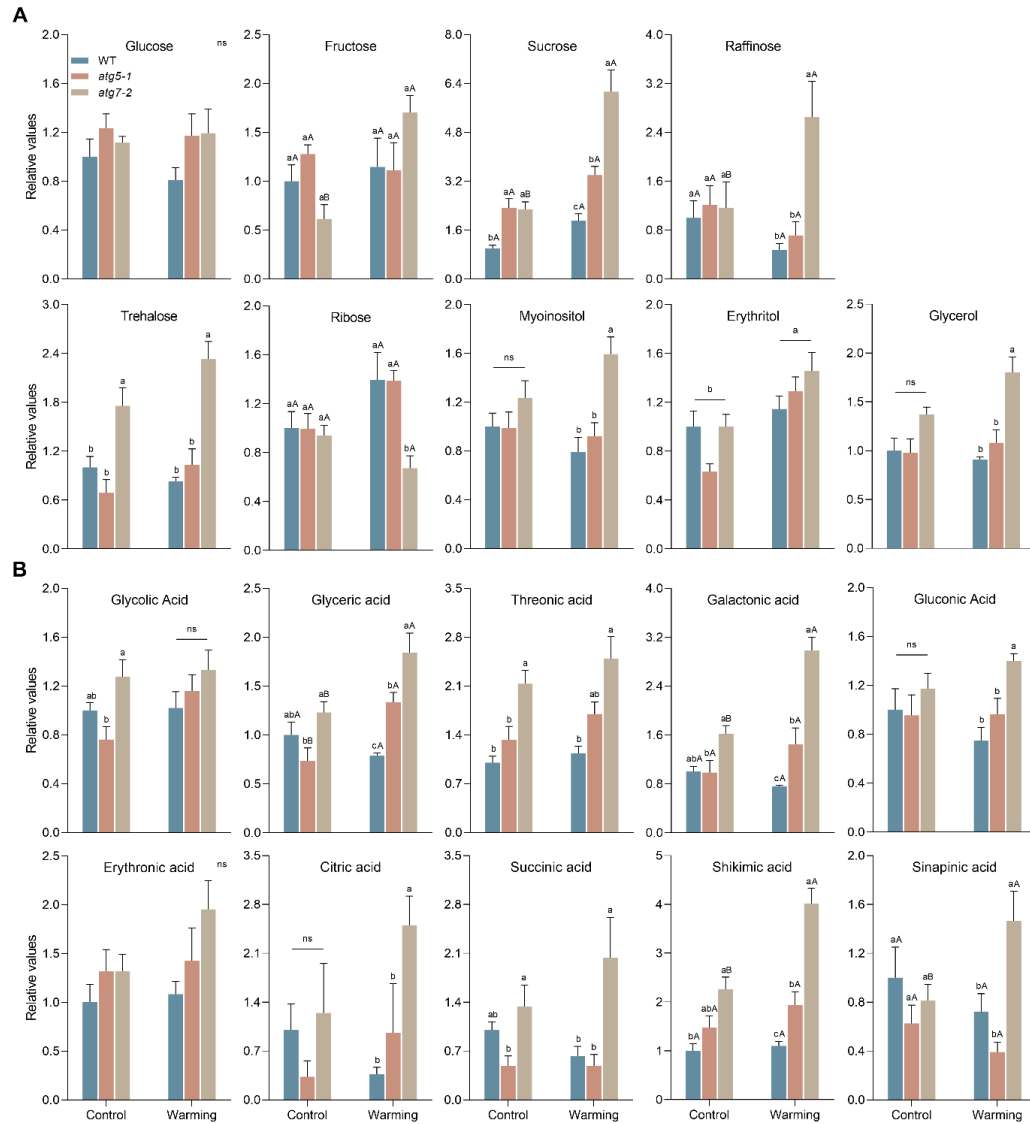


Figure 4. Deficiency of autophagy dysregulates carbon metabolism under prolonged warming in *Arabidopsis thaliana*. Relative abundance of soluble sugars, sugar alcohols (polyols), organic acids, and secondary metabolites measured using full rosette of 4-week-old *Arabidopsis* wild-type (WT) and autophagy-deficient mutants (*atg5-1* and *atg7-2*) plants, after treatment for 7 days in control (22°C) and prolonged warming (28°C). The quantification was based on GC-MS peak areas normalized to internal standard and dry weight. Metabolites are grouped by biochemical class: (A) major soluble sugars and minor sugars and polyols; (B) organic acids and secondary metabolites. Data presented as mean $\pm$ SE ($n = 4$). Statistical analyses were performed using two-way ANOVA with genotype and treatment as fixed factors. For metabolites with a significant genotype vs. treatment interaction ($P < 0.05$), pairwise comparisons among all genotype vs. treatment combinations were conducted using Tukey's test ($P < 0.05$). Different lowercase and uppercase letters indicate significant differences among genotypes within a treatment and between treatments within a genotype, respectively. For metabolites showing only a significant main effect of genotype or treatment, one-way ANOVA followed by Tukey's test was applied separately for the respective factor, and different lowercase letters indicate significant differences ($P < 0.05$). "ns" indicates no significant effects.

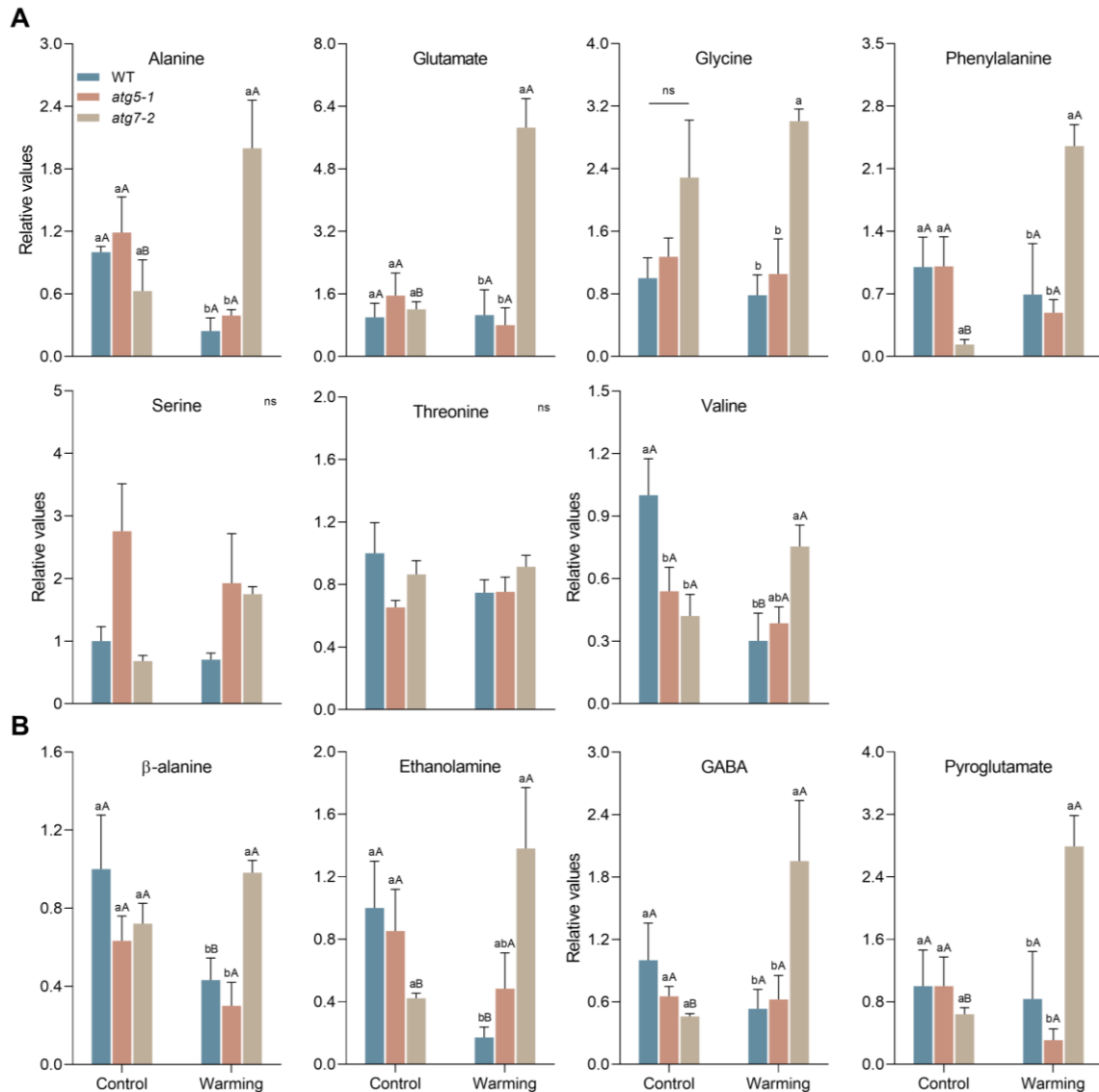


Figure 5. Autophagy keeps the homeostasis of nitrogen metabolism under prolonged warming in *Arabidopsis thaliana*. Relative abundances of some proteinogenic and non-proteinogenic amino acids and related metabolites measured using full rosette of *Arabidopsis* wild-type (WT) and autophagy-deficient mutants (*atg5-1* and *atg7-2*) plants after 7 days under control (22°C) and prolonged warming (28°C). The quantification was based on GC-MS peak areas normalized to internal standard and dry weight. Metabolites are grouped by biochemical class: (A) proteinogenic amino acids; (B) non-proteinogenic amino acids and related metabolites. Data presented as mean $\pm$ SE (n = 4). Statistical analyses were performed using two-way ANOVA with genotype and treatment as fixed factors. For metabolites with a significant genotype vs. treatment interaction ($P < 0.05$), pairwise comparisons among all genotype vs. treatment combinations were conducted using Tukey's test ($P < 0.05$). Different lowercase and uppercase letters indicate significant differences among genotypes within a treatment and between treatments within a genotype, respectively. For metabolites showing only a significant main effect of genotype or treatment, one-way ANOVA followed by Tukey's test was applied separately for the respective factor, and different lowercase letters indicate significant differences ($P < 0.05$). "ns" indicates no significant effects.

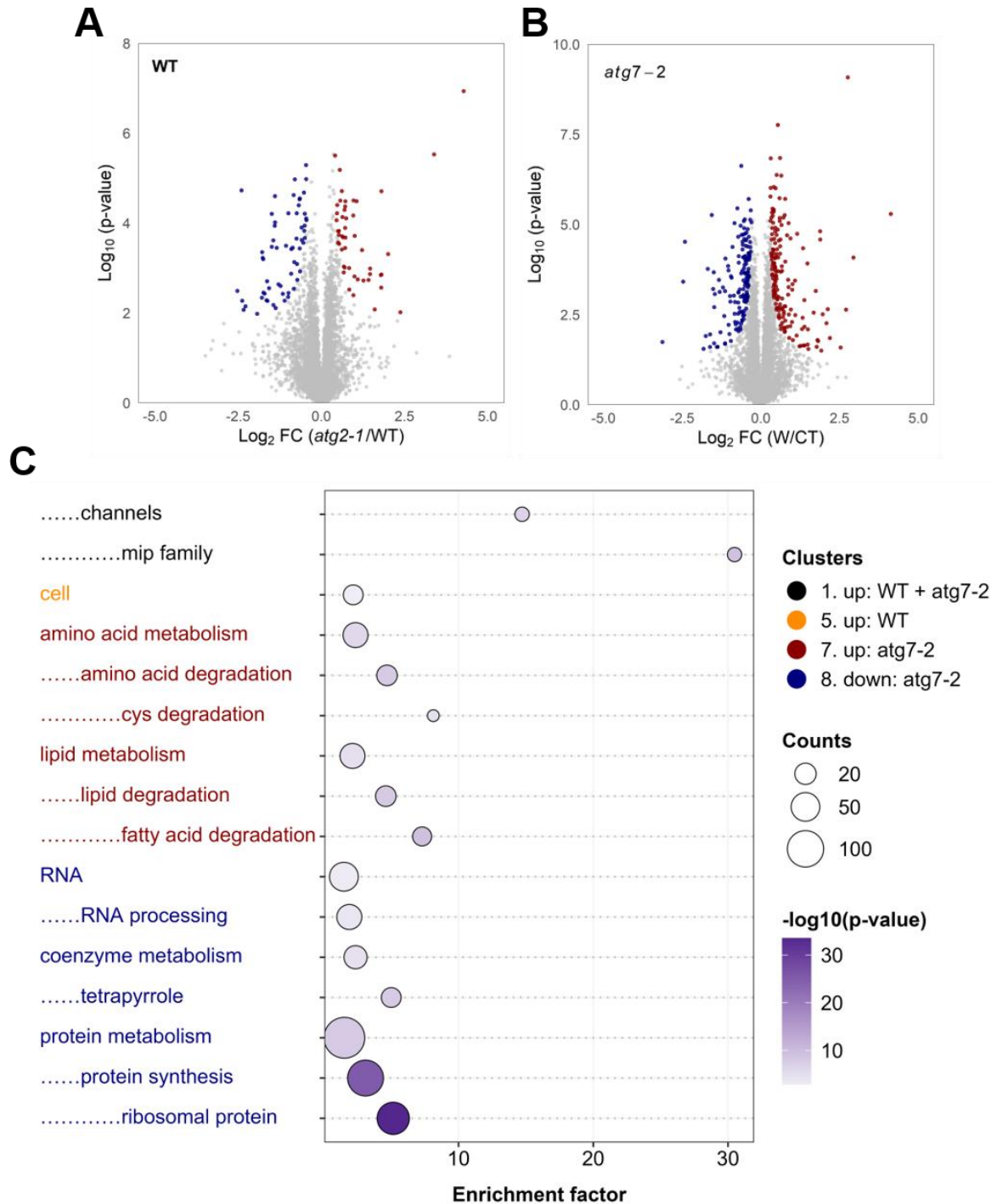


Figure 6. Prolonged warming induces alteration in the proteome of *Arabidopsis thaliana*. Proteome analysis were performed in full rosette of 4-week-old *Arabidopsis* wild-type (WT) and autophagy-deficient mutant (*atg7-2*) plants growth under control conditions (22°C) or exposed to prolonged warming (28°C) during 7 days before collection. (A-B) Volcano plots of differentially abundant proteins (DAPs) in response to prolonged warming relative to control in (A) WT and (B) *atg7-2*. Red and blue dots indicate significantly increased and decreased proteins, respectively. Statistical significance was determined using a two-tailed Student's t-test (FDR < 0.05). (C) Functional enrichment analysis of DAP clusters. in proteins that are significantly increased or decreased in the plant proteome. Bubble size represents the number of proteins assigned to each functional category, whereas color intensity indicates enrichment significance (-log₁₀ P-value). Data is based on at least four biological replicates. Graphs were generated using the ggplot2 package in RStudio.

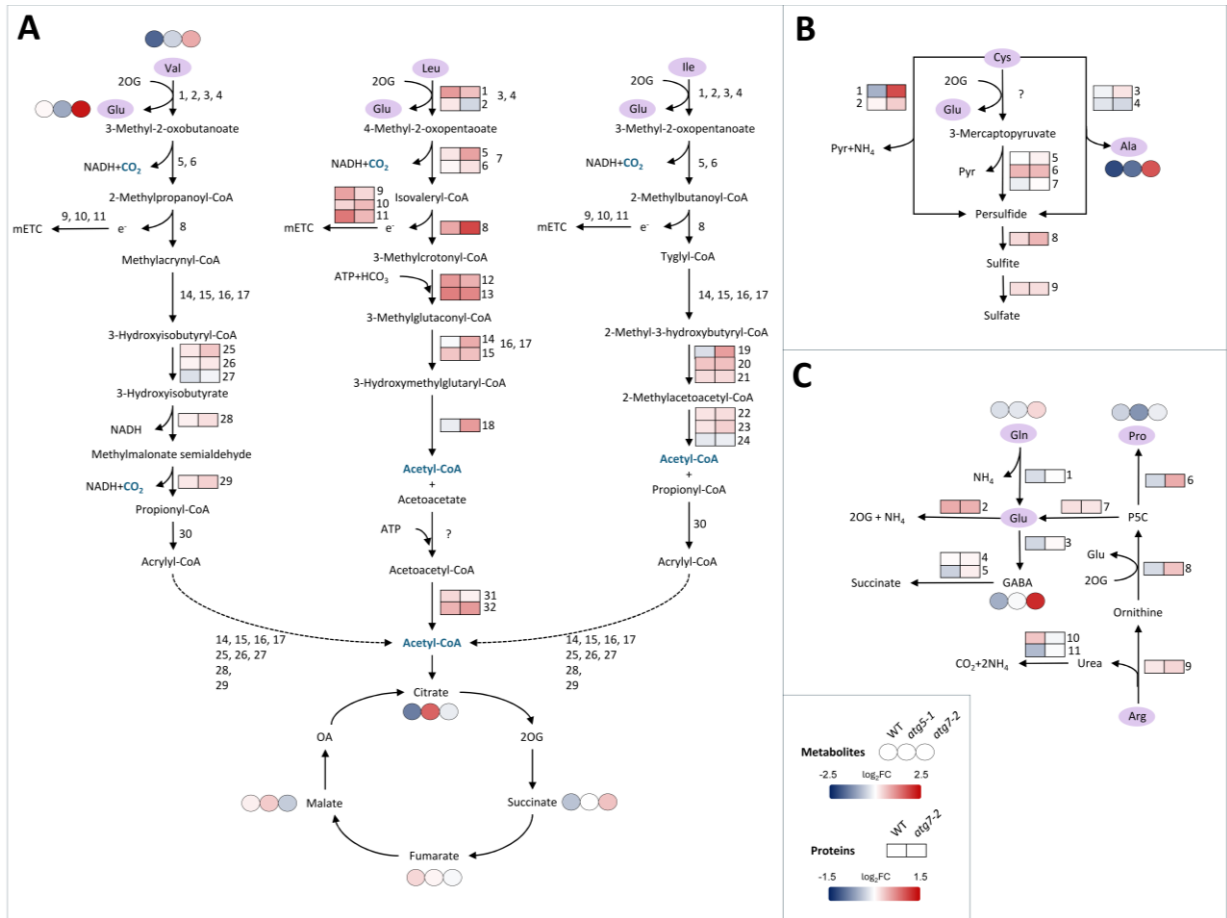


Figure 7. Autophagy deficiency increased catabolism of alternative substrates to support enhanced respiration under prolonged warming.

Overview of selected metabolic pathways affected by prolonged warming (28°C for 7 d) in *Arabidopsis thaliana* wild-type (WT) and autophagy-deficient mutants (*atg5-1* and *atg7-2*) (Adapted from Hildebrandt et al. (2015)). (A) Branched-chain amino acid (BCAA) degradation pathway, including valine, leucine, and isoleucine catabolism and their connection to acetyl-CoA production and the tricarboxylic acid (TCA) cycle. (B) Cysteine degradation pathway. (C) Glutamate-, glutamine-, arginine-, proline-, and GABA-associated metabolic pathways. (D) Proteins associated with organelle quality control and reactive oxygen species (ROS) metabolism. Colored circles indicate metabolite abundance changes, and colored squares indicate protein abundance changes under prolonged warming relative to control conditions. Values are presented as log₂ fold change (warming/control), with red and blue indicating increased and decreased abundance, respectively. Numbers adjacent to protein abundance boxes correspond to protein identifiers in Fig. S7. Dashed arrows indicate multi-step reactions not shown in detail. Abbreviations: 2OG, 2-oxoglutarate; OAA, oxaloacetate; Pyr, pyruvate; NH₄⁺, ammonium; mETC, mitochondrial electron transport chain.

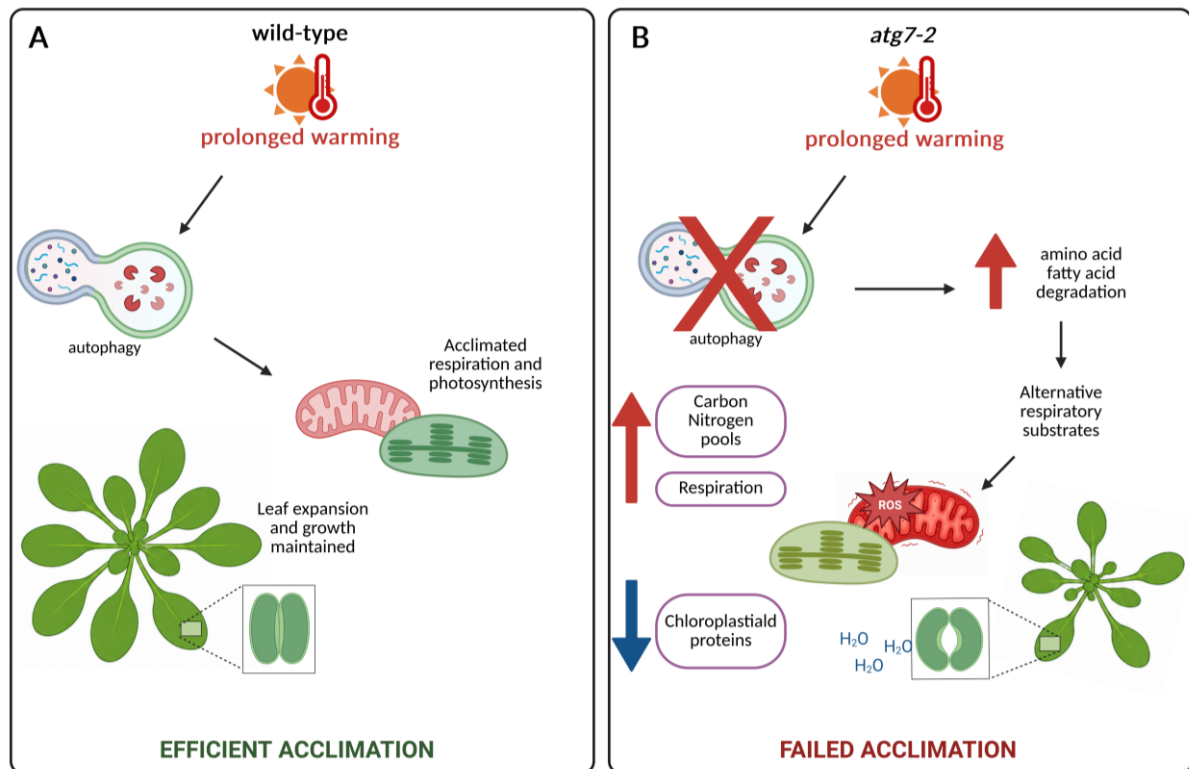


Figure 8. Proposed model for the role of autophagy in acclimation to prolonged warming in *Arabidopsis thaliana*. Under prolonged warming (28°C for 7 d), functional autophagy promotes successful acclimation by supporting organelle quality control, respiratory acclimation, photosynthetic performance, and thermomorphogenic growth. In wild-type plants (A), autophagy contributes to the maintenance of chloroplast and mitochondrial function, allowing sustained leaf expansion and efficient physiological acclimation to elevated temperature. In contrast, autophagy deficiency in *atg7-2* (B) impairs acclimation, resulting in reduced abundance of chloroplast-associated proteins, increased respiration, accumulation of carbon and nitrogen pools, enhanced degradation of amino acids and fatty acids, and greater reliance on alternative respiratory substrates. These metabolic adjustments are accompanied by mitochondrial stress, activation of reactive oxygen species (ROS)-associated responses, increased water loss, and reduced growth, ultimately leading to failed acclimation to prolonged warming.

SUPPLEMENTAL DATA

TABLE

Supplementary Table S1. Primer list.

Primer name	Primer sequence (5' to 3')	Note
At5g17290 Fw	GATTGAAACGAGGAAGAATTTAGAC	For <i>ATG5-1</i> genotyping
At5g17290 Rv	TAATTGATATGCCTAACATCGTCC	
At5g17290 Lb	TCAGAAATGGATAAATAGCCTTGC	
At5g45900 Fw	GACTGTACCTAACTCAGTGGGATG	For <i>ATG7-2</i> genotyping
At5g45900 Rv	GCTCCTGCAATAGGAGCTAGAC	
At5g45900 Lb	ATAATAACGCTGCGGACATCTACATTT	

FIGURES

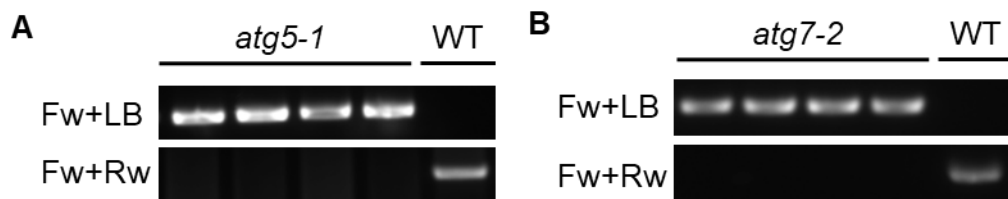


Figure S1. PCR genotyping of *atg5-1* and *atg7-2* mutants. Fw, forward primer; Rv, reverse primer; LB, T-DNA left border primer. Genomic DNA from wild-type (WT) and mutant lines were used as template.

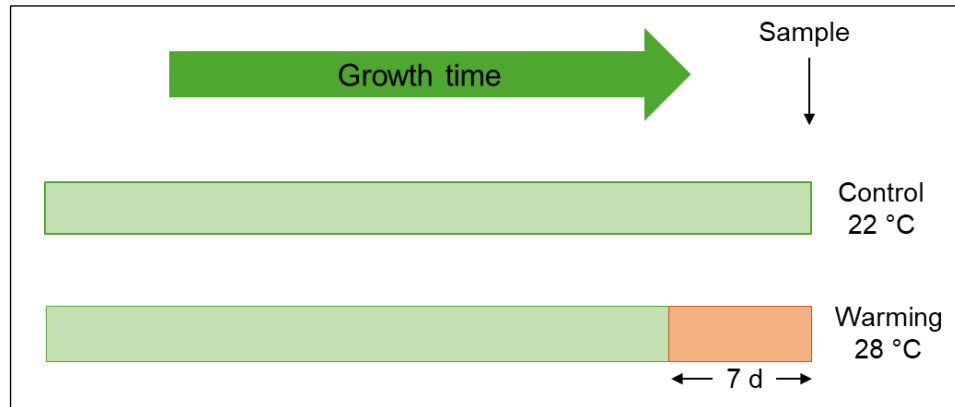


Figure S2. Experimental timeline for control (CT) and prolonged warming (W) conditions. Four-week-old *Arabidopsis thaliana* plants grown under short-day conditions (8h light/16h dark) prior to transfer to the indicated treatments. Plants under control conditions were maintained at 22°C during the whole growth (green), whereas plants under prolonged warming treatment were transferred to 28°C for 7 days (orange). Adapted from Wang et al. (2020).

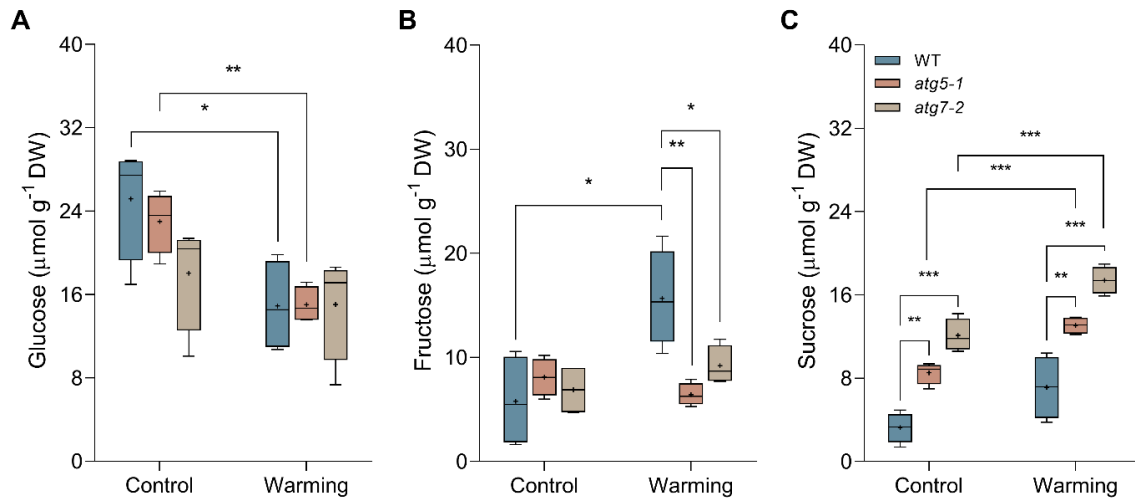


Figure S3. Autophagy deficiency alters sugar pools in *Arabidopsis thaliana* following prolonged warming. (A) Glucose, (B) fructose, and (C) sucrose were measured using whole rosettes of 4-week-old short-day-grown *Arabidopsis* plants after 7 d following control (22°C) and prolonged warming (28°C). Data presented as mean $\pm$ SE ($n = 4$). Asterisks indicate significant differences based on two tailed Student's t-test (* $P < 0.05$). "ns" indicates no significant effects.

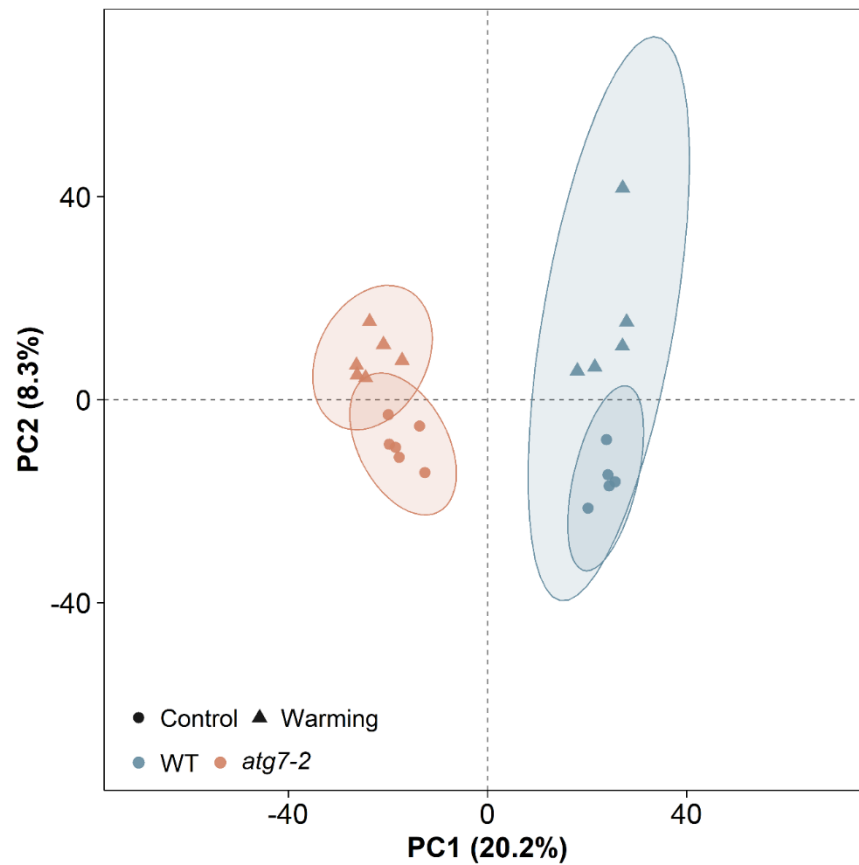


Figure S4. Autophagy deficiency and prolonged warming affect the proteome of *Arabidopsis thaliana*. Proteome analysis was carried out in full rosette of 4-week-old *Arabidopsis* wild-type (WT) and autophagy-deficient mutant (*atg7-2*) plants, after treatment for 7 days in control (22°C) and prolonged warming (28°C). Principal component analysis (PCA) of proteomic samples. Data presented are means of five biological replicates. Ellipses represent 95% confidence intervals.

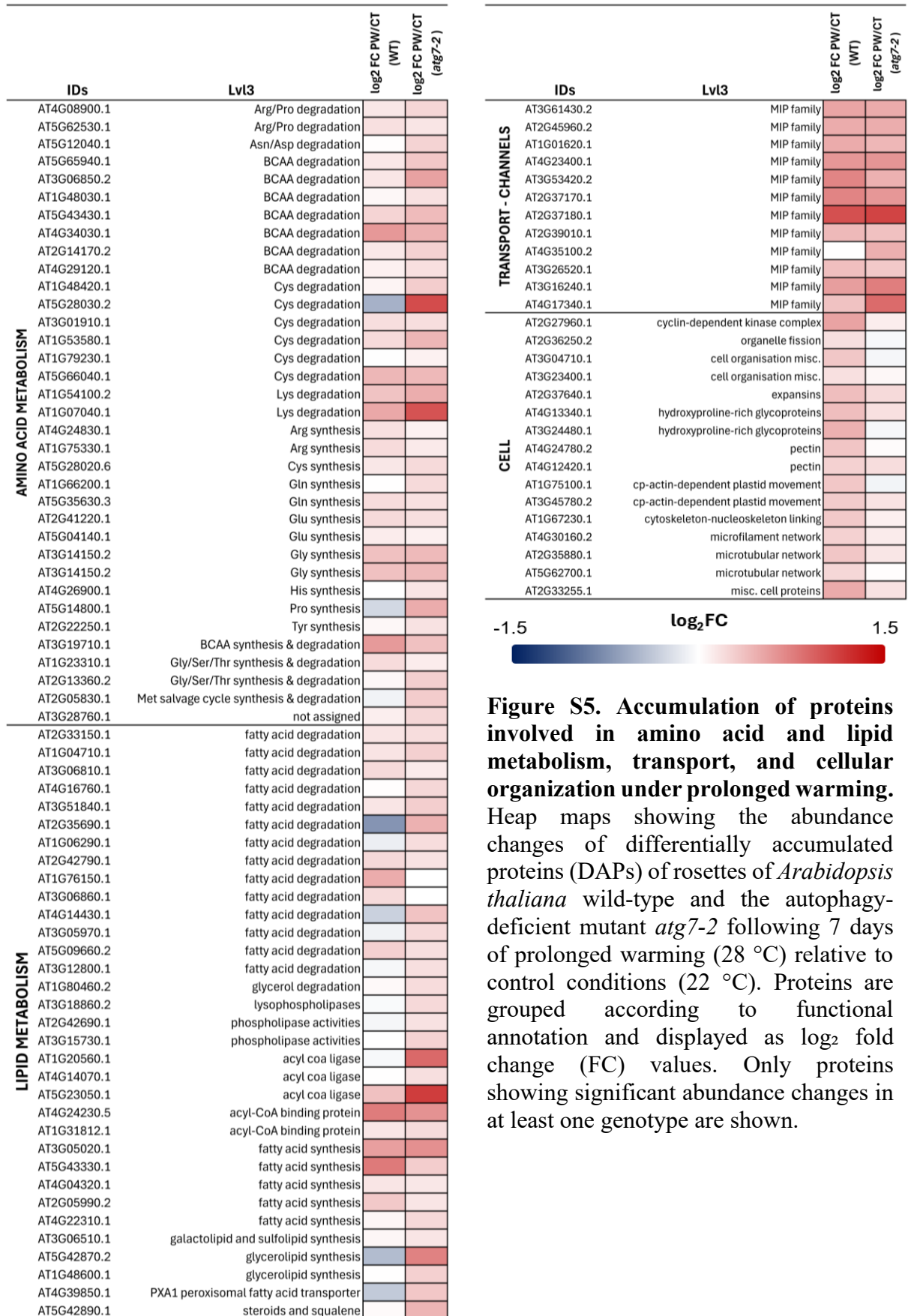


Figure S5. Accumulation of proteins involved in amino acid and lipid metabolism, transport, and cellular organization under prolonged warming. Heat maps showing the abundance changes of differentially accumulated proteins (DAPs) of rosettes of *Arabidopsis thaliana* wild-type and the autophagy-deficient mutant *atg7-2* following 7 days of prolonged warming (28 °C) relative to control conditions (22 °C). Proteins are grouped according to functional annotation and displayed as log₂ fold change (FC) values. Only proteins showing significant abundance changes in at least one genotype are shown.

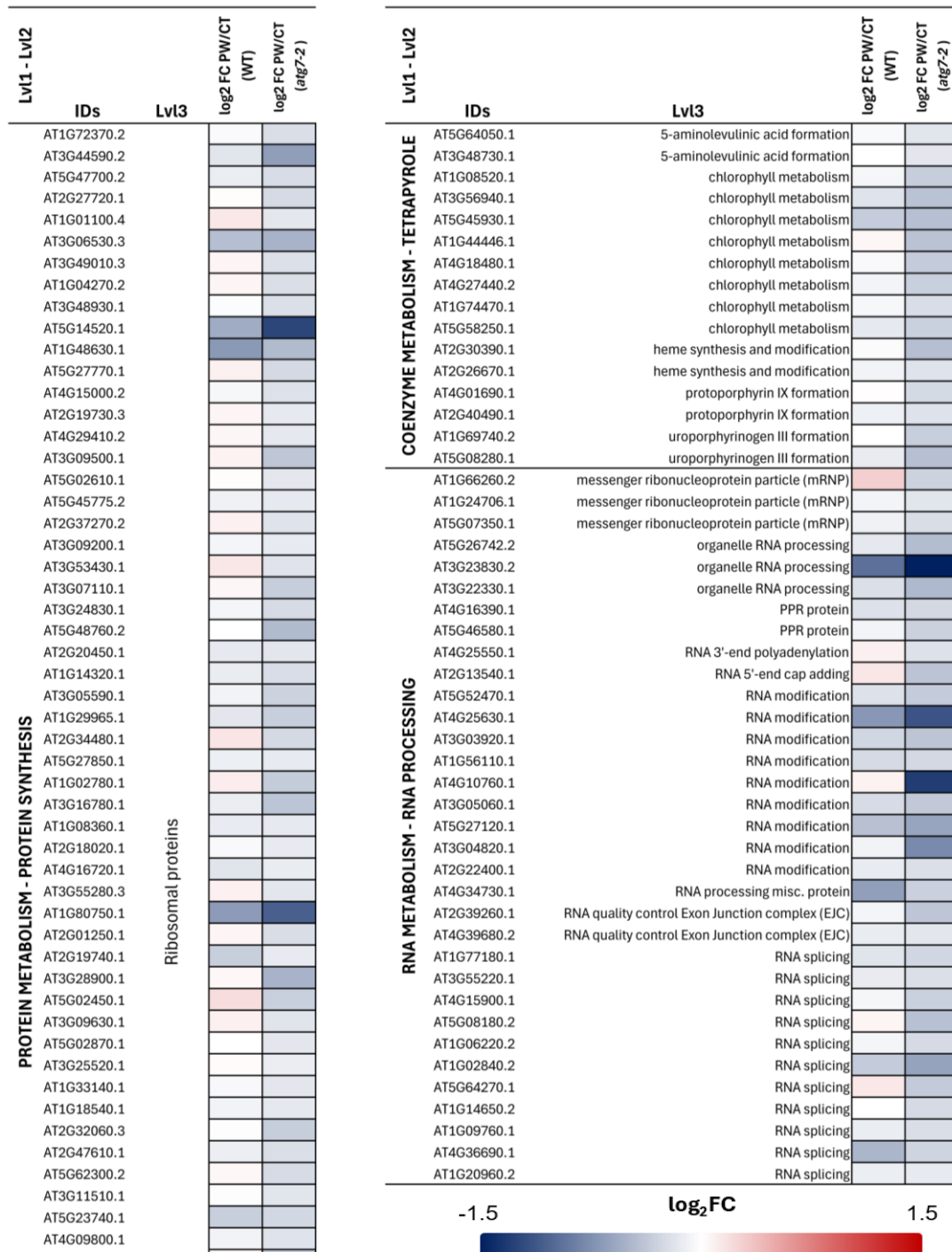


Figure S6. Autophagy deficiency represses protein synthesis, chlorophyll biosynthesis, and RNA metabolism during prolonged warming. Heat maps showing the abundance changes of differentially decreased proteins (DAPs) of rosettes of *Arabidopsis thaliana* wild-type and the autophagy-deficient mutant *atg7-2* following 7 days of prolonged warming (28 °C) relative to control conditions (22 °C). Proteins are grouped according to functional annotation and displayed as log₂ fold change (FC) values. Only proteins showing significant abundance changes in at least one genotype are shown.

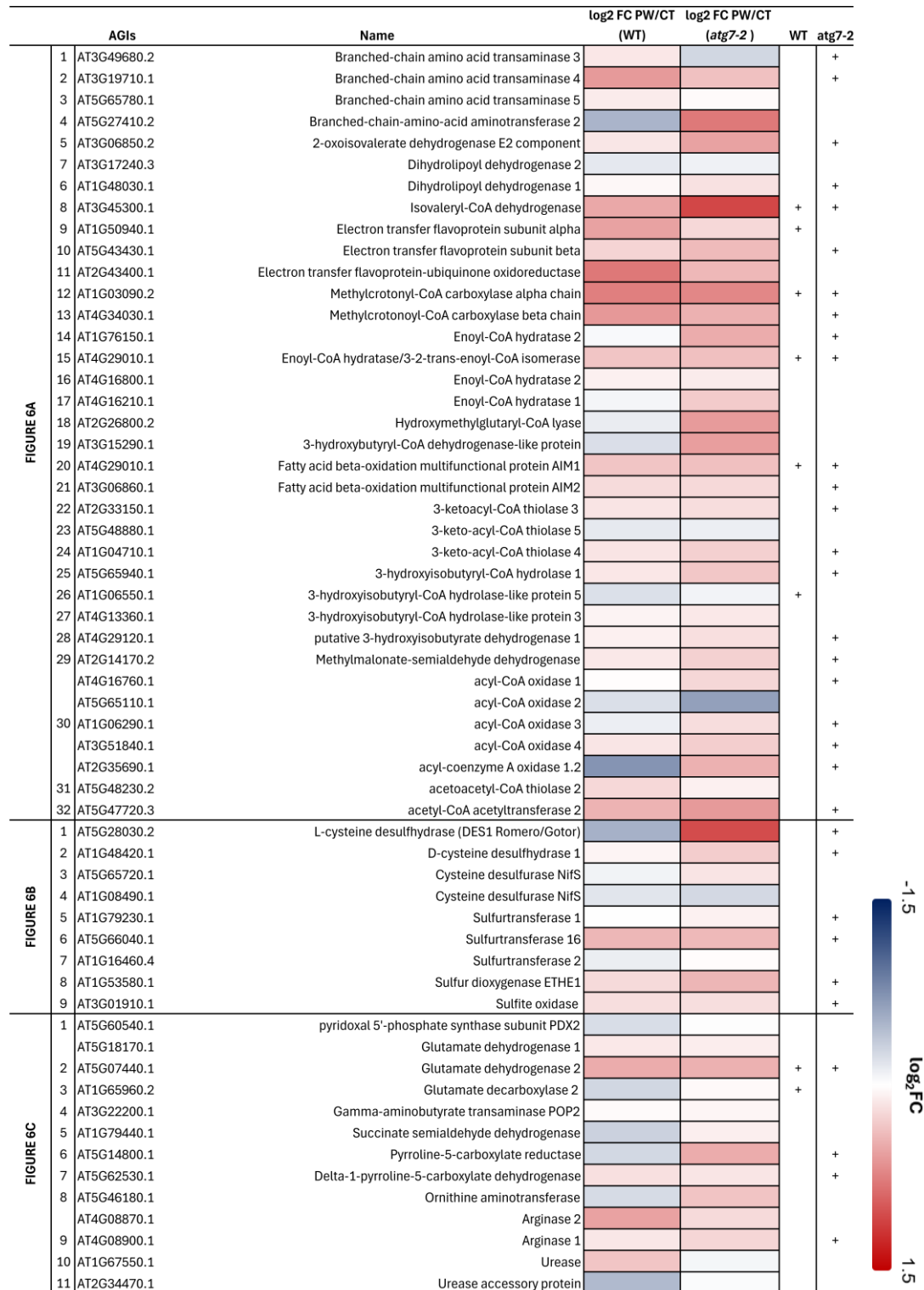


Figure S7. Autophagy deficiency enhanced the abundance of proteins involved in alternative respiratory substrate catabolism during prolonged warming. Heatmap showing abundance changes of proteins associated with (A) branched-chain amino acid (BCAA) degradation, (B) cysteine degradation, and (C) glutamate-, glutamine-, arginine-, proline-, and GABA-associated metabolic pathways in *Arabidopsis thaliana* wild-type (WT) and the autophagy-deficient mutant *atg7-2* following prolonged warming (28°C for 7 d). Protein abundance is presented as log₂ fold change (warming/control), with red and blue indicating increased and decreased abundance, respectively. Plus (+) and minus (-) symbols indicate proteins detected exclusively in warming-treated or control samples, respectively. AGI identifiers and protein names are listed for each protein.

**CHAPTER 3 – Autophagy supports mitochondrial acclimation to phosphate starvation
through mitochondrial proteome remodeling**

Autophagy supports mitochondrial acclimation to phosphate starvation through mitochondrial proteome remodeling

ABSTRACT

Phosphate (Pi) limitation creates an energetic challenge for plants by increasing ATP demand while simultaneously restricting mitochondrial ATP synthesis through reduced Pi availability. Autophagy is increasingly recognized as a component of the Pi-starvation response (PSR), yet its role in mitochondrial acclimation to Pi limitation remains unclear. Here, we combined physiological analysis, mitochondrial respiration assays, and quantitative mitochondrial proteomics to investigate whether autophagy supports mitochondrial function during Pi-starvation in *Arabidopsis thaliana*. Autophagy-deficient mutants (*atg2-1* and *atg5-1*) exhibited impaired growth and accelerated depletion of intracellular Pi pools, consistent with reduced Pi recycling during the early stages of stress. In isolated mitochondria, Pi-starvation stimulated ADP-dependent respiration in wild type (WT), indicating enhanced mitochondrial respiratory capacity during acclimation to Pi limitation. This response was dysregulated in the *atg* mutants, particularly in *atg5-1*. Proteomic analyses revealed extensive mitochondrial remodeling under Pi-starvation, with autophagy mutants displaying broader depletion of proteins involved in oxidative phosphorylation, central carbon metabolism, amino acid catabolism, and mitochondrial gene expression. Together, our results demonstrate that autophagy contributes to mitochondrial acclimation during Pi-starvation by supporting intracellular phosphate maintenance, respiratory adaptation, and mitochondrial proteome remodeling.

Keywords: Autophagy. Pi-starvation; respiratory acclimation; mitochondrial proteome.

INTRODUCTION

Phosphorus (P) is an essential macronutrient required for all living organisms, playing a central role in cellular energy transfer, nucleic acid metabolism, and structural maintenance (Sharkey and Vanderveer 1989; Dissanayaka et al. 2021a). In plant cells, P is primarily acquired as inorganic phosphate (Pi) and is incorporated into organic molecules including nucleotides, phospholipids, and phosphoproteins (Isidra-Arellano et al. 2021; Mehta et al. 2021). However, more than 80% of soil P occurs in unavailable form for plant uptake due to adsorption, precipitation, and conversion to organic forms, making Pi a major limiting factor for plant growth (Schachtman et al. 1998; Ojeda-Rivera et al. 2020; Wei et al. 2021).

To cope with Pi scarcity, plants activate a suite of morphological, physiological, and metabolic adaptations known as phosphate starvation responses (PSR) (Paries and Gutjahr

2023). These responses aim to optimize Pi acquisition, remobilization, and use efficiency. Morphological adaptations include extensive remodeling of root architecture, whereas metabolic adjustments promote the conservation and recycling of intracellular phosphate pools (Chien et al. 2018; Roychowdhury et al. 2023). At the metabolic level, Pi-starvation induces a shift toward Pi-independent pathways, such as inorganic pyrophosphate (PPi)-dependent glycolysis, and enhances alternative respiratory pathways including the alternative oxidase (AOX) pathway (Palma et al. 2000; Sieger et al. 2005). Pi deficiency also induces amino acid accumulation, which reflects both reduced protein synthesis and enhanced protein degradation (Chutia et al. 2021; Roychowdhury et al. 2023). Pi transport is upregulated, and internal phosphate is remobilized through senescence-associated mechanisms, often accompanied by anthocyanin accumulation (Stigter and Plaxton 2015; Liu et al. 2016; He et al. 2021).

A fundamental challenge of PSR is high energy demand. The induction of high-affinity phosphate transporters, plasma-membrane H⁺-ATPase, membrane remodeling processes, and intracellular nutrient recycling mechanisms requires continuous ATP investment (Versaw and Harrison 2002; Misson et al. 2004; Zhang et al. 2025). Consequently, successful acclimation to Pi limitation depends on the maintenance of mitochondrial energy production. In plants, Pi is imported into mitochondria by members of the PHT3 family and serves as an essential substrate for ATP synthesis through oxidative phosphorylation (Wang et al. 2017; Fabiańska et al. 2019). Thus, mitochondrial ATP production is inherently dependent on Pi availability. However, mitochondrial synthesis is intrinsically dependent on phosphate availability (Rychter et al. 1992). In yeast, depletion of cytosolic phosphate rapidly limits mitochondrial respiration by restricting Pi availability for oxidative phosphorylation, revealing phosphate itself as a critical regulator of mitochondrial energy metabolism (Vengayil et al. 2024). This finding indicates that, regardless of the upstream cause (e.g., high glucose or Pi-starvation), the mitochondria is ultimately constrained by its local Pi concentration (Vengayil et al. 2024). Thus, a critical energy dilemma arises: Pi-starvation increases the demand for ATP while simultaneously limiting the substrate (Pi) required to produce it. It is imperative to ascertain the mechanism by which plants successfully navigate this energetic bottleneck to sustain the PSR.

Among the mechanisms that enable plants to adapt to nutrient stress, autophagy has emerged as a critical regulator of cellular homeostasis. Autophagy is a conserved eukaryotic degradation pathway that delivers cytoplasmic constituents to the vacuole for recycling, thereby maintaining metabolic balance and cellular quality control under both optimal and stress conditions (Signorelli et al. 2019a; Sharma et al. 2022a). Under nutrient limitation, autophagy mobilizes intracellular reserves and recovers essential metabolites, allowing cells to sustain

metabolism when external nutrient supply is restricted (Masclaux-Daubresse 2016; Chen et al. 2019). Beyond its role in bulk nutrient recycling, autophagy also contributes to organelle quality control through the selective removal of dysfunctional cellular components, including mitochondria and portions of the endoplasmic reticulum (Izumi et al. 2017; Luong et al. 2022).

Accumulating evidence indicates that autophagy is intimately integrated into the PSR through distinct mechanisms. Early responses to phosphate limitation involve selective autophagy pathways linked to endoplasmic reticulum (ER) homeostasis. Naumann et al. (2019a) demonstrated that Pi limitation activates ER-stress dependent autophagy in root meristems via the PDR2-LPR1 module. Subsequently, Yoshitake et al. (2022) showed that Fe-dependent ER stress triggers a specific ER-phagy pathway that promotes Pi recycling from endomembrane systems and contributes to the regulation of downstream PSR processes, including membrane lipid remodeling. Furthermore, Li et al. (2022) revealed that *atg* mutants accumulated mitochondrial and chloroplast proteins while displaying metabolic imbalance, suggesting that autophagy-mediated organelle turnover contributes to cellular adaptation under Pi limitation. These observations suggest that autophagy may simultaneously support Pi recycling and organelle quality control during Pi-starvation, although the functional consequences for mitochondrial performance remain unclear.

Despite these advances, a major knowledge gap remains regarding the functional relationship between autophagy and mitochondrial performance during Pi-starvation. Although impaired turnover of mitochondrial proteins has been observed in *atg* mutants under Pi limitation (Li et al. 2022a), it remains unknown whether these proteomic alterations translate into defects in mitochondrial respiration and oxidative phosphorylation. In particular, it is unclear whether autophagy contributes to preserving respiratory capacity through adaptive remodeling of the mitochondrial proteome, maintenance of respiratory complex composition, or optimization of Pi utilization within mitochondria. Given the central role of mitochondria in sustaining the energetic requirements of PSR, understanding how autophagy supports mitochondrial function represents a critical step toward resolving the Pi-energy paradox.

Here, we investigated the role of autophagy in maintaining mitochondrial function during Pi-starvation using isolated mitochondria from *Arabidopsis thaliana* suspension cell cultures. To directly assess mitochondrial performance independently of whole-cell metabolic complexity, we isolated mitochondrial and combined respiratory analyses, BN-PAGE, and quantitative mitochondrial proteomics in WT and autophagy-deficient mutants (*atg2-1* and *atg5-1*) grown under Pi-sufficient and Pi-deficient conditions. Our results indicate that autophagy contributes to mitochondrial acclimation during Pi-starvation by supporting

maintenance of intracellular phosphate pools, respiratory adaptation, and remodeling of the mitochondrial proteome.

RESULTS

Autophagy deficiency intensifies phosphate depletion and biomass reduction over time

To investigate the physiological effects of Pi limitation and establish a baseline for mitochondrial analysis prior mitochondrial isolation and investigation (Fig. 1), we first assessed biomass accumulation (fresh and dry weight) and cellular Pi content in intact cells of wild type (WT) and autophagy-deficient mutants (*atg2-1* and *atg5-1*; Fig. S1) over a 7-day time course (Fig. S2 and Fig. S3).

Under control, WT showed a steady increase in fresh and dry weight over time (Fig. S2A, C). While *atg2-1* displayed only a mild reduction in biomass relative to WT, *atg5-1* showed a consistent and pronounced growth penalty, indicating that autophagy deficiency, particularly in *atg5-1*, impairs biomass accumulation even in the absence of nutrient stress. Dry weight differences were already detectable in *atg5-1* from mid-timepoints onward, whereas *atg2-1* largely maintained values comparable to WT (Fig. S2C). Under Pi-starvation, the differences between WT and *atg5-1* became more pronounced (Fig. SB, D). By Day 3, the selected time point for mitochondrial isolation, both *atg2-1* and *atg5-1* showed significantly reduced fresh weight compared to WT, with reductions of approximately 18.5% and 22.3%, respectively (Fig. 2A). However, DW reduction at this stage was most pronounced in *atg5-1*, whereas *atg2-1* showed values comparable to WT (Fig. 2B). Over the full starvation period, *atg5-1* continued to exhibit the strongest cumulative biomass loss, indicating heightened sensitivity to prolonged Pi deprivation (Fig. S2B, D). Although both autophagy mutants display growth sensitivity under Pi limitation, *atg5-1* showed the most severe impairment in biomass accumulation.

Free inorganic phosphate levels revealed strong genotype-specific differences in Pi homeostasis over time (Fig. S3A, B). Under control conditions, both *atg2-1* and *atg5-1* consistently showed lower free Pi compared to WT from the early time point onward, indicating constitutive disruption of intracellular phosphate balance associated with autophagy deficiency (Fig. S3A). At day 3, free Pi levels were reduced by 48% in *atg2-1* and 44% in *atg5-1* relative to WT (Fig. 2C). Under Pi-starvation, free Pi declined rapidly across all genotypes, and differences between WT and *atg* mutants became less pronounced over time, suggesting that prolonged external Pi depletion overrides early genotype-specific effects (Fig. S3B). Total P revealed a different temporal pattern (Fig. S3C, D). Under control, no significant differences in

total P were detected among genotypes (Fig. S3C). Under Pi-starvation, however, WT maintained higher total P during the early phase, reaching a peak at day 3 before declining sharply (Fig. S3D). In contrast, *atg2-1* and *atg5-1* showed comparatively lower total P levels throughout the time course. At day 3, both mutants exhibited significantly lower total P than WT (24% in *atg2-1* and 20% in *atg5-1*) (Fig. 2D). From day 4 onward, total P levels were lower in WT compared to *atg* mutants. The estimated organic Pi pool followed a similar pattern to total P (Fig. S3E, F). Under control, the values were comparable among genotypes (Fig. S3E). Under Pi-starvation, however, *atg2-1* and *atg5-1* exhibited significantly lower organic Pi at day 3 (-22% and -17% vs WT, respectively) (Fig. 2E and Fig. S3F).

Pi-starvation enhances mitochondrial respiratory capacity in WT and *atg2-1*, whereas the response is attenuated in *atg5-1*

To evaluate the impact of Pi limitation on mitochondrial function, we established an experimental setup as outlined in Fig. 1. Mitochondrial respiration was analyzed on day 3 of Pi treatment, corresponding to the earliest point at which significant genotype-dependent reductions in biomass and Pi content were observed. This time point was selected to capture early mitochondrial responses to Pi limitation, before the development of severe physiological impairment.

To further investigate the impact of Pi limitation on mitochondrial respiration, total electron transport chain (ETC)-linked respiration assay was measured in freshly isolated mitochondria supplied with succinate and NADH, followed by sequential addition of ADP, KCN, and SHAM to assess respiratory capacity and the relative contributions of the cytochrome and alternative oxidase pathways, respectively (Fig. 3). Pi-starvation increased mitochondrial respiratory activity in WT and *atg2-1*, whereas *atg5-1* exhibited only a weak response (Fig. 3A-B). When expressed relative to their respective controls, WT and *atg2-1* displayed respiratory induction under Pi-starvation, whereas the response of *atg5-1* remained comparatively limited.

Pi-starvation increased ADP-stimulated respiration in all genotypes, although the magnitude of the response differed among them (Fig. 3C). In WT, ADP-stimulated respiration increased under Pi-starvation relative to control conditions (1.72 vs. 0.73 nmol O₂ s⁻¹ ug⁻¹ protein). Interestingly, *atg2-1* displayed an even more pronounced induction, largely because respiration under control conditions was extremely low (2.46 vs. 0.07 nmol O₂ s⁻¹ ug⁻¹ protein). In contrast, *atg5-1* exhibited only a modest increase under Pi-starvation compared to control (0.35 vs 0.20 nmol O₂ s⁻¹ ug⁻¹ protein).

Importantly, the relative contributions of the cytochrome c (Fig. 3D), and alternative oxidase (AOX) (Fig. E) pathways, estimated from the sequential inhibitor responses following KCN and SHAM addition, remained largely unchanged across genotypes and treatments.

Abundance of proteins and complexes I and V activities are not affected following Pi limitation

To assess the role of respiratory complex reconfiguration during Pi limitation, the respiratory complexes were analyzed performing Blue-Native (BN)-PAGE from freshly isolated mitochondria. After separating the complexes on the native gel, the abundance of proteins and the activities of complexes I and V were determined through Coomassie and in-gel enzyme activity staining (Fig. 4). Neither protein abundance nor the activities of complexes I and V were affected by Pi-starvation in any genotype. The lack of substantial changes in the abundance or activity of respiratory complexes I and V, despite observed alterations in mitochondrial respiration rates, suggests that the changes in mitochondrial respiration may result from shifts in substrate utilization, post-translational modifications of the complexes, or alterations in alternative electron transport pathways, rather than direct modifications to the respiratory complexes themselves.

Importantly, BN-PAGE in-gel activity assays primarily reflect the catalytic performance of fully assembled respiratory complexes under specific assay conditions. In contrast, quantitative proteomics captures changes in the abundance of individual proteins, which may not necessarily translate into detectable differences in in-gel activity. Thus, the absence of changes in complex activity does not preclude functionally meaningful remodeling of mitochondrial respiratory capacity.

Autophagy deficiency enhances alterations in the mitochondrial proteome in response to Pi limitation

To investigate mitochondrial proteome alterations in WT and autophagy-deficient mutants (*atg2-1* and *atg5-1*) under control and Pi-starvation, we performed Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS) analysis on three independent mitochondrial isolations per genotype and treatment. To assess the purity of our isolated mitochondria we used a subcellular location database (SUBA5) (Hooper et al. 2014, 2017). Out of 4,374 identified proteins, 1,300 (29.7%) were annotated as mitochondrial or dual targeted, while the remainder were primarily cytosolic or plastidial. Quantitative analysis of protein abundance based on normalized iBAQ values indicated that 77-82% of the total protein

abundance originated from mitochondrial proteins (Fig. S4), consistent with reported purity levels for *Arabidopsis* mitochondrial proteomes (Fuchs et al. 2020; Rugen et al. 2024).

Principal component analysis (PCA) revealed strong genotype-dependent clustering of mitochondrial proteome profiles, with WT separating from both *atg2-1* and *atg5-1* under both conditions (Fig. 5A). In contrast, control and Pi-starved samples within the same genotype showed substantial overlap, indicating that genotype explained more proteomic variance than Pi status alone. Importantly, divergence between WT and *atg* mutants became stronger under Pi-starvation, suggesting a role for autophagy in mitochondrial proteome remodeling during nutrient stress.

Consistently, significantly altered protein sets were highly correlated between *atg2-1* and *atg5-1* under both control ($r = 0.9$ and $R^2 = 0.81$) and Pi-starvation ($r = 0.95$ and $R^2 = 0.9$) (Fig. 5B, C). Differential abundance analysis using volcano plots ($p\text{-value} < 0.05$; $\text{FDR} < 0.05$), showed limited proteomic disruption under control conditions (*atg2-1*: 0.31% up, 0.46% down; *atg5-1*: 1.15% up, 2.62% down; Fig. 6A, C). In contrast, Pi-starvation triggered a substantially larger proteomic response, particularly in *atg5-1* (*atg2-1*: 6% up and 31% down; *atg5-1*: 10% up and 35% down in *atg5-1*; Fig. 6B, D), indicating amplified remodeling under stress.

Venn diagram analysis identified 50 commonly increased and 341 commonly decreased proteins in both mutants under Pi-starvation (Fig. 6E, F). Among genotype-specific responses, *atg5-1* showed the largest number of uniquely altered proteins under Pi-starvation (66 increased and 88 decreased proteins). Under control conditions, both mutants showed reduced abundance of proteins associated with mitochondrial respiration and amino acid metabolism, including proline dehydrogenase 1 (ProDH1), branched-chain amino acid aminotransferase 2 (BCAT2), succinate dehydrogenase subunit 7 (SDH7), SDH assembly factor 2 (SDHAF2), NAD(P)H dehydrogenase B4 (NDB4), alternative oxidase 1A (AOX1A), and ATP-Mg/Pi carrier protein 2 (APC2) (Fig. 8A). Reduced abundance of HSP23.5 was also observed. In contrast, photorespiratory enzymes such as alanine-glyoxylate aminotransferase 2 (AGT2) and glutamate-glyoxylate aminotransferase 1 (GGAT1), together with UDP-3-O-acetyl-N-acetylglucosamine deacetylase 1 (LPXC1) and pentatricopeptide repeat (PPR) proteins, accumulated in both mutants.

By contrast, Pi limitation induced a broad proteomic reprogramming, with alterations in proteins involved in cellular respiration, amino acid, protein, and RNA metabolism, as well as transport (Fig. 6). Functional enrichment analysis reveals repression of energy-related pathways in *atg* mutants, most severe in *atg5-1* (Fig. 7E). In this genotype, proteins associated with oxidative phosphorylation (complexes II and IV), the TCA cycle (pyruvate dehydrogenase

(PDH) complex), amino acid degradation, RNA metabolism (PPR proteins), and MC-type solute transporter were reduced (Fig. 8B; 9; 10; 11). The *atg2-1* mutant displayed a milder and more selective reduction, consistent with its attenuated respiratory phenotype.

Under Pi-starvation, both mutants exhibited marked reductions in proteins associated with amino acid degradation and alternative respiratory substrate metabolism, including 2-oxoisovalerate dehydrogenase subunits E1 α 2 and E1 β 1, and alanine-glyoxylate aminotransferase 3 (AGT3), LPD1, and LPD2. At the same time, proteins involved in stress mitigation, organelle maintenance, and mitochondrial gene expression accumulated, including glutamate decarboxylase 1 (GAD1), serine hydroxymethyltransferase 3 (SHMT3), HSP70 family proteins, mitochondrial ribosomal protein L17 (RPL17), and several PPR proteins.

Among genotype-specific responses, *atg5-1* showed coordinated reductions in multiple electron transport chain components, including subunits of complexes I, II, and IV, together with proteins involved in cytochrome c maturation. In contrast, *atg2-1* retained or increased the abundance of proteins associated with mitochondrial maintenance and stress responses, including TOM20-1, SDH1-2, FeSOD1, and the small heat shock protein sHSP23.6.

DISCUSSION

Autophagy is a central recycling pathway that supports quality control and metabolic adaptation, particularly under nutrient limitation (Li et al. 2015; Barros et al. 2017a, 2021; Chiu et al. 2023b). Pi-starvation poses an energetic dilemma: ATP demand increases to sustain uptake and remodeling programs, while Pi limitation restricts oxidative phosphorylation by limiting availability of a key substrate for ATP synthesis (Dissanayaka et al. 2021b; Vengayil et al. 2024). Here, we place mitochondria at the center of this Pi-energy paradox and demonstrate that autophagy is an important determinant of mitochondrial acclimation during Pi limitation. Autophagy-deficient mutants exhibited disrupted Pi homeostasis already under Pi-sufficient conditions and failed to sustain Pi pools during Pi-starvation, accompanied by altered respiratory response and remodeling of the mitochondrial proteome. Collectively, our results support a model in which autophagy helps sustain mitochondrial performance under Pi limitation by buffering intracellular Pi availability and enabling adaptive proteome and metabolic reprogramming required for respiratory flexibility.

Autophagy as a gatekeeper of basal mitochondrial metabolic adaptability and phosphate homeostasis

The physiological and proteomic phenotypes of the *atg* mutants under Pi sufficient conditions reveal a state of metabolic vulnerability. This aligns with evidence across eukaryotes that autophagy preserves mitochondrial function by maintaining metabolic homeostasis and preventing the accumulation of damaged organelles and proteins (Nakamura et al. 2021; He 2022; Kacprzak and Van Aken 2022). Loss of core ATG genes can lead to accumulation of dysfunctional mitochondria and bioenergetic impairment even under nutrient-replete conditions (Graef and Nunnari 2011; Hill et al. 2012). In plants, accelerated senescence under sugar deprivation provided genetic support for autophagy-dependent turnover of mitochondrial proteins and mitochondrial-derived vesicles (Li et al. 2014).

In our system, both *atg2-1* and *atg5-1* exhibited lower free Pi under control conditions, indicating constitutive disruption of intracellular Pi balance in the absence of canonical autophagy. In parallel, mitochondrial physiology was perturbed, most clearly reflected by altered baseline respiratory capacity (including an exceptionally low ADP-stimulated rate in *atg2-1* under control conditions), suggesting that autophagy supports robust respiratory control even before nutrient limitation. One possibility is that impaired autophagy-dependent turnover limits mitochondrial renewal and/or constrains the availability of substrates and cofactors required for efficient oxidative phosphorylation. Consistently, mitophagy has been proposed to operate proactively during developmental or metabolic transitions to maintain organelle quality (Ma et al. 2021). Although we did not quantify mitophagy flux here, the overall phenotype supports the idea that impaired basal recycling may pre-condition cells to respond less efficiently to subsequent nutrient stress.

Proteomic profiles under Pi sufficiency further support the idea of constrained metabolic flexibility in the *atg* mutants. The depletion of proteins contributing to alternative substrate entry into mitochondrial metabolism, including ProDH1 and BCAT2, suggests a reduced potential to sustain TCA-cycle inputs from non-carbohydrate substrates. Whether this translates into lower flux through proline and branched-chain amino acid catabolism remains to be experimentally determined. Given that ProDH-mediated proline catabolism can enhance mitochondrial ROS production and suppress respiratory performance (Hancock et al. 2016; Nagano et al. 2017; Renzetti et al. 2025), its depletion may reflect a compensatory shift that limits oxidative burden under impaired mitochondrial quality control. Notably, APC2, a mitochondrial ATP-Mg/Pi carrier implicated in regulating adenine nucleotide exchange and phosphate-linked energy balance, was also reduced. Given that APC transport activity can

contribute to maintaining mitochondrial ATP availability and coordinating Pi flux with respiratory demand, its depletion could further constrain oxidative phosphorylation efficiency and intracellular Pi buffering. While these data do not establish causality, they are consistent with reduced free Pi in the *atg* mutants and with models proposing that constitutive recycling contributes to maintaining accessible intracellular Pi pools (Yoshitake and Yoshimoto 2023). Related concepts have emerged from yeast, where perturbation of vacuolar/lysosomal function alters cytosolic Pi availability (Shirahama et al. 1996; Bru et al. 2025), reinforcing the idea that degradative compartments contribute to Pi homeostasis.

Furthermore, accumulation of PPR proteins and other RNA-associated factors is consistent with altered mitochondrial gene-expression capacity and compensatory retrograde signaling. In Arabidopsis, similar patterns occur in mitochondrial mutants, where accumulation of PPR proteins has been linked to attempts to stabilize transcripts and support respiratory complex assembly (Hsieh et al. 2015; Wang et al. 2022). Increased abundance of photorespiratory aminotransferases (e.g., GGAT1, AGT2) further indicates compensatory adjustments in carbon-nitrogen coupling, potentially supporting mitochondrial inputs when canonical respiratory metabolism is constrained. Accumulation of LPXC1 also suggests membrane remodeling and/or stress-responsive lipid signaling associated with organellar dysfunction (Li et al. 2011).

Our results suggest that autophagy deficiency drives a constitutive compensatory state even under nutrient-sufficient conditions. Because dysfunctional components cannot be efficiently removed, compensation may become metabolically inefficient, increasing energetic costs associated with maintenance of cellular homeostasis. This pre-existing energetic fragility and elevated metabolic overhead likely reduce reserve capacity and resilience to subsequent Pi limitation, reinforcing autophagy as a gatekeeper of basal mitochondrial adaptability and Pi homeostasis.

Autophagy supports mitochondrial energy demands during Pi limitation

The PSR presents a metabolic paradox: Pi limitation increases ATP demand to sustain high-affinity Pi uptake and others energy-intensive processes, while simultaneously limiting the substrate (Pi) required for oxidative phosphorylation. Our findings indicate that autophagy contributes to attenuating this constraint, and that loss of autophagy compromises mitochondrial acclimation during Pi limitation.

In WT, Pi-starvation induced a pronounced increase in ADP-stimulated mitochondrial respiration, indicating an enhanced respiratory capacity during Pi-starvation. This is consistent

with evidence that mitochondrial function is reconfigured under Pi limitation to maintain electron flow when phosphorylation is constrained, including engagement of alternative oxidase (AOX) and non-phosphorylating NAD(P)H dehydrogenases (Vijayraghavan and Soole 2010; Plaxton and Tran 2011; Dissanayaka et al. 2021b). In contrast, this respiratory induction was severely attenuated in *atg5-1* and dysregulated in *atg2-1*, suggesting that autophagy contributes to respiratory plasticity during nutrient stress. Notably, the relative contributions of the cytochrome c and alternative oxidase pathways, remained largely unchanged across genotypes and treatments, indicating that the observed differences in ADP-stimulated respiration are unlikely to be explained by a switch between cytochrome and AOX capacity alone, but instead could reflect changes in overall respiratory capacity, coupling efficiency, or metabolic regulation under Pi-starvation conditions, potentially linked to autophagy-dependent metabolic remodeling.

A central outcome of our proteomic analysis is the coordinated repression of mitochondrial energy metabolism in *atg* mutants under Pi-starvation. Both *atg2-1* and *atg5-1* exhibited reduced abundance of proteins associated with oxidative phosphorylation, pyruvate dehydrogenase activity, and central carbon metabolism, consistent with a systemic restriction of mitochondrial metabolic capacity. This effect was pronounced in *atg5-1*, where additional depletion of complex IV subunits and proteins involved in cytochrome c maturation suggests more severe impairment of respiratory-chain maintenance. A shared feature of both mutants was the reduced abundance of NAD⁺- and NADP⁺-dependent isocitrate dehydrogenase (IDH) isoforms, potentially constraining NADH supply to the ETC and limiting NADPH regeneration required for mitochondrial redox buffering (Schertl and Braun 2014). Given the central role of NAD(H)/NADP(H) pools in coordinating respiratory flux and redox balance, reduced IDH provides a plausible mechanistic link to impaired respiratory flexibility (Bakalova et al. 2022; Yang et al. 2025). The resulting metabolic bottleneck mirrors emerging evidence from other systems in which Pi limitation impairs mitochondrial ATP synthesis (Vengayil et al. 2024).

The rapid depletion of both free and organic Pi in intact cells of *atg2-1* and *atg5-1* is consistent with a role for autophagy in mobilizing intracellular Pi reserves during Pi-starvation. Our data are consistent with a model in which autophagy contributes to PSR through two complementary metabolic functions: (i) mobilization of intracellular phosphate reserves; and (ii) provision of carbon skeletons and amino acids that support respiratory metabolism.

Consistent with this model, our results indicate broader disruption of metabolic connectivity in *atg* mutants. Downregulation of pyruvate dehydrogenase subunits, aminotransferases, mitochondrial carriers, and enzymes supporting BCAA and proline

oxidation suggests restricted alternative respiratory substrate supply. This likely constrains compensatory fluxes, including anaplerotic TCA inputs and oxidative pentose phosphate pathways (PPP) activity, which have been shown to support mitochondrial redox balance and function under stress (Moon et al. 2020). As a result, central metabolism may become constrained into a low-flux state insufficient to meet energetic and biosynthetic demands imposed by Pi limitation.

Beyond metabolic restriction, our results indicate that autophagy supports the mitochondrial proteome integrity and renewal. Reduced abundance of Fe-S cluster assembly proteins and respiratory complex assembly factors is consistent with compromised ETC maintenance. The enrichment of TOM20-1 may reflect altered mitochondrial quality-control processes and potentially reduced mitochondrial turnover (Nakamura et al. 2021; Kacprzak and Van Aken 2022; Duckney et al. 2025).

Notably, PPR proteins were depleted under Pi-starvation, suggesting a compromised mitochondrial RNA metabolism and transcript processing capacity. Given that PPR proteins are essential for RNA editing, transcript stabilization, and coordinated expression of respiratory chain subunits (Rovira and Smith 2019), their depletion would be expected to further constrain OXPHOS biogenesis and reinforce the observed decline in respiratory performance.

An alternative interpretation is that the widespread depletion of mitochondrial proteins observed in the *atg* mutants reflects a failure to maintain mitochondrial proteome homeostasis during Pi-starvation. Reduced abundance of proteins involved in oxidative phosphorylation, central carbon metabolism, and mitochondrial gene expression suggests that autophagy contributes not only to nutrient recycling but also to preservation of mitochondrial protein composition under stress. Whether these changes arise through altered retrograde signaling, impaired protein turnover, defects in organelle renewal, or a combination of these mechanisms remains unresolved and will require further investigation.

The coordinated depletion of OXPHOS components, TCA-cycle enzymes, and other proteins associated with mitochondrial energy metabolism suggests that autophagy deficiency affects not only mitochondrial turnover but also the maintenance of the mitochondrial proteome during Pi-starvation. Similar feedback effects have been described in systems with severe mitochondrial dysfunction, where impairment of mitochondrial function results in reduced abundance and activity of nuclear-encoded mitochondrial enzymes involved in respiration and central carbon metabolism (Mueller et al. 2012). In this context, the reductions in IDH, PDH-associated proteins, and respiratory-chain components observed in *atg* mutants may reflect secondary consequences of impaired mitochondrial quality control and altered organelle

homeostasis rather than primary defects in the expression of these pathways. Consistent with this interpretation, the accumulation of stress-associated proteins together with the depletion of energy-metabolism proteins indicates extensive mitochondrial remodeling in response to autophagy deficiency during Pi limitation.

Finally, because mitochondria were analyzed after three days of Pi-starvation, our results may capture an early stage of mitochondrial acclimation to Pi stress. Together with previous evidence that autophagy participates in early phases of the PSR (Naumann et al. 2019a; Yoshitake et al. 2022; Lin et al. 2023), our findings suggest that autophagy contributes to mitochondrial adaptation from the onset of Pi limitation, before the development of severe physiological impairment. This highlights autophagy as an integral component of the early coordination between phosphate availability and mitochondrial metabolism during nutrient stress.

CONCLUSIONS

We provide evidence that autophagy contributes to mitochondrial acclimation during Pi-starvation by supporting respiratory flexibility, intracellular phosphate homeostasis, and maintenance of mitochondrial proteome integrity. Autophagy-deficient mutants exhibited accelerated Pi depletion, impaired respiratory responses, and extensive mitochondrial proteome reprogramming, including depletion of proteins associated with oxidative phosphorylation, mitochondrial gene expression, and central carbon metabolism. Together, these findings indicate that autophagy is an important component of the mitochondrial response to Pi limitation, linking intracellular Pi recycling with respiratory acclimation and metabolic flexibility during nutrient stress.

METHODS

Plant material and nutrient solution

Arabidopsis (*Arabidopsis thaliana*) accession Columbia (Col-0) and loss-of-function mutants of the autophagy pathway, *atg2-1* (SALK_076727) and *atg5-1* (SAIL_129_B07) were previously described (Inoue et al. 2006; Yoshimoto et al. 2009a; Barros et al. 2017a).

The nutrient solution contained 5 mM KNO₃, 2.5 mM KH₂PO₄, 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 40 µM Fe-EDTA, 70 µM H₃BO₃, 14 µM MnCl₂, 0.5 µM CuSO₄, 1 µM ZnSO₄, 0.2 µM NaMoO₄, 10 µM NaCl, and 0.01 µM CoCl₂. In addition, 4.7 mM MES and 0.5% (w/v) Suc were included. This medium is referred to as +Pi or control medium. For -Pi or Pi-starvation medium, KH₂PO₄ was omitted from the above nutrient solution (Naumann et al. 2019b).

Establishment of *Arabidopsis thaliana* cell suspensions and induction of phosphate starvation stress

The establishment of *A. thaliana* cell culture was conducted as described by May and Leaver (1993). Briefly, *A. thaliana* seeds were surface sterilized and imbibed for 2 days at 4 °C in the dark on 0.8% [w/v] agar plates containing half-strength MS medium Murashige and Skoog (1962) (MS) medium at pH 5.7. Seeds were subsequently germinated under long-day photoperiod conditions (16 h light/8 h dark) at an irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 10 days, seedlings were cut into small pieces (3 cm²) and laid out on plates with B5 medium (0.316% [w/v] B5, 0.75 g [w/v] agar, 3% sucrose [w/v], 0.0001% [w/v] 2,4-dichlorophenoxyacetic acid and 0.00001% kinetin [w/v], pH 5.7/KOH) for callus induction. The B5 plates were stored in darkness for 3 weeks. Afterward, callus was transferred into liquid B5 medium (B5 medium without agarose) for establishing a cell suspension culture. Cells were cultivated in 500 mL flasks containing 100 mL of the medium at 25 °C under a rotary shaker (90 rpm) in the dark. To maintain the cells in optimal condition, the medium was renovated every 7 days. Phosphate starvation was induced using the nutrient solution described in Section 2.1.

Measurement of total, free, and esterified phosphate

Total and free Pi content were determined as previously described by Knowles and Plaxton (2013) with minor modifications. Dry material was homogenized with extraction buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, and 1mM b-mercaptoethanol, pH 8.0) at a ratio of 5 mg of sample to 250 mL of extraction buffer. A total of 100 mL of homogenized sample was mixed with 900 mL of 1% glacial acetic acid and incubated at 42°C for 30 min. Then, 100 mL of the mixture was transferred to a glass tube for total P assay. The rest of the solution was centrifuged at 13,000g for 5 min, and 300 mL of the supernatant aliquot was used in the Pi assay. The reaction contained 700 mL of assay solution (0.35% NH₄MoO₄, 0.86 N H₂SO₄, and 1.4% ascorbic acid) and 300 mL of sample was incubated at 42°C for 30 min. Pi content was measured at 820 nm. For the total P assay, 30 mL of 10% Mg(NO₃)₂ in 95% ethanol was added to 100 mL of the aliquot. The samples were then dried and flamed to ash. After cooling, 300 mL of 0.5 N HCl was added to dissolve the samples at 65°C for 30 min. Total P content was measured as described for Pi by the addition of 700 mL of assay solution. Esterified phosphate was estimated as the difference between Total Pi and Free Pi. Phosphate concentrations were determined using a calibration curve based on known concentrations of inorganic phosphate standards and normalized by dry weight of biomass.

Isolation of mitochondria by Percoll gradient ultracentrifugation

Preparations of mitochondria were performed according to Schertl et al., (2014). The starting material was ~40g cells per condition. In brief, *Arabidopsis* cells were disrupted in a Waring blender in an extraction buffer containing 450 mM sucrose, 15 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 1.5 mM ethylene glycol tetra acetic acid (EGTA), 0.6 [w/v] polyvinylpyrrolidone (PVP-40), 0.2% BSA, 10 mM sodium ascorbate, 10mM cysteine, and 0.2% phenylmethylsulphonyl fluoride (PMSF), pH 7.4 (KOH). To remove cell debris the suspensions were centrifuged twice for 5 min at 2,700 g and once for 10 min at 8,300 g. Afterward, the supernatants containing the mitochondrial fractions were transferred to a new tube for high-speed centrifugation at 17.000g for 10 min. The mitochondrial pellets were resuspended in 3 mL of washing buffer containing 300 mM sucrose, 10 mM MOPS, 1 mM EGTA, 0.2 mM PMSF, pH 7.2 (KOH). A discontinuous gradient made of three layers was prepared with Percoll concentrations of 40% (v/v), 23% (v/v), and 18% (v/v) and the mitochondrial suspensions were carefully placed on the gradients and centrifuged for 90 min at 70,000 g using an ultracentrifuge. After centrifugation, the bands between 40% and 23% layer representing the mitochondrial fractions were transferred into new tubes using a Pasteur pipette. The mitochondrial fractions were washed three times in resuspension buffer containing 400 mM mannitol, 10 mM Tricine, 1 mM EGTA, 0.2 mM PMSF, pH 7.2 (KOH) by centrifugation at 14.300 g for 10 min. All steps were carried out at 4 °C. Isolated mitochondria were divided into aliquots and stored at -80 °C.

Total ETC-Linked Respiration

Dark respiration of the cell cultures was evaluated in an oxygen electrode following a protocol detailed previously Jacoby et al. (2015). Measurements using 60 mg of protein from fresh mitochondria took place in a 2 ml reaction vessel at 25°C in the dark in respiration medium containing 300 mM sucrose, 10 mM NaCl, 5 mM KH₂ PO₄, 2 mM MgSO₄, 0.1 % (w/v) bovine serum albumin (BSA), 10 mM TES, pH 7.2. The combination of succinate (5 mM) and NADH (1 mM) was added to directly supply reductants to the electron transport chain (ETC) and external NAD(P) dehydrogenases, bypassing soluble enzymes of the TCA cycle. ATP (500 µM) was included to activate succinate dehydrogenase, while ADP (300 µM) was added to prevent any limitation imposed by the proton motive force across the inner membrane. Potassium cyanide (1 mM) was used to inhibit cytochrome c oxidase, and salicylhydroxamic acid (2 mM) was applied to inhibit the alternative oxidase. Oxygen depletion was maintained below 20% of the initial value. In addition to measuring total oxygen consumption, ADP-

stimulated respiration was calculated as the difference between the rates measured after ADP addition and after substrate addition. The relative contributions of the AOX and cytochrome pathways were estimated based on the sequential inhibitor response, using background-corrected values to exclude residual non-mitochondrial respiration after KCN addition. These parameters allowed comparison of mitochondrial respiratory capacity, coupling, and pathway engagement between genotypes and treatments.

Sample preparation and blue native (BN)-PAGE

For BN-PAGE, pellets from freshly isolated mitochondrial (150 µg mitochondrial protein) were resuspended in 100 µL digitonin solubilization buffer [30 mM HEPES, 150 mM potassium acetate, 10% (v/v) glycerol, and 5% (w/v) digitonin; protocol according to Klodmann et al. (2011). BN-PAGE was carried out according to Wittig et al. (2006). Separation gels consisted of linear gradients of 4.5-16% polyacrylamide. On the resulting gels, proteins and protein complexes were separated in a molecular mass range of 50-3,000 kDa. For Coomassie staining the respective BN gel lanes were first incubated in fixing solution (15% ethanol, 10% acetic acid, 75% ddH₂O) for 2 h and then transferred to the Coomassie staining solution (Neuhoff Volker, Norbert A, Taube Dieter 1988). The staining solution consists of 20% ethanol, 78.4% [v/v] solution A (2% [v/v] ortho-phosphoric acid, 10% [v/v] ammonium sulphate, 88% ddH₂O), 1.6% solution B (5% Coomassie Brilliant Blue G-250, 95% ddH₂O)). Solution A and B were mixed and shook overnight, whereas the ethanol was added immediately before the staining. The staining was carried out overnight. For better contrast, the gel was thoroughly washed several times with tap water.

Activity staining of respiratory complexes in BN-PAGE

Activity staining methods for complexes I, and complex V of the mETC were done as described in Jung et al. (2000) and Jha et al. (2016). To analyze complex I activity, the gel was incubated in 100 ml 0.1 M Tris-HCl (pH 7.4), 0.14 mM NADH, and 1.0 mg/mL nitro blue tetrazolium (NBT). To analyze complex V activity, the gel was incubated in 100 ml 35 mM Trism, 270 mM glycine, 14 mM MgSO₄, 10 mM ATP and 0.2% (w/v) Pb(NO₃)₂ overnight (~16 hr). Since the bands are transparent, the gel was inverted (to black background). Reactions were carried out at room temperature and stopped by transferring the gel into fixing solution (15% ethanol, 10% acetic acid, 75% ddH₂O). The gels were scanned with an Epson Perfection V10 scanner (Seiko Epson Corp., Nagano, Japan). The colors of the gel scan were inverted (to black

background) after scanning, since the bands indicating complex V activity are transparent. The pictures were saved in TIFF format, and the band areas were calculated with ImageJ software.

Protein extraction, digestion, and sample preparation for proteome analysis via mass spectrometry

The procedure is based on the single-pot solid-phase-enhanced sample preparations (SP4) protocol for proteomics experiments of Johnston et al. (2022). In brief, proteins were solubilized with sodium dodecyl sulfate (SDS) from lyophilized plant powder. Disulfide bridges were reduced with dithiothreitol (DTT) and subsequently alkylated with iodoacetamide (IAM). Glass beads were used to bind the denatured proteins. The beads were washed multiple times with 80% ethanol to get rid of interfering components for the digestion. Finally, the proteins were digested overnight with trypsin (Mass Spectrometry Grade, Promega). The peptide-containing supernatants were collected and desalted on 50 mg Sep-Pak tC18 columns (Waters). The purified peptides were quantified with a quantitative Peptide Assay Kit (Thermo Scientific) and adjusted to 400 ng μl^{-1} in 0.1 % formic acid.

Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS)

400 ng of peptides were injected via a nanoElute 2 UHPLC (Bruker Daltonik, Bremen, Germany) and separated on an analytical reversed-phase C18 column (Aurora Ultimate 25 cm x 75 μm , 1.6 μm , 120 Å; IonOpticks). Using MS grade water and a multi-staged gradient acetonitrile containing 0.1 % formic acid (0 min, 2%; 54 min, 25%; 60 min, 37%; 62 min, 95%; 70 min, 95%), peptides were eluted and ionized by electrospray ionization with a CaptiveSpray 2 ion source and a flow rate of 300 nl min^{-1} . The ionized peptides were separated, fragmented and analyzed with a standard data-dependent acquisition parallel accumulation-serial fragmentation application method (DDA-PASEF) of the system with the following settings: Ion mobility window of 0.7-1.5 V s cm^{-2} , 4 PASEF ramps, target intensity 14,500 (threshold 1,200), and a cycle time of ~0.53 s. The analysis was performed on a timsTOF-HT mass spectrometer (Bruker Daltonik, Bremen, Germany).

Data processing and functional annotation

The ion mobility spectrometry (IMS-MS/MS) spectra from both experiments were analyzed with MaxQuant software (Cox and Mann 2008) using default search parameters and TAIR10 (Arabidopsis.org) as database for protein identification. The calculation of label-free quantification (LFQ) values and intensity-based absolute quantification (iBAQ) values were

both enabled. Data evaluation was performed using Perseus software (Tyanova et al. 2016). Proteins were excluded from further analysis if they were not detected in at least n-1 biological replicates in at least one of the sample groups. Subsequently, missing values were replaced with randomly chosen low values from a normal distribution. Significant changes were calculated from the LFQ values using Student's t-tests (p -value < 0.05 ; FDR < 0.05).

Statistical analysis

The experiment was performed in a completely randomized design, with three replicates per treatment. The complete set-up was repeated at least three times with similar phenotypes observed each time. Statistical analysis of the MS dataset was performed in Perseus using two-sample t tests ($P < 0.05$). Statistical analysis of growth, and P content was compared using Tukey test ($P < 0.05$) in R per Exp.Des package (RDC TEAM 2013; Ferreira et al. 2014). STRING association network analysis was performed in Cytoscape (version 3.10.1; <https://cytoscape.org/>) using the String DB plugin (<https://string-db.org/>). The minimum edge threshold was set to 0.7 for the network. The data were plotted using GraphPad Prism (version 8 for Windows, GraphPad Software, Boston, Massachusetts USA; <https://www.graphpad.com>) or RStudio (Posit team 2025).

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FIGURES

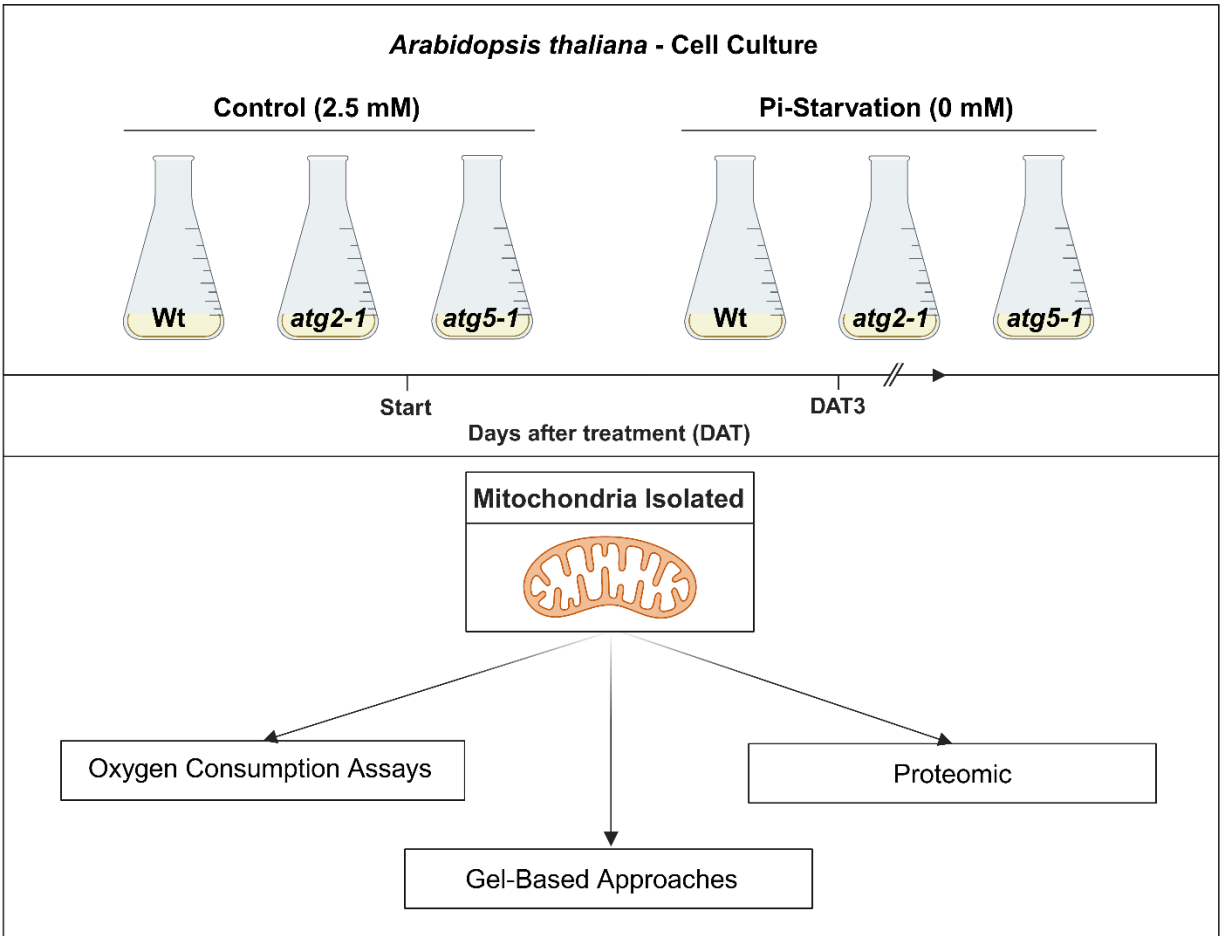


Figure 1. Experimental setup for investigating effects of autophagy deficiency in *Arabidopsis thaliana* mitochondria following Pi limitation. Cell suspensions of wild-type and autophagy-deficiency were grown under control (2.5 mM KH_2PO_4), and Pi-starvation (0 mM KH_2PO_4) conditions. After three days under the conditions described, mitochondria isolation and further analyses were conducted. Adapted from (Cavalcanti et al. 2017).

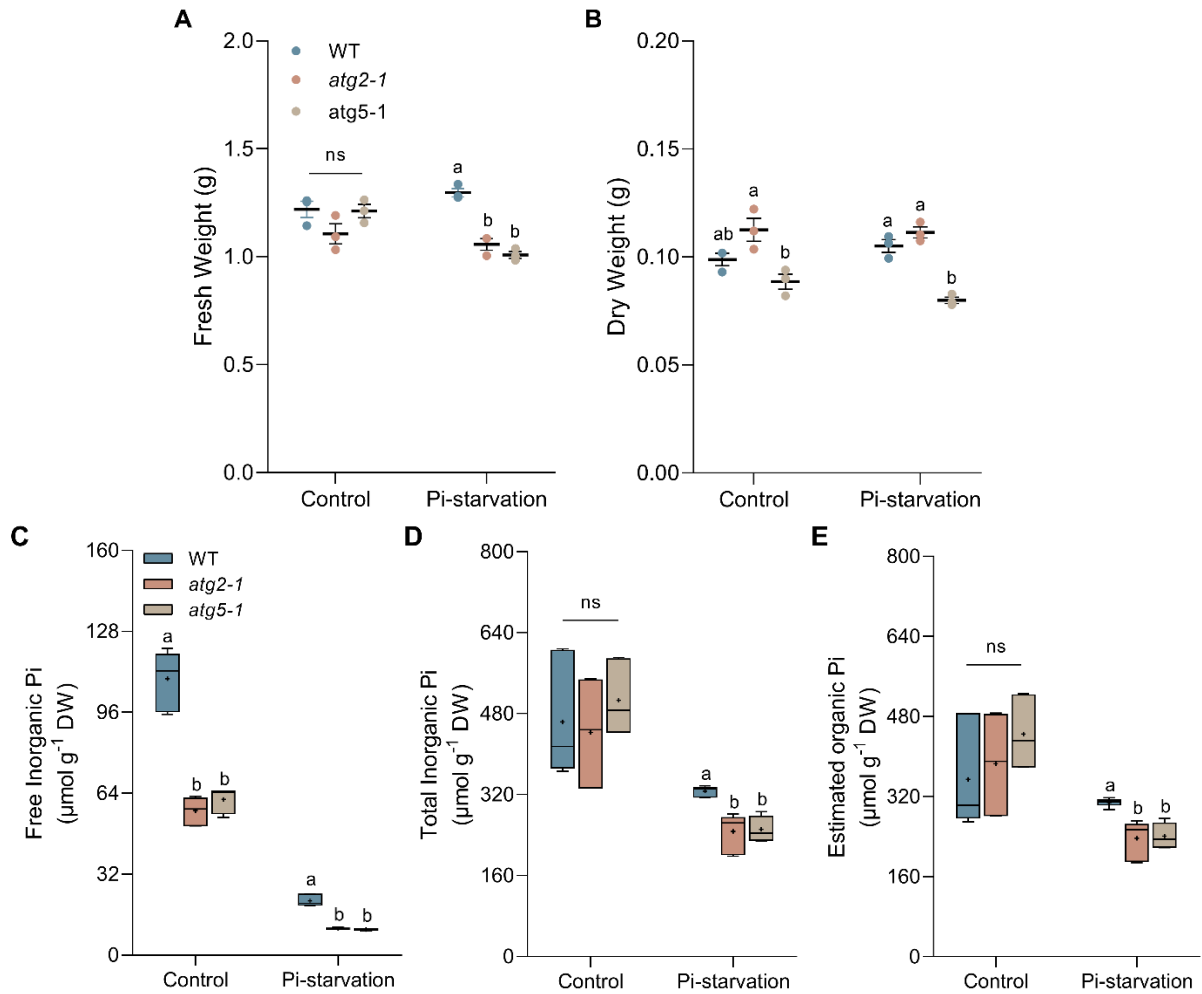


Figure 2. Lack of autophagy impairs biomass accumulation and phosphate accumulation in intact cells of *Arabidopsis thaliana* following phosphate starvation. The growth measurements were based on (A) fresh weight and (B) dry weight of intact cells after 3 days, both expressed in grams. (C) Free Pi, (D) total Pi, and (E) estimated organic Pi were quantified and normalized by dry weight. All the parameters were measured after three days under control and Pi limited conditions. Data are presented as means $\pm$ SE ($n = 3-4$ biological replicates). The letters compare the genotypes within the same treatment using Tukey's test (p -value < 0.05). ns: no significant.

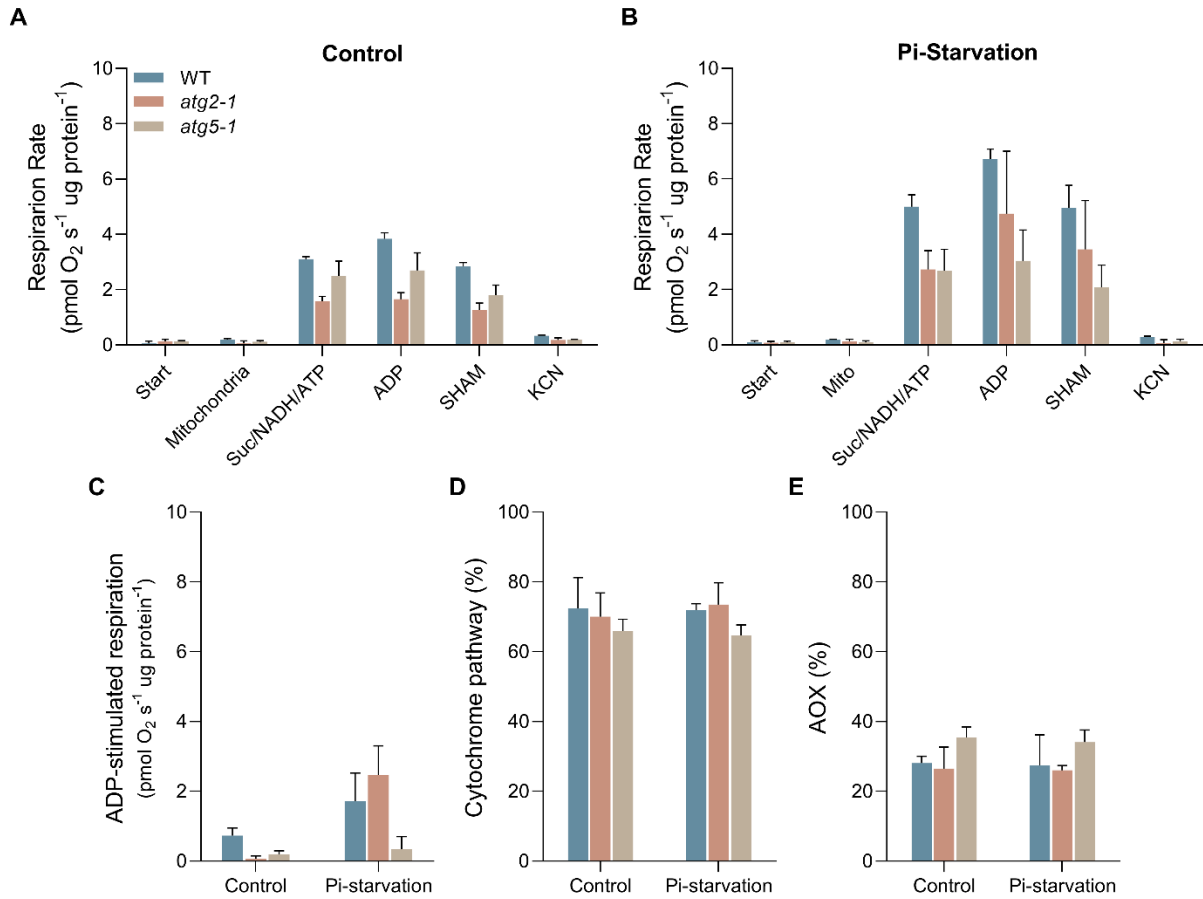


Figure 3. Rate of ETC-linked respiration of isolated mitochondria from wild-type and *atg* mutants *Arabidopsis* lines is stimulated under Pi limitation and impaired by the lack of autophagy. Respiration rates were measured by O₂ consumption per time using a Clark-type oxygen electrode. The measurements were performed in the darkness after 3 days under (A) control and (B) Pi limited conditions for wild-type, *atg2-1* and *atg5-1*. Freshly mitochondria were supplemented with the substrates succinate and NADH to deliver reductant directly to the ETC at complex II and external NAD(P) dehydrogenases and bypasses soluble enzymes of the TCA cycle. ATP was included to activate succinate dehydrogenase. ADP was added to relieve any limitation from a proton motive force across the inner membrane and achieve the maximal respiration rates. SHAM and KCN were included to inhibit alternative oxidases (AOX) and cytochrome c oxidase activity, respectively. (C) ADP-stimulated respiration (reflecting oxidative phosphorylation capacity) and the relative contributions of the (D) cytochrome (E) AOX pathways were calculated based on inhibitor response. Data are presented as means $\pm$ SD (n = 3 replicates).

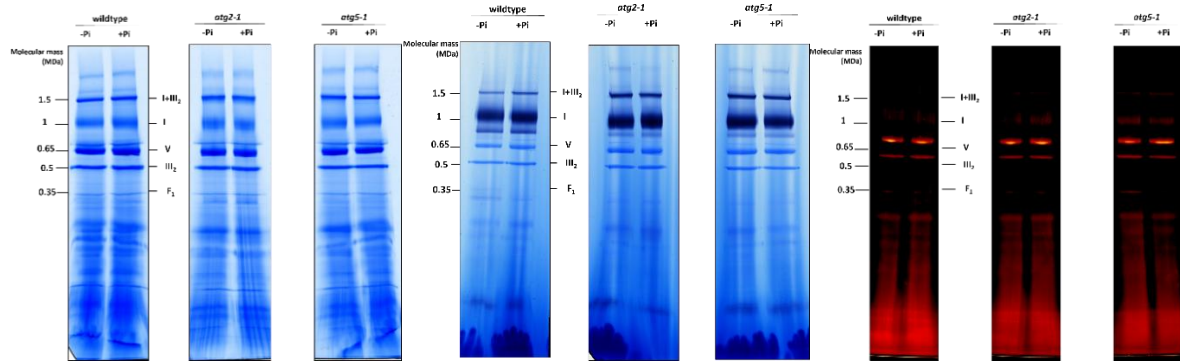


Figure 4. Blue Native PAGE gel followed by OXPHOS complex *in gel* activity assays. (A) Coomassie stained gel. (B) Complex I activity, and (C) Complex V activity. Mitochondrial membrane proteins were solubilized by 5% digitonin and subsequently separated by one-dimensional blue native PAGE. +Pi, control; -Pi, Pi-starvation.

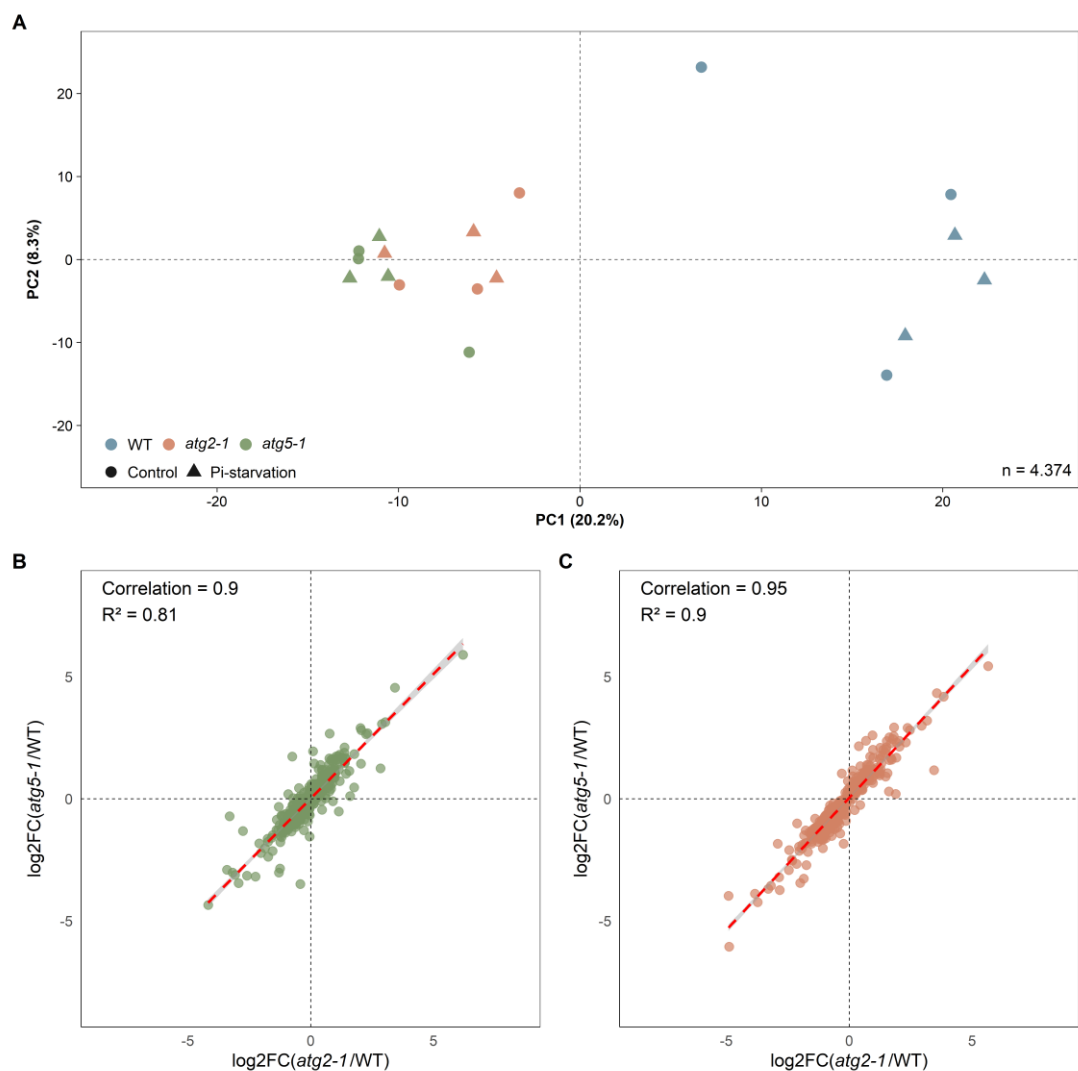


Figure 5. Autophagy deficiency enhances alterations in the mitochondrial proteome in response to Pi limitation. Proteomic analyses were performed on isolated mitochondria following 3 days of Pi-starvation. (A) Principal component analysis (PCA) of mitochondrial protein abundance. Ellipses represent 95% confidence intervals. (B, C) Pairwise comparison of log₂ fold changes (log₂FC) in mitochondrial protein abundance between *atg2-1*/WT and *atg5-1*/WT under (B) control and (C) Pi-starvation conditions, with Pearson correlation coefficients and coefficients of determination (R^2) indicated. The total number of proteins analysed in each panel are 630. The dashed lines show a corr = 1. Data presented are based on three independent mitochondria isolations. All plots were generated using the ggplot2 package in R.

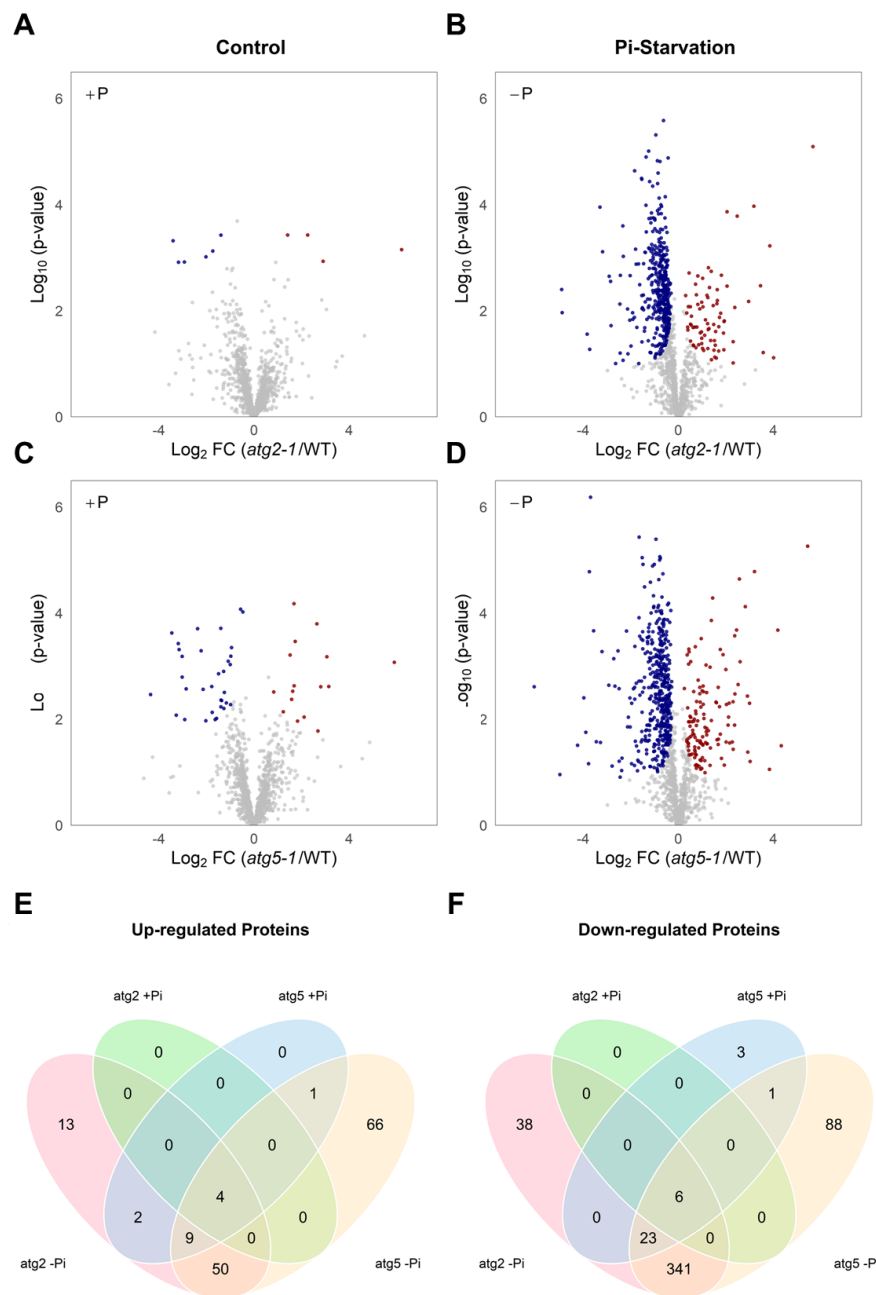


Figure 6. Autophagy deficiency enhances alterations in the mitochondrial proteome in response to Pi limitation. (A-D) Volcano plots illustrates the protein abundance changes between mitochondrial fraction of *atg2-1* vs WT under (A) control, and (B) Pi-starvation; *atg5-1* vs WT under (C) control, and (D) Pi-starvation. The x-axis displays log₂ fold changes (log₂FC) in relative protein abundance of mitochondrial fractions in mutants relative to WT, the y-axis shows $-\log_{10}(\text{p-value})$. Red and blue dots represent proteins with significant changes that increased or decreased abundance significantly, respectively ($P < 0.05$, $\text{FDR} < 0.05$). Only proteins annotated as mitochondrial according to SUBA5 localization were included. (E-F) Four-way Venn diagram showing the overlap in the numbers of proteins that were consistently (E) up- or (F) down-regulated in both mutants relative WT under control and Pi-starvation. Data presented are based on three independent mitochondria isolations. All plots were generated using the ggplot2 package in R.

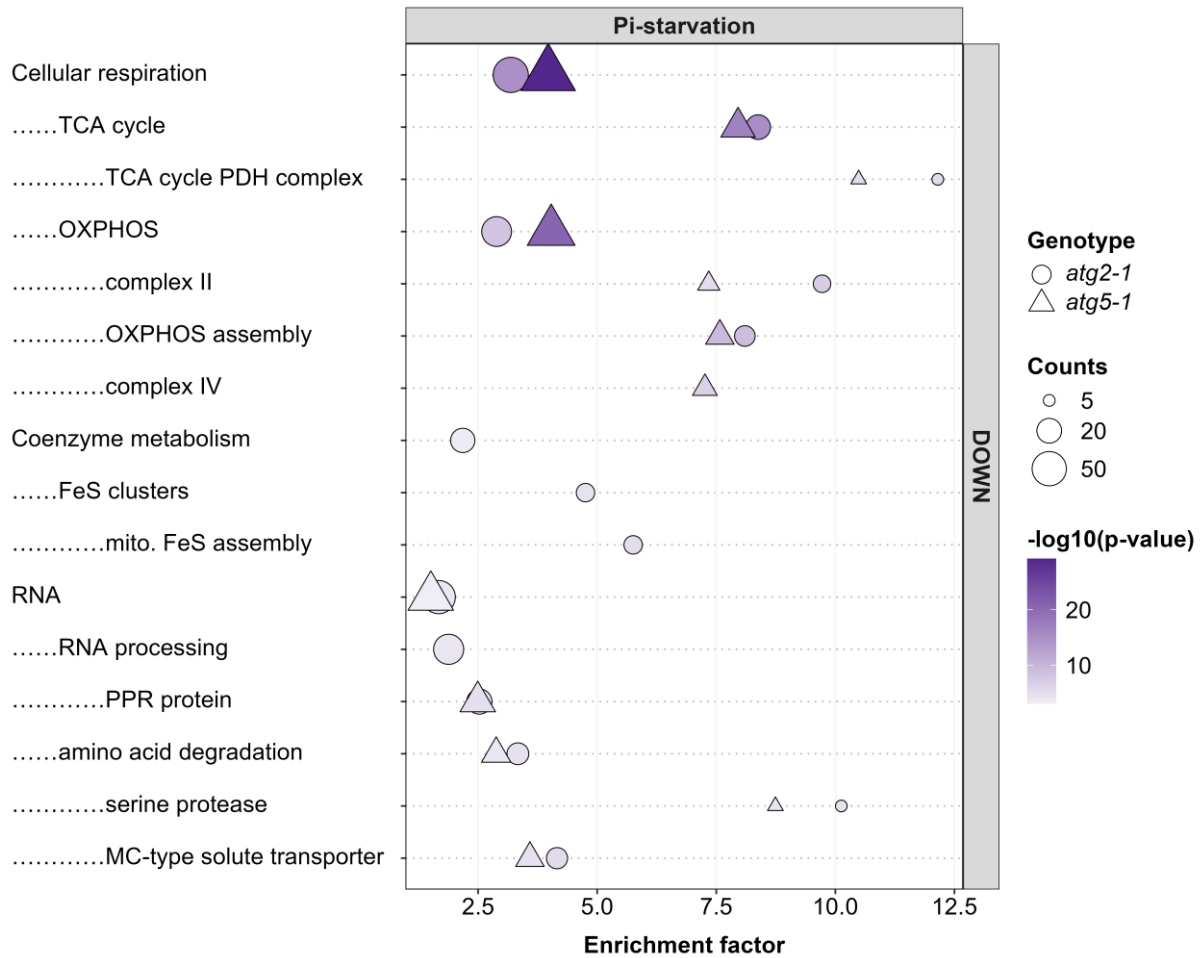


Figure 7. Autophagy deficiency enhances alterations in the mitochondrial proteome in response to Pi limitation. (A-D) Venn diagram showing the overlap in the numbers of proteins that were consistently up- or down-regulated in *atg* mutants relative to wild-type (WT) under control and Pi-starvation. Up-regulated in *atg2-1* (A), and *atg5-1* (B). Down-regulated in *atg2-1* (C), and *atg5-1* (D). (E) Enrichment of functional categories in proteins that are significantly increased or decreased in the mitochondrial proteome of autophagy-deficient mutants compared to the WT under Pi limitation. The plot displays significantly enriched protein categories (y-axis) in *atg2-1* (circles) and *atg5-1* (triangles) compared to the WT. The x-axis represents the enrichment factor. Dot size correlates with the number of proteins identified in each category, while color intensity indicates the significance of the enrichment ($-\log_{10}(\text{p-value})$). Proteins are categorized as "Up" (accumulated) or "Down" (reduced). Data presented are based on three independent mitochondria isolations.

A

AGIs	Annotatio TAIR10	Control		Pi-Starvation		Control		Pi-Starvation	
		<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>
AT3G30775.1	PDH1/ProDH1						+	+	+
AT3G08860.1	AGT3					+	+	+	+
AT1G23310.1	GGAT1						+	+	+
AT5G14780.1	FDH					+	+	+	+
AT1G23800.1	ALDH2B7						+		+
AT5G20250.4	RFS6						+	+	+
AT1G22360.2	UGT85A2						+	+	+
AT5G51040.2	SDHAF2						+	+	+
AT2G20800.1	NDB4						+	+	+
AT3G22370.1	AOX1A						+	+	+
AT1G25145.1	LPXC4					+	+	+	+
AT2G21640.1	unkown protein					+	+	+	+
AT3G45740.1	HAD					+	+	+	+
AT5G27760.1	HRP					+	+	+	+
AT2G39725.2	LYR/ISD11						+	+	+
AT2G03780.1	TSN						+		
AT1G20430.1	unknown protein						+	+	+
AT5G07090.2	RPS4y						+		
AT5G10360.1	RPS6y						+		
AT1G71060.1	TPR					+	+	+	+
AT1G26900.1	PPR					+	+	+	+
AT4G14850.1	PPR						+		+
AT5G51440.1	HSP23.5-M					+	+	+	+
AT5G51050.1	APC2						+	+	+

B

AGIs	Identification	Control		Pi-starvation		Control		Pi-starvation	
		<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>
AT3G08580.2	AAC1							+	+
AT5G13490.2	AAC2							+	+
AT4G28390.1	AAC3							+	+
AT3G48850.1	PHT3-2/MPT2							+	+
AT5G14040.1	PHT3-3/MPT3							+	+
AT3G54110.1	PUMP1/UCP1							+	+
AT1G14140.1	UCP3							+	+
AT4G24570.1	UCP4								+
AT5G19760.1	DTC							+	+
AT5G01340.1	SFC1							+	+
AT1G74240.1	MSCP							+	+
AT1G07030.1	MSCP/MIT2							+	
AT5G07320.1	APC3								+
AT5G51050.1	APC2						+	+	+
AT5G61810.1	APC1								+
AT4G01100.1	ADNT1							+	+
AT5G48970.1	TPC2/MHC9.10							+	+
AT1G53760.1	KHE/KEA							+	+
AT5G58270.1	ASCB25/ATM3							+	+
AT4G01660.1	ABC1							+	+
AT2G40090.1	ABCL							+	+
AT1G25380.1	NDT2							+	+
AT1G51610.1	MTPC4							+	+
AT1G57610.1	MCU5						+	+	

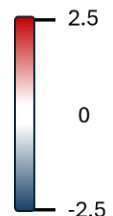


Figure 8. Heatmap visualization of relative mitochondrial protein abundance changes in *atg2-1* and *atg5-1* mutants under control and Pi-starvation conditions. (A) Differentially abundant proteins showing significant changes under control conditions as well as Pi-starvation. (B) Differentially abundant proteins specifically assigned to the transport functional category. The color scale indicates the $\log_2$ fold change of protein abundance relative to the wild type (red: up-regulated, blue: down-regulated, white: no significant change). Statistically significant protein abundance changes for each specific genotype and condition are indicated by a plus sign (+).

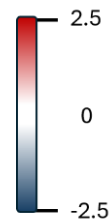
A

AGIs	Identification	Control		Pi-Starvation		Control		Pi-Starvation	
		<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>
AT4G08900.1	ARGAH1								+
AT5G62530.1	P5CDH							+	+
AT3G30775.1	PDH1/ProDH1					+		+	+
AT5G09300.2	BCKDH α 2							+	+
AT1G55510.1	BCKDH β 1							+	+
AT3G13450.1	BCKDH β 2							+	+
AT4G29120.1	HIBDH1								+
AT3G60510.3	CHY1							+	
AT4G31810.1	CHYL2								+
AT1G48030.1	mtLDP1							+	+
AT3G17240.3	mtLDP2							+	+
AT1G50940.1	ETFA							+	+
AT5G43430.1	ETFB							+	+
AT4G34030.1	MCCB							+	+
AT5G65720.1	NFS1							+	+
AT1G53580.1	ETHE1							+	+
AT3G01910.1	SO								+
AT1G79230.1	STR1							+	+
AT1G79440.1	SSADH							+	+
AT2G37500.1	ArgJ							+	+
AT5G28020.6	CASD2							+	+
AT3G59760.3	OAS-TL							+	+
AT3G13110.1	SAT3								+
AT5G17330.1	GAD1							+	+
AT3G60880.1	DHDP51							+	+
AT5G20980.2	METS3								+
AT1G17290.1	ALAAT1							+	+
AT1G72330.3	ALAAT2							+	+
AT2G30970.2	ASP1							+	+
AT1G10070.1	BCAT2							+	+
AT3G61440.1	CASC1								+
AT5G18170.1	GDH1							+	+
AT5G07440.1	GDH2							+	+
AT4G39660.1	AGT2.1							+	+
AT2G38400.1	AGT2.2							+	+
AT3G08860.1	AGT3					+	+	+	+
AT1G23310.1	GGAT1						+	+	+

B

AGIs	Identification	Control		Pi-Starvation		Control		Pi-Starvation	
		<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>	<i>atg2-1</i>	<i>atg5-1</i>
AT5G57590.1	BIO3							+	
AT2G43360.1	BIO2							+	+
AT1G51390.1	NFU5							+	+
AT4G12130.1	transferase								+
AT5G06410.1	HSCB							+	+
AT5G09590.1	HSC70-10							+	+
AT4G37910.1	HSC70-9							+	+
AT4G28630.1	ABCB23/ATM1								+
AT5G58270.1	ABCB256/ATM3							+	+
AT4G03240.1	FH							+	+
AT4G32360.1	AR								+
AT4G21090.3	ADX2							+	
AT5G03905.1	ISCA2							+	+
AT4G22220.1	ISCU1/ISC1							+	
AT2G20860.1	LIP1							+	+
AT1G01290.2	CNX2							+	+
AT2G31955.1	CNX3								+
AT1G19140.1	COQ							+	+
AT2G03690.1	COQ4								+
AT4G01660.1	ABC1							+	+
AT2G34630.2	SPS3							+	+
AT3G24200.1	COQ6							+	+
AT5G57300.3	COQ5							+	+
AT4G30000.2	HPPK/DHPS							+	+
AT5G64050.1	GLURS							+	+
AT1G45110.1	SUMO/UPM1								+

Figure 9. Heatmap visualization of relative mitochondrial protein abundance changes in *atg2-1* and *atg5-1* mutants under control and Pi-starvation conditions. (A) Differentially abundant proteins specifically assigned to the amino acid metabolism functional category. (B) Differentially abundant proteins specifically assigned to the coenzyme metabolism functional category. The color scale indicates the log₂ fold change of protein abundance relative to the wild type (red: up-regulated, blue: down-regulated, white: no significant change). Statistically significant protein abundance changes for each specific genotype and condition are indicated by a plus sign (+).



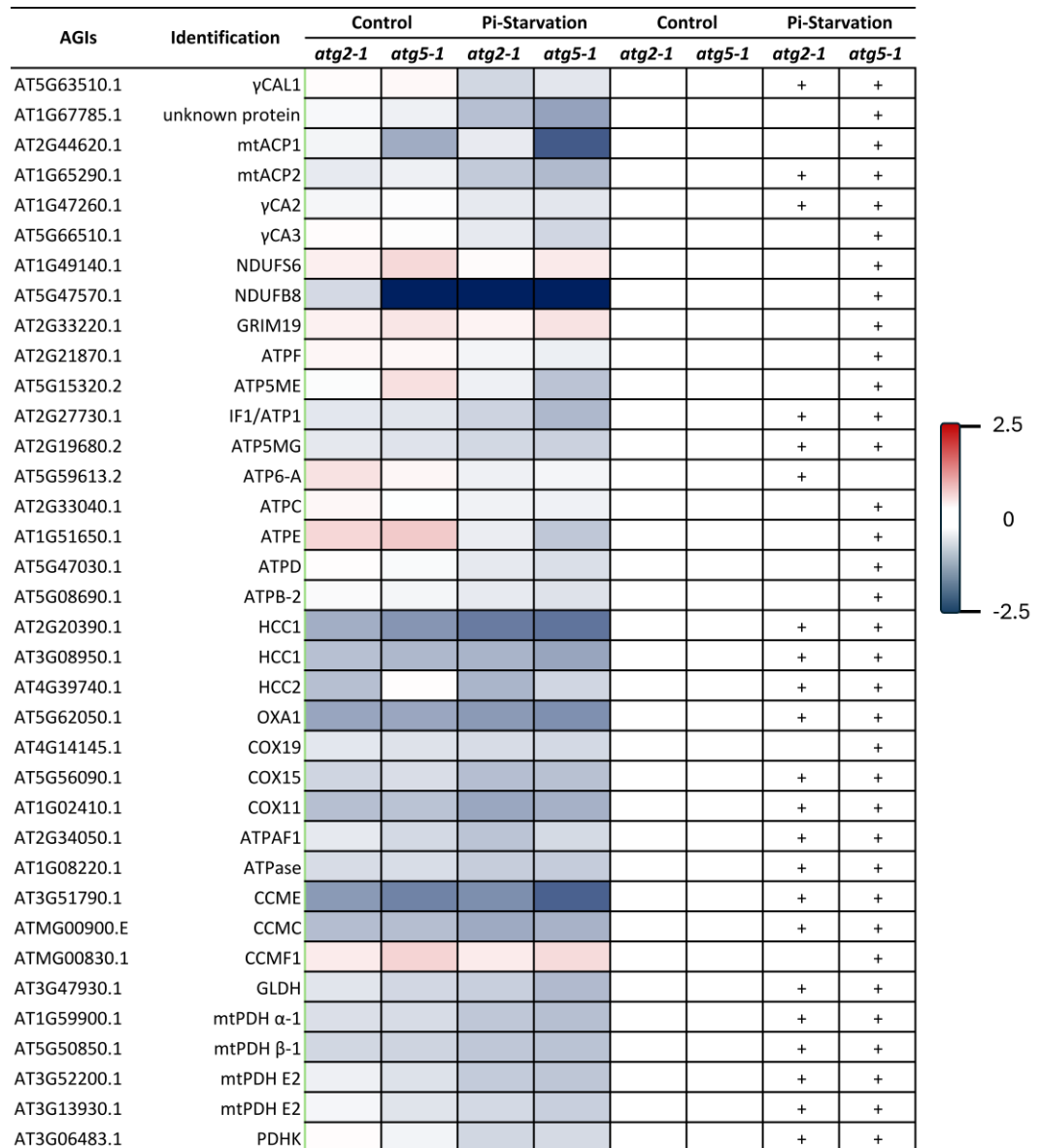


Figure 10. Heatmap visualization of relative mitochondrial protein abundance changes in *atg2-1* and *atg5-1* mutants under control and Pi-starvation conditions. Differentially abundant proteins specifically assigned to the cellular respiration functional category, comprising proteins related to OXPHOS (complex II, IV, and assembly), and TCA cycle (PDH complex). The color scale indicates the log₂ fold change of protein abundance relative to the wild type (red: up-regulated, blue: down-regulated, white: no significant change). Statistically significant protein abundance changes for each specific genotype and condition are indicated by a plus sign (+).

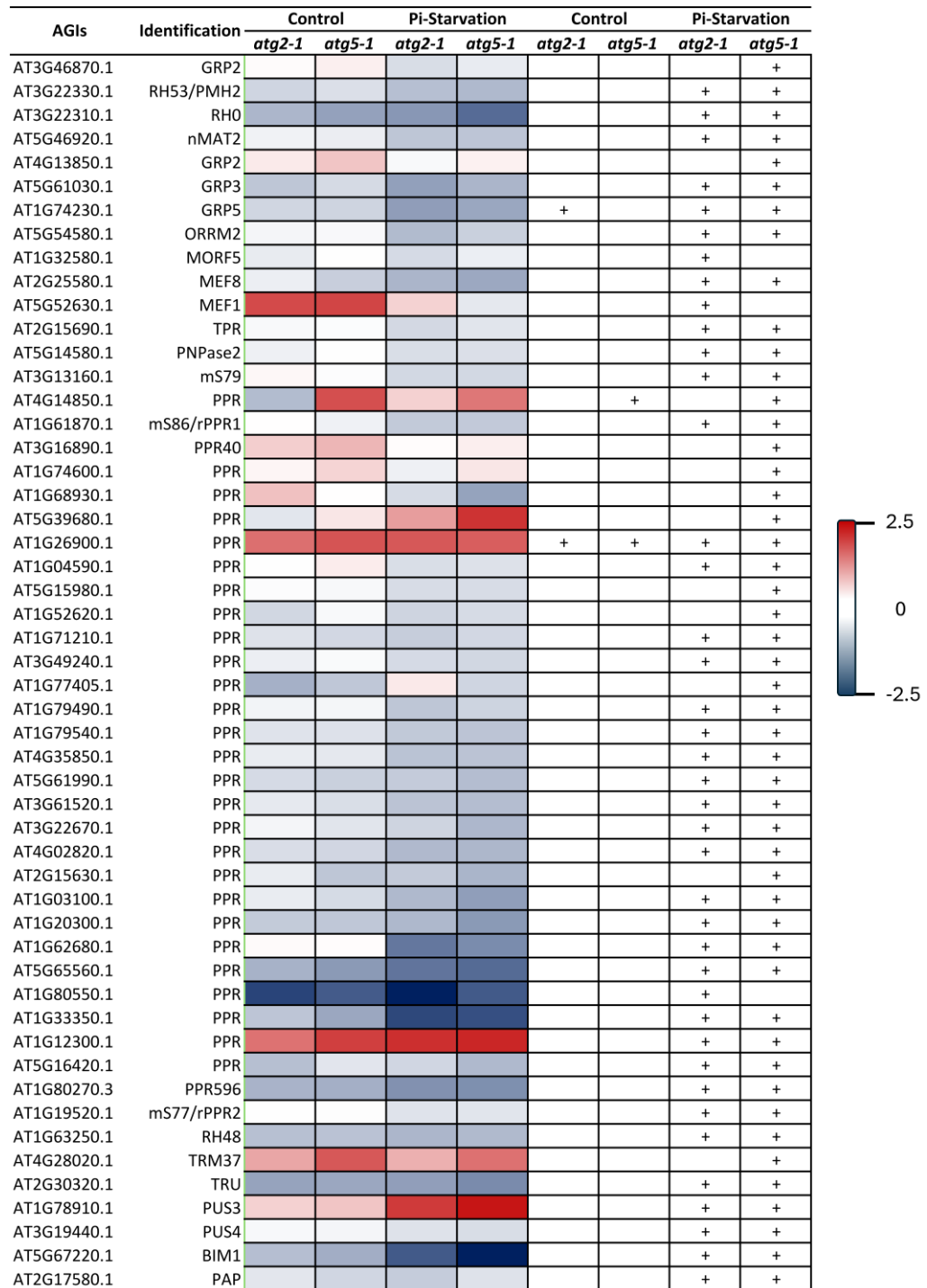


Figure 11. Heatmap visualization of relative mitochondrial protein abundance changes in *atg2-1* and *atg5-1* mutants under control and Pi-starvation conditions. Differentially abundant proteins specifically assigned to the RNA metabolism functional category. The color scale indicates the log₂ fold change of protein abundance relative to the wild type (red: up-regulated, blue: down-regulated, white: no significant change). Statistically significant protein abundance changes for each specific genotype and condition are indicated by a plus sign (+).

SUPPLEMENTAL DATA

TABLE

Supplementary Table S1. Primer list.

Primer name	Primer sequence (5' to 3')	Note
At3g19190 Fw At3g19190 Rv At3g19190 Lb	GCGTGGACCGCTTGCTGCAACT TCCTACGGCCGAGATCGTACA GCACTTTCCATCAGCTACTCGC	For <i>ATG2-1</i> genotyping
At5g17290 Fw At5g17290 Rv At5g17290 Lb	GATTGAAACGAGGAAGAATTTAGAC TAATTGATATGCCTAACATCGTCC TCAGAAATGGATAAATAGCCTTGC	For <i>ATG5-1</i> genotyping

FIGURES

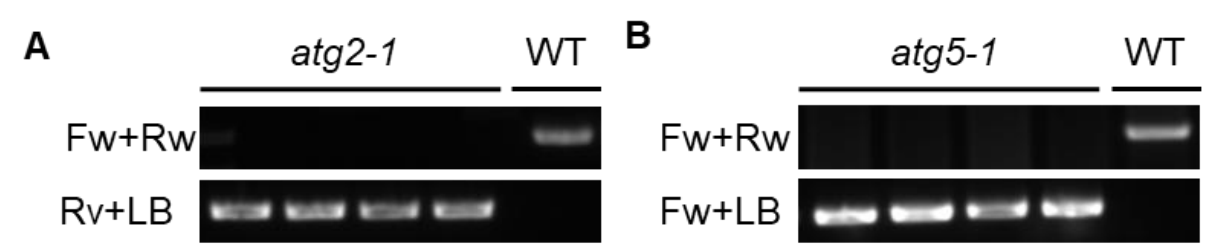


Figure S1. PCR genotyping of *atg2-1* and *atg5-1* mutants. Fw, forward primer; Rv, reverse primer; LB, T-DNA left border primer. Genomic DNA from wild-type (WT) and mutant lines were used as template.

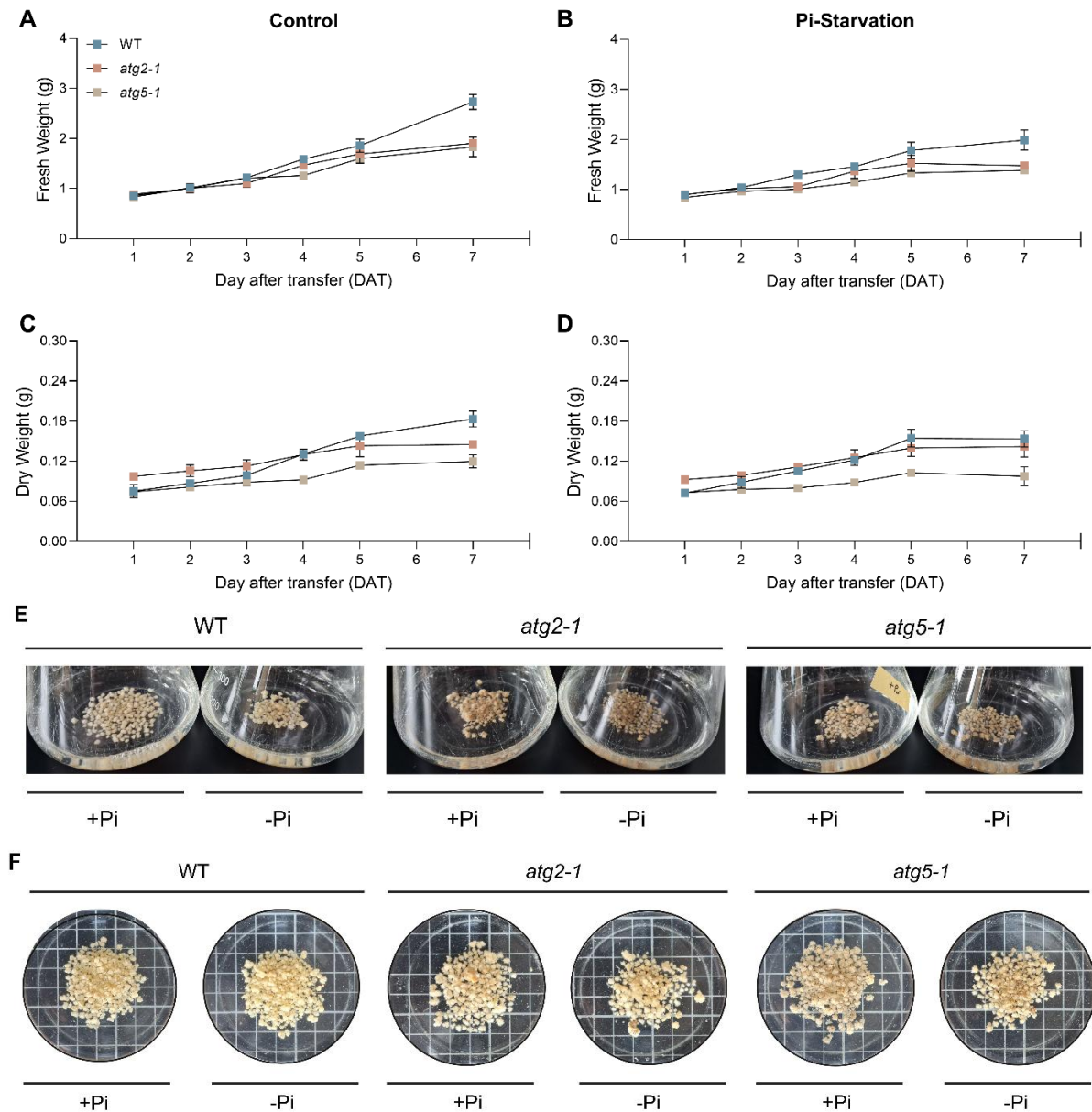


Figure S2. Growth dynamics and phenotypes responses of wild-type (WT) and autophagy-deficient mutants (*atg2-1* and *atg5-1*) under control and Pi-starvation conditions. Time-course measurements over 7 days after transfer (DAT) to the respective media for fresh weight (g) under (A) control and (B) Pi-starvation conditions, and dry weight (g) under (C) control and (D) Pi-starvation conditions. (E, F) Visual phenotypes of cell suspension cultures are shown for the time point, used for mitochondrial isolation and subsequent analysis, (E) DAT3 and the final time point at (F) DAT7 in media containing sufficient phosphate (+Pi) or lacking phosphate (-Pi). Data are presented as means $\pm$ SE ($n = 3$ biological replicates).

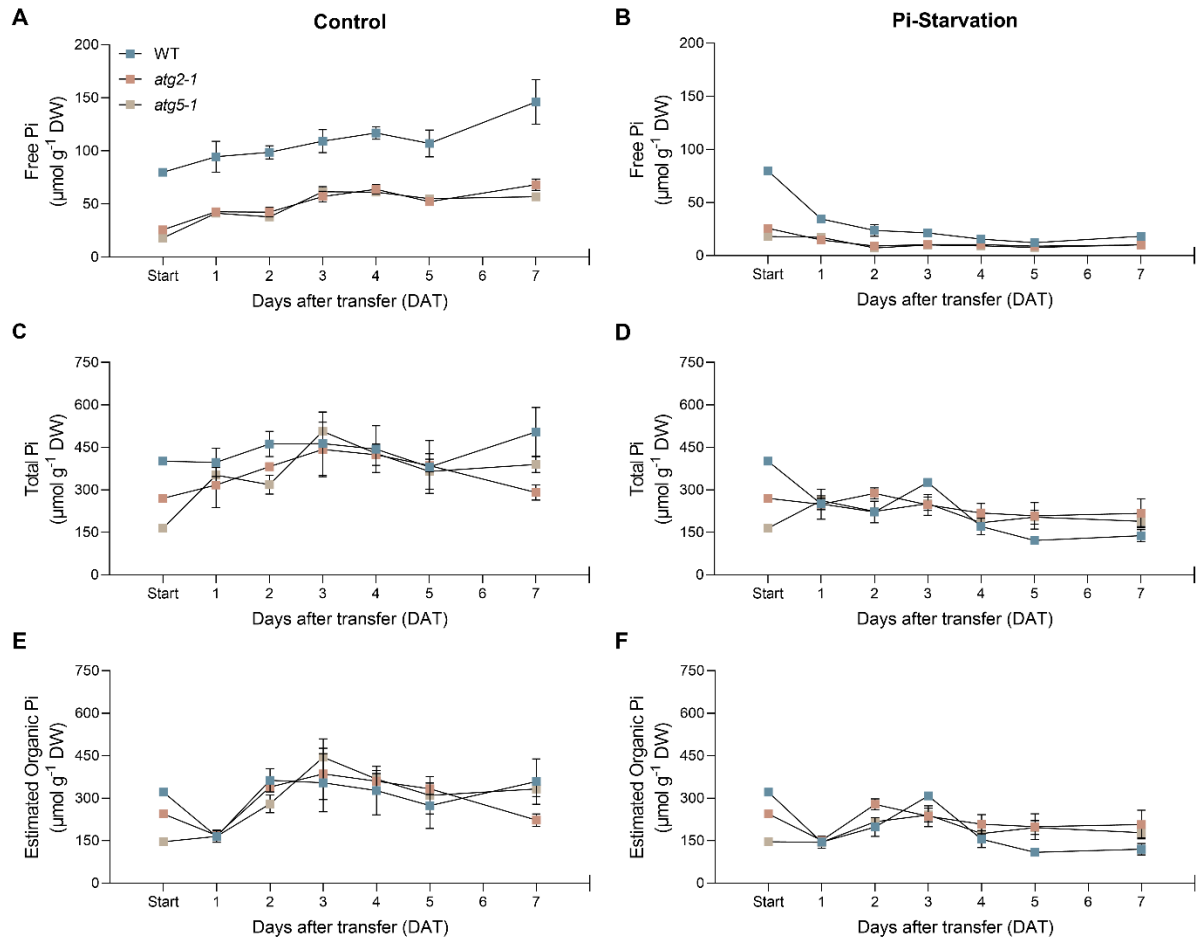


Figure S3. Autophagy deficiency alters phosphate pool dynamics during acclimation to phosphate limitation. (A, B) Time-course analysis of Pi contents in wild-type (WT) and autophagy-deficient mutants (*atg2-1* and *atg5-1*) under control and Pi-starvation conditions. (C, D) Time-course of free Pi ($\mu\text{mol g}^{-1}$ DW) for (A) control and (B) Pi-starvation; total Pi ($\mu\text{mol g}^{-1}$ DW) for (C) control and (D) Pi-starvation; and (E, F) estimated organic Pi for (E) control and (F) Pi-starvation ($\mu\text{mol g}^{-1}$ DW). Data are presented as means $\pm$ SE (n = 3 biological replicates).

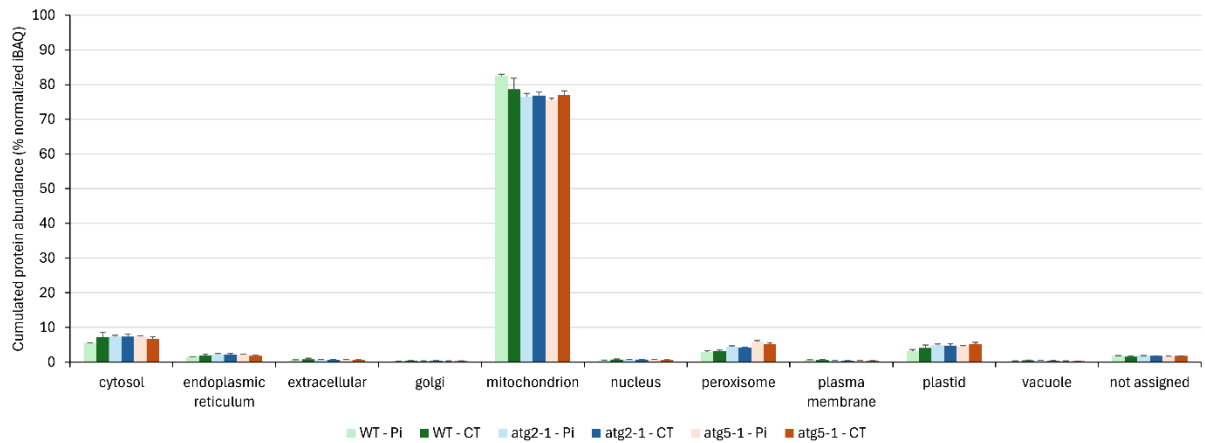


Figure S4. Purity estimation of the mitochondrial fraction. All proteins identified by all our samples were assigned to subcellular localizations using the SUBA5 annotation. The bar chart indicates the average of three isolations of mitochondria for wild-type and autophagy-deficient mutants, *atg2-1* and *atg5-1* under control and Pi-starvation conditions. Data presented are based on three independent mitochondria isolations.

**CHAPTER 4 - Exploring tissue- and time-dependent roles of autophagy in phosphate
starvation responses of *Arabidopsis thaliana***

Exploring tissue- and time-dependent roles of autophagy in phosphate starvation responses of *Arabidopsis thaliana*

ABSTRACT

Phosphate (Pi) is a critical determinant of plant energy metabolism because it constrains ATP synthesis, carbon flux, and redox balance across plastidial and mitochondrial pathways. During Pi limitation, plants undergo extensive physiological and metabolic reprogramming to sustain growth and survival. Autophagy, a conserved intracellular recycling pathway, contributes to early phosphate starvation responses (PSR), but its role in coordinating systemic Pi allocation and tissue-specific metabolic acclimation during prolonged Pi deprivation remains unclear. To investigate the functional importance of autophagy across time and tissues, we performed time-resolved physiological and organ-specific quantitative proteomic analyses in *Arabidopsis thaliana* autophagy-deficient mutants (*atg2-1* and *atg5-1*). Under Pi-sufficient conditions, autophagy deficiency altered basal proteome organization and Pi partitioning, resulting in elevated root Pi pools and reduced shoot Pi availability. During progressive Pi-starvation, *atg* mutants exhibited impaired root-to-shoot Pi redistribution, premature repression of photosynthetic and plastidial functions, and enhanced accumulation of proteins associated with glycolysis, mitochondrial respiration, and amino acid catabolism, particularly in *atg5-1*. These changes were accompanied by altered Pi transporter abundance, extensive remodeling of vesicle trafficking pathways, and tissue-specific activation of ABA-associated responses in shoots and SnRK1-associated energy-stress signaling in roots. Collectively, our findings demonstrate that autophagy is an integrator of Pi-starvation acclimation, coordinating Pi homeostasis, proteome remodeling, and metabolic adaptation across tissues. By maintaining Pi allocation and metabolic flexibility during nutrient limitation, autophagy may support whole-plant resilience to prolonged Pi stress.

Keywords: Autophagy; Pi limitation; phosphate starvation response; root-shoot; proteomics.

INTRODUCTION

Phosphorus (P) is an essential macronutrient that plays fundamental roles in plant physiology, including ATP-dependent energy metabolism, reversible protein phosphorylation, and the synthesis of nucleic acids and membrane phospholipids (Morcuende et al. 2007; Plaxton and Tran 2011; Dissanayaka et al. 2021a). Plants acquire P predominantly as inorganic phosphate (Pi), a form that is often limiting in soil due to its low solubility and strong fixation to metal cations such as Fe^{3+} , Al^{3+} , and Ca^{2+} (Dao et al. 2005; Borch and Fendorf 2007; Gerke

2010). Given the finite nature of Pi rock reserves and the environmental costs associated with fertilizer overuse, elucidating endogenous mechanisms that govern Pi acquisition, intracellular redistribution, and internal recycling is a major priority in plant biology and sustainable agriculture (Scholz et al. 2013; Ueda et al. 2021).

To cope with Pi scarcity, plants activate a coordinated set of morphological, physiological, and molecular responses collectively termed phosphate starvation response (PSR) (Paries and Gutjahr 2023). These responses include the remodeling of root system architecture to enhance soil exploration, the secretion of phosphatases and organic acids to mobilize external Pi, extensive transcriptional reprogramming, and metabolic adjustments that improve Pi uptake, transport, and utilization efficiency (Nussaume et al. 2011; Ueda et al. 2021; O’Gallagher et al. 2022). At the metabolic level, Pi limitation imposes a direct energetic constraint, because Pi is required for ATP synthesis and for sustaining phosphorylated intermediates that support photosynthesis and respiration. Accordingly, plants reconfigure central carbon metabolism, including induction of Pi- and ATP-independent bypass reactions that sustain flux when adenylate availability is restricted (Plaxton and Tran 2011; Dissanayaka et al. 2021b).

The PSR is governed by a complex regulatory network that integrates local Pi sensing at the root apex with long-distance shoot-root signaling. Key components include the PHOSPHATE DEFICIENCY RESPONSE 2-LOW PHOSPHATE ROOT 1 (PDR2-LPR1) module, which modulates primary root growth in response to Pi availability, and SPX-domain proteins that sense intracellular Pi status via binding to inositol pyrophosphates (Ticconi et al. 2009; Müller et al. 2015). PSR transcription factors coordinate systemic induction of Pi-starvation-responsive gene programs (Thibaud et al. 2010; Prathap et al. 2022). Importantly, Pi sensing and response outputs are strongly dependent on tissue. Roots act as the primary site of local sensing and uptake, whereas shoots integrate systemic status, adjust photosynthetic capacity, and mobilize intracellular Pi reserves. These functional differences are mirrored by distinct, and often asynchronous, transcriptional and metabolic trajectories between organs (Wu et al. 2003; Crombez et al. 2019; Guo et al. 2022).

Autophagy, a conserved catabolic pathway that delivers cytoplasmic material to the vacuole for degradation and recycling, has emerged as a central regulator of cellular homeostasis during nutrient limitation and abiotic stress (Marshall and Vierstra 2018). In plants, autophagy is executed by a conserved AUTOPHAGY-RELATED (ATG) machinery that coordinates autophagosome formation, cargo sequestration, and vacuolar degradation (Ohsumi 2014; Petersen et al. 2024b; Gross et al. 2025). Increasing evidence indicates that autophagy is

tightly integrated into the PSR. Under Pi limitation, activation of the PDR2-LPR1 module links local Pi sensing to ER stress, triggering a cytoprotective autophagy response required to preserve root meristem integrity (Naumann et al. 2019b). Subsequently, LPR1 was identified as a high-affinity ferroxidase that mediates local Pi sensing through Fe-dependent signaling in the root apoplast, ultimately influencing root meristem maintenance under Pi-starvation (Naumann et al. 2022). During early Pi-starvation, autophagy-deficient (*atg*) mutants display accelerated Pi depletion and reduced shoot growth, supporting a requirement for autophagy in maintaining Pi homeostasis (Yoshitake et al. 2022). Mechanistically, an IRE1-dependent ER-phagy pathway is activated by iron-mediated lipid ROS during early Pi-starvation; by recycling Pi from ER membrane phospholipids, ER-phagy transiently refills intracellular Pi pools and delays onset of late PSR such as membrane lipid remodeling (Yoshitake et al. 2022). In parallel, impaired turnover of specific mitochondrial and chloroplast proteins in *atg* mutants during early Pi limitation associates with metabolic dysregulation that compromises ATP-generating capacity and redox balance (Li et al. 2022a). Autophagy also contributes to intracellular Pi recycling during the initial phase of starvation, while its roles during prolonged Pi limitation and at the whole-plant level remain less resolved (Yoshitake and Yoshimoto, 2023). Furthermore, the role of autophagy in Pi-starvation has been expanded beyond intracellular recycling to include functions in Pi homeostasis, including Pi transporter regulation (e.g. PHOSPHATE TRANSPORTER 1 (PHT1) (Chiu et al. 2023a; Liu et al. 2023), modulation of root developmental responses (Liu et al. 2023), and maintenance of autophagic flux through vacuolar processes (Guan et al. 2025).

Despite this progress, the spatiotemporal organization of autophagy during Pi limitation remains poorly defined. Autophagy activity varies across tissues and cell types during nutrient limitation (Cheng et al. 2022; Dong et al. 2022). In Pi-starvation, the central transcriptional regulator PHR1 modulates ATG8 isoform expression in a tissue-specific manner, inducing ATG8a, ATG8f, ATG8g, and ATG8h in shoots, but predominantly ATG8f and ATG8h in roots (Lin et al. 2023). The metabolic responses vary with the temporal parameter and the severity of Pi-starvation, indicating that autophagy fulfills distinct functions across tissues and stages of starvation. Recent organ-specific proteomic profiling underscores this complexity, revealing that even the recovery phase from Pi stress is highly asynchronous between tissues (Smith et al. 2025). This study demonstrated that shoots and roots employ distinct proteomic trajectories to re-establish homeostasis, with rapid shifts in glycolytic enzymes (e.g., FBA5), and energy-related proteins (e.g., AOX1A) occurring in a tissue-specific manner (Smith et al. 2025). Notwithstanding these findings, a key question remains unresolved: how autophagy coordinates

tissue-specific Pi allocation with metabolic remodeling across early and prolonged phases of Pi-starvation?

Here, we investigate how autophagy shapes whole-plant acclimation to Pi limitation through time-resolved, organ-specific physiological and quantitative proteomic analyses in *Arabidopsis thaliana*. Using wild type (WT) and *atg* mutants (*atg2-1* and *atg5-1*), we dissect early and late Pi-starvation responses in roots and shoots (Fig. 1). Our results reveal that autophagy is required for proper coordination of Pi homeostasis and metabolic acclimation during Pi limitation. Autophagy deficiency altered basal proteome organization prior to stress, disrupted root-to-shoot Pi partitioning, and promoted distinct tissue-specific proteome remodeling during Pi-starvation. These changes were associated with impaired maintenance of shoot Pi pools and extensive reprogramming of carbon and energy metabolism, highlighting autophagy as a key regulator of nutrient redistribution and metabolic flexibility under Pi limitation.

RESULTS

Autophagy deficiency alters biomass accumulation and shoot:root balance following Pi-starvation

To assess biomass allocation in WT and *atg* mutants following Pi-starvation, shoot and root phenotypes were first observed, followed by quantification of fresh weight (FW) and dry weight (DW) under control and Pi-starvation conditions at early (DAT3) and late (DAT7) time points (Fig. 2). Based on previously described temporal dynamics of Pi depletion under Pi-starvation (Yoshitake et al. 2022), DAT3 and DAT7 were selected as representative early and late stages, respectively. In WT shoots, Pi content at DAT3 remained at 54.6% of DAT0, declining further to 25.3% at DAT7 (Fig. S1).

At the early stage of Pi-starvation, shoot biomass remained unchanged across genotypes, with no significant differences in relative FW or DW between WT and *atg* mutants (Fig. 2C). In contrast, *atg5-1* showed a significant decrease in root FW and DW compared to WT, while *atg2-1* showed an intermediate response (Fig. 2E). Consequently, shoot-to-root DW ratios were not significantly altered at DAT3 (Fig. 2G).

At the late stage of Pi-starvation (DAT7), biomass allocation diverged more clearly among genotypes (Fig. 2D-F). In shoots, WT and *atg2-1* showed a significant reduction in relative biomass under Pi-starvation (approximately -25% compared to control), whereas *atg5-1* maintained higher shoot FW and DW values (Fig. 2D). In roots, WT and *atg2-1* exhibited moderate decreases (approximately -10%), while *atg5-1* remained largely unaffected (Fig. 2F).

The shoot-to-root ratio increased significantly in *atg5-1* under prolonged Pi-starvation, while *atg2-1* remained comparable to WT (Fig. 2H).

Autophagy deficiency alters Pi accumulation and remobilization dynamics across tissues and time

To investigate the role of autophagy in Pi homeostasis and remobilization during Pi limitation, free inorganic Pi and total P content were quantified in shoots and roots. We additionally analyzed free Pi-to-total Pi ratios, shoot:root distribution, and relative changes between control and Pi-starvation to assess remobilization dynamics (Fig. 3; Fig. 4).

At the onset of the experiment (DAT0), shoots showed no genotypic differences in free or total Pi content (Fig. 3A, B). However, *atg5-1* roots exhibited a ~65% increase in free Pi compared to WT (Fig. 3D). The free Pi-to-total P ratio in shoots was comparable across genotypes (Fig. 3C). In contrast, roots of both *atg* mutants exhibited higher values than WT (27% in WT vs. 35% in *atg2-1* and *atg5-1*), indicating a higher proportion of Pi in free form. Additionally, the shoot:root ratio of free Pi was elevated in *atg5-1* compared to WT, while *atg2-1* showed no difference (Fig. 3F). These initial differences indicate that autophagy contributes to basal Pi partitioning and homeostasis under control conditions, with *atg5-1* showing enhanced root free Pi accumulation, consistent with altered internal Pi homeostasis even under Pi sufficiency.

At DAT3, a significant decrease in shoot free Pi content was observed across all genotypes (Fig. 4A). However, both *atg2-1* and *atg5-1* accumulated significantly less free Pi in shoots than WT under Pi-starvation, with reductions of approximately 68% and 48% relative to WT, respectively. These differences were already evident under control conditions, where both mutants displayed ~20% lower shoot free Pi than WT, indicating a reduced basal shoot Pi pool. In contrast, root free Pi levels at DAT3 were not significantly affected by Pi-starvation in any genotype, and no statistically significant differences were detected between WT and mutants under either condition. Consequently, relative changes in free Pi (% -Pi / +Pi) revealed a significantly stronger decline in shoot free Pi in both *atg* mutants compared to WT, most pronounced in *atg5-1* also in roots (Fig. 4B). Pi partitioning analyses further supported altered remobilization dynamics. The free Pi-to-total P ratios in shoots were comparable among genotypes under control and Pi-starvation (Fig. 4C). Conversely, in roots, both mutants exhibited reduced free Pi-to-total Pi ratios under Pi-starvation compared to WT. Accordingly, shoot-to-root ratios of free Pi decreased under Pi-starvation in all genotypes; however, these

ratios remained significantly lower in *atg2-1* and *atg5-1* compared to WT, consistent with reduced root-to-shoot Pi allocation in the mutants (Fig. 4D).

At DAT7, genotypic differences in Pi allocation became more pronounced. Under Pi-sufficient conditions, shoot free Pi followed the pattern observed in DAT3 (Fig. 4A). Under Pi-starvation, both *atg2-1* and *atg5-1* exhibited a marked decrease in shoot free Pi relative to WT, with reductions of approximately 36% and 47%, respectively. In contrast to shoots, roots of *atg* mutants accumulated substantially more free Pi than WT under Pi-starvation, with *atg5-1* showing the strongest accumulation. Relative change analysis reinforced these trends, while WT showed a strong decline in free Pi between DAT3 and DAT7, *atg5-1* exhibited a modest decrease, suggesting sustained Pi retention over time (Fig. 4B). This divergence indicates that autophagy deficiency modifies the temporal dynamics of Pi depletion during prolonged starvation. The free Pi-to-total P ratio in shoots was significantly reduced in *atg5-1* under both control and Pi-starvation, whereas WT maintained higher ratios (Fig. 4C). In roots, *atg5-1* exhibited a tendency of increased free Pi-to-total P ratio under Pi-starvation compared to WT and *atg2-1*. These tissue-specific changes culminated in altered shoot-to-root Pi distribution. Under Pi-starvation, WT plants showed an elevated shoot-to-root ratio of free Pi relative to control, while *atg5-1* exhibited a significant decrease (Fig. 4D), indicating enhanced Pi retention in roots and impaired root-to-shoot Pi remobilization.

Autophagy shapes time- and tissue-specific proteome remodeling during Pi-starvation

To investigate whether the altered biomass allocation and Pi partitioning observed in *atg* mutants are associated with changes in proteome organization, we performed Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS) in shoots and roots across Pi-starvation progression. Given that *atg* mutants already exhibit impaired growth and Pi distribution under Pi-sufficient conditions, we included a baseline proteomic assessment prior to starvation onset (DAT0). This design enabled separation of constitutive genotype effects from Pi-starvation-induced remodeling.

Autophagy deficiency reshapes basal shoot and root proteomes under Pi-sufficient conditions

To define constitutive effects of impaired autophagy prior to Pi depletion, we compared shoot and root proteomes of *atg2-1* and *atg5-1* with WT at DAT0. Principal component analysis (PCA) showed clear separation of WT and *atg* mutants in both tissues (Fig. 5A, C). In shoots, PC1 (23.7% variance) separated WT from *atg* mutants (Fig. 5A), whereas in roots, PC1 (23.3%) and PC2 (11.2%) resolved WT, *atg2-1*, and *atg5-1* into discrete clusters (Fig. 5C), indicating a

stronger basal impact of autophagy deficiency in below-ground tissues. Direct comparison of mutant-versus-WT fold changes revealed strong concordance between *atg2-1* and *atg5-1* in shoots ($r = 0.87$, $R^2 = 0.76$; Fig. 5B) and roots ($r = 0.82$, $R^2 = 0.67$; Fig. 5D), consistent with a shared autophagy-dependent proteome signature.

Volcano-plot analyses identified extensive, tissue-dependent protein abundance changes in both mutants (Fig. 6A-D), with the most pronounced remodeling in *atg5-1* roots (Fig. 6C, D). Overlap analyses confirmed that a substantial fraction of differentially abundant proteins was shared between *atg2-1* and *atg5-1* in shoots (329 commonly increased; 219 commonly decreased; Fig. 6E, F) and especially in roots (661 commonly increased; 814 commonly decreased; Fig. 6G, H). Integrating genotype and tissue identified a core set shared across tissues (up: 206; down: 98) as well as tissue-specific protein sets (roots: up 344, down 572; shoots: up 59, down 94; Fig. 6I, J).

Functional enrichment analyses showed that proteins increased in abundance in both shoot and root were enriched for lipid metabolism (including fatty-acid degradation), redox, secondary metabolism, and components of endomembrane system, including peroxisomes, cytosol, ER, and COPII coatomer components (Fig. 7). In contrast, proteins involved in amino acid biosynthesis reduced in both tissues and genotypes.

In shoots, decreased proteins were enriched for photosynthesis-related categories, including plastid functions, photophosphorylation, and coenzyme metabolism, including tetrapyrrole metabolism. Additional reductions were observed in *atg2-1* for chlororespiration, Calvin cycle, and photosystem II components, whereas *atg5-1* showed reduced abundance of proteins related to chloroplast biogenesis, organellar protein translation and translocation, and selected amino acid biosynthetic pathways, including Lys and Arg. Conversely, increased proteins in shoots, particularly in *atg5-1*, were enriched in carbohydrate metabolism, vesicle trafficking (e.g. membrane tethering and fusion regulation, and 26S proteasome), protein N-linked glycosylation, and aminoacyl-tRNA synthetases.

In roots, proteins increased in abundance were enriched for mitochondrial and respiratory functions, including the TCA cycle, oxidative phosphorylation, and amino acid and nucleotide degradation. In parallel, decreased proteins were enriched for multiple compartments and trafficking-related functions, including nucleus, plasma membrane, vacuole, extracellular space, and clathrin-mediated trafficking modules (including the TPLATE AP-2 co-adaptor complex and clathrin-coated vesicle machinery), together with transporters/channels (including MIP family proteins) and RNA/DNA-associated categories (RNA splicing, histones, and chromatin-related functions). Genotype-specific features included

enrichment of porins in *atg2-1* and protein S-glutathionylation/ deglutathionylation in *atg5-1*, suggesting differential redox and membrane-associated adjustments.

A genotype-dependent metabolic feature was enrichment of sucrose synthesis in both shoots and roots of *atg5-1*, indicating altered carbohydrate partitioning independent of tissue identity. Hormone-associated functions were enriched in roots of both mutants and selectively in shoots of *atg2-1*, whereas thioredoxin-related functions were enriched in roots of both mutants and selectively in shoots of *atg5-1*, indicating genotype-specific redox and signaling signatures.

Interestingly, proteins associated with PSR were already altered at DAT0 in a tissue specific manner. In roots, the high-affinity phosphate transporter PHT1-1 showed reduced abundance in both mutants, whereas no changes were detected in shoots. In addition, root phosphatase 12 and phosphatase 26 were also reduced in both mutants, while Multiple Inositol Polyphosphate Phosphatase 1 (MIPP1) was increased in shoots.

Global proteome structure reveals tissue-specific remodeling under short Pi-starvation

To assess early Pi-starvation responses, we compared shoot and root proteomes of *atg*-mutants with WT at DAT3. Multivariate analyses indicated that early Pi limitation did not override the autophagy-dependent proteome configuration established under Pi-sufficient conditions; instead, it reinforced and stratified this configuration in a tissue- and genotype-dependent manner.

In shoots, PCA indicated that autophagy deficiency was the primary source of variance (PC1, 27.4%), separating WT from both mutants largely independent of Pi availability (Fig. 8A). The responses between *atg2-1* and *atg5-1* remained highly correlated under both control ($r=0.89$, $R^2=0.80$; Fig. 8B) and Pi-starvation ($r=0.88$, $R^2=0.78$; Fig. 8C) conditions. Volcano plots revealed extensive differential abundance in both mutants relative to WT under control and Pi-starvation (8D-G). Venn analyses identified a large constitutive core shared across mutants and conditions (up: 715; down: 927) alongside a smaller set was specifically induced in both mutants under Pi-starvation (up: 113; down: 79) (Fig. 8H, I).

Functional enrichment analysis showed that proteins increased in abundance retained strong signatures of lipid catabolism, carbohydrate metabolism, secondary metabolism, and redox processes, together with sustained enrichment of vesicle trafficking, especially endomembrane and secretory pathway components, and membrane tethering/fusion (Fig. 9A). Within this shared framework, genotype-specific patterns emerged: in *atg2-1*, increased proteins were enriched for phenylpropanoid metabolism, ABA, nucleotide sugar biosynthesis,

and oxidoreductase activities under both conditions, whereas TCA-cycle was restricted to control. Under Pi-starvation, *atg2-1* showed enrichment of COPI coatomer and Golgi tethering factors, whereas *atg5-1* exhibited enrichment of autophagy-related processes, including the ATG8/ATG12 conjugation system, together with reduced abundance of proteins related to photorespiration. Proteins with decreased abundance consistently reflected repression of plastidial/photosynthetic and anabolic functions across genotypes and conditions. Additional depletion in *atg5-1* under Pi-starvation included photorespiration (Fig. 9B).

In roots, PCA revealed a clear separation of genotypes and treatments (Fig. 10A). PC1 (22.3%) primarily distinguished WT from *atg* mutants, whereas PC2 (9.0%) captured variance related to Pi availability, most evident in *atg5-1*. The responses between *atg2-1* and *atg5-1* remained strongly correlated under control ($r = 0.76$, $R^2 = 0.80$; Fig. 10B) and Pi-starvation ($r = 0.83$, $R^2 = 0.78$; Fig. 10C) conditions. Volcano plots revealed extensive remodeling in both mutants (Fig. 10D-G), with Pi-starvation amplifying changes in *atg2-1* (1,161 up; 1,683 down) compared to control (706 up; 1,072 down), whereas *atg5-1* displayed substantial alterations already under control conditions. Venn analyses showed a similar altered number of proteins conserved across mutants and conditions (up: 713; down: 756), compared to DAT0 (Fig. 10H, I). Consistent with the volcano plots, *atg2-1* showed a larger Pi-specific response (up: 253; down: 376), compared to *atg5-1* (up: 91; down: 127).

Functional enrichment analyses of proteins increased in abundance revealed signatures of lipid catabolism, carbohydrate metabolism, amino-acid turnover, redox, and aminoacyl-tRNA synthetase activities, associated with vesicle trafficking (COPII and autophagosome formation), and 26S proteasome for both genotypes and conditions (Fig. 11A). Genotype-specific Pi-responsive features included enrichment of SnRK1 metabolic regulator system and respiratory/catabolic categories (TCA cycle, and carbohydrate and sucrose synthesis) in *atg2-1*, whereas *atg5-1* showed sustained mitochondrial enrichment under control (OXPHOS-complexes I, III and IV) and additional induction of glycolysis, carbohydrate degradation, secondary metabolism, acyltransferase activities, and N-linked glycosylation under Pi-starvation. Decreased proteins broadly reflected repression of transport capacity, including MIP family, V-type ATPase, and proteins associated with cell wall, and DNA/RNA-related processes for both genotypes and conditions (Fig. 11B); additional genotype-specific losses included repression of BCAA synthesis in *atg2-1*, cell cycle and sulfur assimilation in *atg5-1*, and carbohydrate handling and nutrient uptake in both mutants.

At the level of Pi acquisition, early Pi-starvation was associated with reduced abundance of PHT1-1 in both mutants under both conditions, while PHT1-4 was specifically reduced under

Pi-starvation. Conversely, PHT1-1 and PHT1-4 increased just in shoots of *atg2-1* under Pi-starvation.

Global proteome structure reveals differential tissue remodeling under prolonged Pi-starvation

To determine how prolonged Pi limitation reshapes shoot proteome organization, we compared shoot and root proteomes of *atg2-1* and *atg5-1* with WT at DAT7 under control and Pi-starvation. PCA indicated that autophagy deficiency remained the dominant determinant of proteome structure in shoots (PC1 ~26%; Fig. 12A) and roots (PC1 ~21%; Fig. 14A) largely independent of Pi status. Pi-starvation increased secondary dispersion, most notably in *atg5-1* shoots, consistent with enhanced condition-dependent divergence over time. Scatter plots showed continued correlation between *atg2-1* and *atg5-1* DAPs at DAT7 in shoots (control: $r=0.82$, $R^2=0.80$; Pi-starvation: $r=0.82$, $R^2=0.78$) (Fig. 12B, C) and roots (control: $r=0.90$, $R^2=0.80$; Pi-starvation: $r=0.90$, $R^2=0.78$) under both conditions (Fig. 14B, C), indicating a conserved autophagy-dependent component despite progressive stress.

In shoots, volcano plots revealed extensive remodeling of the proteome that intensified under Pi-starvation, particularly in *atg5-1* (up: 1,882; down: 2,333) (Fig. 12.D-G). Venn diagram analyses reinforced this pattern, with Pi-starvation triggering a pronounced response in *atg5-1* (up: 702; down: 574) compared to *atg2-1* (up: 105; down: 126) (Fig. 12H, I).

Functional enrichment analyses showed sustained activation of lipid catabolism, carbohydrate and amino acid degradation, redox, aminoacyl-tRNA synthetases, and extensive proteostasis and vesicle trafficking (ATG8/ATG12 conjugation system, 26S proteasome, membrane tethering and fusion, and COPII) under both genotypes and conditions (Fig. 13A). Notably, *atg5-1* displayed a Pi-dependent shift toward mitochondrial/respiratory metabolism (e.g., glycolysis, TCA cycle, OXPHOS, and BCAA degradation), together with enrichment of mevalonate pathway, ABA, RAB-GTPase activities, PDIL, protein translocation ER, and the ATG9-ATG2-ATG18 complex. Decreased proteins showed enrichment in categories related to photosynthetic and anabolic functions, including Calvin-cycle, photophosphorylation, and chlorophyll metabolism, together with amino acid and fatty acid synthesis, primary active transport, amino acid synthesis and RNA processing (e.g. PPR proteins) in both genotypes and conditions (Fig. 13B). Under Pi-starvation, chloroplast redox homeostasis and channels categories were enriched in both mutants, while photorespiration, starch synthesis and degradation, and cell wall (e.g. pectin) were just enriched in *atg5-1*.

In roots, prolonged Pi limitation further intensified proteome remodeling, with a more pronounced response in *atg5-1* (up: 1,058; down:1,782) compared to *atg2-1* (up:532; down:

919) (Fig. 14D-G). Venn analysis identified a conserved set across mutants and conditions (458 upregulated and 749 downregulated DAPs) (Fig. 14H, I). In addition, Pi-starvation induced a larger condition-specific response in *atg5-1* (up: 401; down: 438) compared to *atg2-1* (up: 108; down: 85).

Functional enrichment revealed a consolidated catabolic and proteostatic state across mutants and conditions, characterized by lipid metabolism (lipid and amino acid degradation), protein metabolism (26S proteasome), redox, and vesicle trafficking (autophagosome formation) (Fig. 15A-D). In *atg2-1*, proteins associated with protein synthesis, nucleotide and carbohydrate metabolism were enriched under both conditions, with additional enrichment of nucleotide degradation and metal handling pathways under Pi-starvation. In contrast, *atg5-1* displayed enrichment in TCA cycle, glycine synthesis, and porins under both conditions, and BCAA degradation specifically under Pi-starvation. SnRK1 kinase complexes were enriched in both mutants specifically under Pi-starvation.

Proteins with decreased abundance in roots reflected broad repression of RNA (RNA processing and biosynthesis), DNA (histone), transport (primary active transport) and cell (e.g. cell cycle) (fig. 15B). Under Pi-starvation, proteins associated with cell wall decreased in both *atg* mutants. Additional genotype-specific losses included repression of amino acid metabolism and nutrient uptake in *atg2-1*, while ribosome biogenesis, RNA modification and splicing, DNA synthesis/chromatin structure, and Golgi decreased in *atg5-1* under Pi-starvation.

Consistently with these changes, Pi transport capacity was strongly reduced in roots, with PHT1-1, PHT1-2, PHT1-3, and PHT1-4 decreased in both mutants under Pi-starvation, whereas in shoots PHT1-1 increased specifically in *atg5-1*.

DISCUSSION

Plants respond to Pi limitation through coordinated adjustments in growth, metabolism, nutrient acquisition, and internal nutrient redistribution that optimize resource use and sustain essential cellular functions, requiring extensive metabolic and cellular reprogramming (Müller et al. 2015; Li et al. 2022a; Smith et al. 2025). Although transcriptional regulation has long been considered a primary driver of the PSR, increasing evidence indicates that proteome remodeling is also a critical component of acclimation (Dissanayaka et al. 2021a; Li et al. 2022a; Smith et al. 2025).

Here, by integrating time-resolved physiological analyses with organ-specific quantitative proteomics, we demonstrate that autophagy is a major determinant of the temporal and tissue-specific organization of the PSR. Autophagy deficiency established distinct basal

proteome configurations in shoots and roots prior to Pi deprivation, indicating that the autophagy machinery contributes to the maintenance of cellular homeostasis even under Pi-sufficient conditions. These constitutive differences were accompanied by altered biomass allocation, Pi partitioning, and tissue-specific proteome remodeling during subsequent Pi-starvation, suggesting that the physiological response to Pi limitation is strongly influenced by the metabolic state established before stress onset. Similar constitutive metabolic and proteomic alterations have been reported in *atg* mutants under nitrogen limitation and carbon starvation (Guiboileau et al. 2012; Avin-Wittenberg et al. 2015; Masclaux-Daubresse 2016; Havé et al. 2019; McLoughlin et al., 2020). Our results extend this concept to Pi-starvation and further reveal that the consequences of autophagy deficiency are not uniform across the plant but instead manifest through distinct shoot and root responses that become increasingly pronounced as Pi deprivation progresses.

Importantly, the constitutive proteomic differences observed at DAT0 indicate that autophagy deficiency establishes a distinct physiological state before the onset of Pi limitation. Consequently, the responses observed during Pi-starvation likely reflect both direct defects in autophagy-dependent nutrient recycling and indirect effects arising from altered basal homeostasis.

Autophagy deficiency perturbs growth and biomass allocation under prolonged Pi stress

During the early phase of Pi-starvation, the reduction in root biomass observed in *atg5-1* suggests that functional autophagy supports root growth. This interpretation is consistent with evidence that autophagy is constitutively active in root meristematic and elongation zones and contributes to basal root development, cell division and elongation (Inoue et al. 2006; Chiu et al. 2023b). Moreover, Pi limitation rapidly activates autophagy in the root apex as part of the local stress response (Naumann et al. 2019a), indicating that disruption of basal autophagic activity may impair growth under Pi deficiency.

More pronounced divergence emerged during prolonged Pi-starvation. The maintenance of shoot biomass in *atg5-1*, in contrast to the decline observed in WT and *atg2-1*, reflects an altered growth adjustment to nutrient limitation. The increased shoot:root ratio in *atg5-1* indicates that biomass allocation between above- and below-ground tissues differs from the pattern observed in WT plants. Whereas WT and *atg2-1* exhibited reductions in both shoot and root biomass under Pi limitation, *atg5-1* maintained biomass in both organs, resulting in a relative increase in shoot investment. These findings suggest that disruption of autophagy affects the coordination of growth responses during prolonged Pi-starvation rather than simply

reducing growth capacity. Because shoot and root growth are regulated through the integration of nutrient status, carbon availability, and long-distance signaling pathways, altered biomass partitioning in *atg5-1* may reflect changes in how resource allocation decisions are coordinated under nutrient stress (Wang and Ruan 2016; Fañanás-Pueyo et al. 2025). Consistent with the established role of autophagy in nutrient recycling and metabolic homeostasis (Guiboileau et al. 2013; Masclaux-Daubresse et al. 2014, 2017), impaired autophagic flux may alter the balance between growth maintenance and resource conservation during prolonged Pi limitation.

Autophagy governs systemic Pi remobilization and prevents root Pi sequestration

Our analysis of Pi homeostasis provides a mechanistic basis for the altered growth phenotypes of *atg* plants (Fig. 3; Fig. 4). A key observation is the accumulation of free Pi in roots of *atg* mutants under Pi-sufficient conditions, indicating that autophagy contributes to basal Pi partitioning even in the absence of external stress (Yoshitake et al. 2022; Yoshitake and Yoshimoto 2023). Thus, autophagy deficiency establishes a pre-existing imbalance in Pi distribution that predisposes plants to maladaptive responses once external Pi availability declines.

A central outcome of this study is that autophagy deficiency leads to progressive alterations in Pi allocation between roots and shoots, characterized by reduced shoot Pi accumulation and, during prolonged Pi-starvation, increased retention of Pi in roots, particularly in *atg5-1*. This imbalance cannot be explained by uniform depletion across tissues and is consistent with altered root-to-shoot Pi redistribution. The observed changes in shoot-to-root Pi ratios, together with contrasting Pi dynamics between roots and shoots, suggest that autophagy contributes to the coordination of whole-plant Pi distribution during nutrient limitation. Given that maintenance of shoot Pi pools is essential to sustain photosynthetic metabolism and growth under Pi deficiency, the progressive depletion of shoot Pi observed in *atg* mutants likely contributes directly to their physiological and metabolic phenotypes. Consistent with this interpretation, *atg* mutants exhibit reduced shoot-to-root Pi ratios and altered Pi mobilization during Pi limitation (Naumann et al. 2019a; Chiu et al. 2023b). Mechanistically, autophagy contributes not only to intracellular Pi recycling but also to the maintenance of Pi transporter homeostasis, as loss of autophagy accelerates the degradation of key PHT1 transporters and perturbs long-distance Pi allocation (Chiu et al. 2023b; Fang et al. 2025).

Already during early Pi-starvation, both *atg* mutants exhibited reduced free Pi levels in shoots relative to WT, indicating that autophagy contributes to the maintenance of shoot Pi

pools from the onset of Pi limitation. As starvation progressed, these differences became more pronounced. WT plants maintained substantially higher shoot-to-root Pi ratios, whereas *atg* plants, particularly *atg5-1*, displayed a marked decline in this ratio driven by reduced shoot Pi and increased root Pi accumulation. This divergence highlights the strong tissue specificity of the autophagy-dependent response to Pi limitation. While roots of *atg* mutants exhibited reduced abundance of Pi transporters together with repression of transport-related functions and enrichment of catabolic pathways, shoots experienced progressive Pi depletion accompanied by repression of photosynthetic and carbon-assimilation processes. These observations suggest that autophagy contributes not only to intracellular nutrient recycling but also to the coordination of nutrient allocation between organs during Pi stress. This interpretation is consistent with previous studies showing that Pi-starvation activates autophagy in roots, particularly in the root apex, where it contributes to local nutrient recycling and adaptive responses to phosphate deprivation (Naumann et al. 2019a). Our data extends these observations by indicating that disruption of autophagy compromises both local Pi homeostasis and whole-plant Pi distribution during prolonged Pi limitation.

Several mechanisms supported by our proteomic data may contribute to this phenotype. Most notably, roots of both *atg* mutants exhibited progressive reductions in the abundance of high-affinity phosphate transporters, including PHT1-1, PHT1-2, PHT1-3, and PHT1-4, particularly during prolonged Pi-starvation. In parallel, extensive alterations in proteins associated with vesicle trafficking, including COPI/COPII components, clathrin-associated pathways, and membrane tethering machinery, indicate disruption of endomembrane processes involved in transporter localization and turnover. Together, these changes are consistent with reduced capacity to maintain efficient Pi allocation during Pi limitation. In addition, the reduction in abundance of proteins related to photosynthetic and carbon-assimilation pathways in shoots may reduce metabolic demand for Pi, further contributing to altered tissue partitioning during prolonged stress. The elevated free Pi-to-total P ratio observed in roots of *atg5-1* further suggests altered utilization of intracellular Pi pools when autophagy-dependent recycling is compromised, consistent with metabolic phenotypes reported under Pi limitation in *atg* plants (Li et al. 2022a).

The more pronounced phenotype of *atg5-1* compared with *atg2-1* highlights functional specialization and hierarchy within the core autophagy machinery. The ATG5-ATG12 conjugation system is indispensable for autophagosome biogenesis, because it is required for ATG8 lipidation and phagophore expansion; its disruption results in a near-complete loss of canonical autophagic flux (Liu and Bassham 2012). In contrast, ATG2 primarily functions in

phagophore membrane expansion and lipid transfer, such that autophagosome formation may be impaired but not fully abolished in *atg2-1* mutants. This distinction is particularly relevant for processes that rely on continuous membrane trafficking, including the regulation of Pi transporters and associated trafficking machinery. Consistent with this view, the more severe defects in Pi partitioning observed in *atg5-1* coincide with pronounced alterations in vesicle trafficking and changes in the abundance of Pi transporters, suggesting compromised regulation of transporter pools and their dynamic subcellular distribution. By contrast, partial preservation of trafficking capacity in *atg2-1* may allow limited transporter redistribution, thereby mitigating defects in systemic Pi allocation. Together, these findings support a model in which ATG5-dependent autophagy couples membrane trafficking to transporter homeostasis to sustain root-to-shoot Pi redistribution under nutrient limitation.

Given that Pi availability constrains ATP production and redox balance, impaired shootward Pi allocation is expected to have profound consequences for photosynthetic and respiratory metabolism.

Autophagy coordinates photosynthetic carbon fixation and respiratory energy production under Pi-starvation

Phosphate availability imposes a fundamental constraint on plant energy metabolism by directly limiting ATP synthesis, adenylate balance, and carbon flux through both plastidial and mitochondrial pathways, thereby necessitating extensive metabolic reprogramming under Pi limitation (Plaxton and Tran 2011; Lambers et al. 2015). Our results indicate that autophagy is a critical determinant of this metabolic reprogramming and that its disruption promotes a progressive and tissue-specific reorganization of energy metabolism during Pi-starvation.

Under Pi-sufficient conditions (DAT0), *atg* plants already exhibited reduced abundance of proteins associated with photosystems I and II, photophosphorylation, and plastidial metabolism. These changes indicate that autophagy contributes to maintenance of the basal photosynthetic proteome, consistent with its established role in chloroplast quality control and turnover under non-stress conditions (Izumi et al. 2017; Havé et al. 2019). Thus, *atg* mutants enter Pi-starvation with a pre-existing reduction in photosynthetic capacity.

During early Pi-starvation (DAT3), the shoot proteome remained largely characterized by this constitutive repression of plastidial functions, suggesting that the initial response to Pi limitation occurs on top of an already altered metabolic baseline. A notable exception was observed in *atg5-1*, where proteins associated with photorespiration were depleted under Pi-starvation. Beyond its role in carbon metabolism, photorespiration functions as a major hub

linking chloroplast, mitochondria, and peroxisomes through coordinated exchange of metabolites, reducing equivalents, and ATP demand (Peterhanse and Maurino 2011; Krone et al. 2025). Early repression of this pathway in *atg5-1* therefore suggests a reduced capacity to buffer fluctuations in chloroplastic redox state and ATP/NADPH balance during the early phase of Pi-starvation. The early impairment of photorespiratory capacity in *atg5-1* may also influence mitochondrial metabolism. Photorespiration also supplies mitochondrial NADH through glycine decarboxylation and contributes to the integration of chloroplastic and mitochondrial metabolism (Niessen et al. 2012; Keech et al. 2016). Consequently, reduced photorespiratory capacity could further restrict the ability of *atg5-1* to coordinate energy production between organelles during the early stages of Pi limitation.

By contrast, prolonged Pi-starvation (DAT7) induced a pronounced and coordinated repression of plastidial metabolism in both *atg* mutants under Pi-starvation. Proteins involved in RuBisCO function, photorespiration, and starch metabolism, including proteins associated with both synthesis and degradation, were markedly depleted, indicating a functional collapse of Calvin-Benson cycle capacity with downstream constraints on carbon export and storage metabolism. This response is consistent with the biochemical sensitivity of photosynthesis to Pi availability, as Pi is required to sustain photophosphorylation, adenylate balance, and Calvin cycle operation (Plaxton and Tran 2011). RuBisCO activation depends on ATP, whereas accumulation of ADP inhibits catalytic activity (Amaral et al. 2024). Accordingly, constrained photophosphorylation and adenylate imbalance under Pi-starvation are expected to limit carbon fixation. In addition, Pi depletion restricts regeneration of Calvin-cycle intermediates such as ribulose-1,5-bisphosphate and glyceraldehyde-3-phosphate, the latter also serving as a precursor for starch biosynthesis (Dissanayaka et al. 2021a). Impaired Calvin-cycle flux therefore propagates into reduced starch turnover, consistent with the proteomic depletion of starch metabolic enzymes observed at DAT7. Together, these changes indicate that *atg* shoots progressively lose the capacity to sustain plastidial carbon assimilation under prolonged Pi-starvation.

Concomitant with this decline in photosynthetic investment, prolonged Pi-starvation triggered a strong induction of mitochondrial metabolism in shoots, particularly in *atg5-1*. At DAT7, proteins associated with oxidative phosphorylation, the TCA cycle, BCAA degradation, and respiratory electron transport accumulated specifically in *atg5-1* under Pi-starvation. This shift is consistent with a compensatory attempt to maintain ATP production as photophosphorylation becomes increasingly constrained, matching established respiratory reprogramming responses to Pi-starvation (Plaxton and Tran 2011; Dissanayaka et al. 2021a).

Notably, proteins associated with cytosolic glycolysis, including phosphofructokinase isoforms, increased in *atg* plants under Pi-starvation (PFK3 in roots at DAT3, and PFK3, PFK4, and PFK5 in shoots at DAT7). Because phosphofructokinase catalyzes a major regulatory and rate-limiting step in glycolysis, its induction is consistent with increased investment in glycolytic capacity aimed at sustaining cytosolic carbon flux under ATP and Pi limitation. Similar glycolytic reinforcement under Pi limitation has been reported as part of the canonical metabolic PSR, in which plants attempt to maintain respiratory substrate supply despite reduced adenylate and free Pi availability (Dissanayaka et al. 2021a). However, while PFK abundance suggests increased enzymatic capacity, actual pathway flux may remain constrained by ATP, ADP, and cytosolic Pi pools, implying that this response may represent partial compensation rather than full restoration of glycolytic throughput.

An important aspect emerging from our proteomic analysis is that metabolic reprogramming under Pi-starvation differed markedly between shoots and roots. In shoots, autophagy deficiency was primarily associated with progressive repression of photosynthetic and carbon-assimilation processes, indicating reduced capacity to sustain plastidial energy production. In contrast, roots showed stronger enrichment of catabolic and respiratory pathways together with repression of transport- and growth-related functions. These contrasting responses likely reflect the distinct physiological roles of each organ during Pi limitation. While shoots depend on maintaining Pi supplies to support photosynthesis, roots function as the primary site of Pi acquisition and local nutrient recycling, requiring extensive metabolic adjustment to sustain nutrient uptake and stress adaptation. The stronger activation of catabolic pathways in roots therefore suggests that autophagy contributes to the metabolic flexibility required to support these tissue-specific demands during Pi-starvation.

BCAA degradation provides alternative respiratory substrates that bypass glycolytic constraints and supply carbon skeletons directly to the TCA cycle (Hildebrandt et al. 2015). Its selective induction in *atg5-1* suggests enhanced reliance on internal carbon and nitrogen reserves to sustain respiration in shoots under prolonged Pi stress. Concomitant enrichment of ABA-related functions in shoots of *atg5-1* suggests activation of stress-signaling pathways associated with metabolic adjustment and growth restraint under energy-limiting conditions (Baena-González and Sheen 2008; Belda-Palazón et al. 2022).

Consistent with the emergence of late-stage energy stress in roots, prolonged Pi-starvation was associated with enrichment of the SnRK1 pathway in *atg* plants. SnRK1 functions as a conserved cellular energy sensor that is activated under ATP depletion and carbon limitation, promoting catabolic metabolism while restraining biosynthetic investment (Baena-

González and Sheen 2008; Feng et al. 2025). SnRK1 promotes autophagy in *Arabidopsis* under energy stress, acting as a positive regulator of stress-induced autophagic flux (Soto-Burgos and Bassham 2017). At the same time, Pi-starvation can activate autophagy through TOR-independent, ER-stress-associated pathways in roots (Naumann et al. 2019a), indicating convergence of upstream signals on autophagy under Pi limitation. In this context, SnRK1 enrichment in *atg* roots likely reflects persistent energy stress and compensatory catabolic reprogramming, reinforcing growth restraint and linking impaired autophagy to systemic energy-stress signaling.

Together, our findings support a model in which autophagy acts as a metabolic buffer during Pi-starvation. In shoots, autophagy helps maintain photosynthetic carbon assimilation and delays the transition toward respiratory metabolism. In roots, it contributes to metabolic homeostasis and limits activation of energy-conservation pathways. Loss of autophagy therefore promotes tissue-specific metabolic reprogramming characterized by progressive photosynthetic repression in shoots and enhanced catabolic responses in roots, ultimately compromising whole-plant energetic homeostasis during prolonged Pi limitation.

CONCLUSION

We demonstrate that autophagy is a central regulator of Pi-starvation acclimation that coordinates phosphate redistribution and tissue-specific metabolic adaptation. Beyond its established role in intracellular recycling, autophagy preconditions the proteomic and metabolic state of the plant prior to stress and preserves metabolic flexibility as Pi limitation intensifies. In *atg* plants, impaired Pi allocation is associated with progressive shoot Pi depletion, root Pi retention, repression of photosynthetic metabolism, and enhanced reliance on glycolysis, respiration, and amino acid catabolism.

Collectively, our findings support a model in which autophagy safeguards Pi homeostasis and energetic stability during prolonged Pi limitation by coordinating nutrient allocation, transporter homeostasis, and tissue-specific metabolic reprogramming. Future studies addressing the mechanistic links between autophagy, membrane trafficking, Pi transporter regulation, and energy-stress signaling pathways such as SnRK1 will provide further insight into how plants integrate nutrient redistribution with metabolic acclimation under nutrient stress.

METHODS

Plant material and growth conditions

Arabidopsis thaliana accession Columbia (Col-0) and autophagy-deficiency knockout mutants, *atg2-1* (SALK_076727) and *atg5-1* (SAIL_129_B07), previously described by Inoue et al. (2006) and Yoshimoto et al. (2009b), were used in this study. Plants were cultivated under sterile hydroponic conditions using specialized glass jars equipped with a stainless steel wire mesh platform (125 μm mesh size), as described by Schlesier et al. (2003).

Seeds (10 mg) were sterilized with 70% (v/v) ethanol for 1 min followed by 6% (v/v) sodium hypochlorite for 5 min and washed six times with sterile water. Sterilized seeds were suspended in 0.15% (w/v) sterile agarose (Sigma-Aldrich, Hamburg, Germany) and adjusted to a final concentration of 5 mg seeds mL^{-1} . One milliliter of the seed suspension was dispensed onto each wire mesh platform, and glass jars were filled to the height of the platform with approximately 150 mL of nutrient solution.

The phosphate-sufficient (+Pi, control) nutrient solution contained 5 mM KNO_3 , 2.5 mM KH_2PO_4 , 2 mM MgSO_4 , 2 mM $\text{Ca}(\text{NO}_3)_2$, 50 μM Fe-EDTA, 70 μM H_3BO_3 , 14 μM MnCl_2 , 0.5 μM CuSO_4 , 1 μM ZnSO_4 , 0.2 μM NaMoO_4 , 10 μM NaCl , and 0.01 μM CoCl_2 , supplemented with 4.7 mM MES and 0.5% (w/v) Suc. For phosphate starvation (-Pi, Pi-starvation), KH_2PO_4 was omitted from the nutrient solution (Ticconi et al. 2001; Naumann et al. 2019a).

After stratification at 4°C for 2 to 3 days, seeds were germinated and cultivated in a controlled environmental chamber at 23 $\pm$ 2°C and a humidity of 60 $\pm$ 5% in long-day conditions (16 hr light [130 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$] and 8 hr dark). Ten days after sowing, seedlings were transferred to fresh nutrient solution containing either +Pi or -Pi medium and maintained for 3 or 7 days, corresponding to early and late responses to phosphate limitation, respectively, as illustrated in the experimental design (Fig. 1). Seedlings were transferred to Pi-free medium for 3 or 7 days, corresponding to early and late phases of Pi-starvation, respectively, as defined by time-dependent depletion of shoot Pi in WT plants (Fig. S1). Shoots and roots were harvested separately in the middle of the light period. For each genotype, treatment, and time point, material from four independent glass jars was pooled, frozen in liquid nitrogen, and stored at -80°C until further use.

Measurement of total, free, and esterified phosphate

Total and free Pi content were determined as previously described by Knowles and Plaxton (2013) with minor modifications. Dry material was homogenized with extraction buffer

(10 mM Tris, 1 mM EDTA, 100 mM NaCl, and 1mM b-mercaptoethanol, pH 8.0) at a ratio of 5 mg of sample to 250 mL extraction buffer. A total of 100 mL homogenized sample was mixed with 900 mL 1% glacial acetic acid and incubated at 42°C for 30 min. Then, 100 mL of the mixture was transferred to a glass tube for total P assay. The rest of the solution was centrifuged at 13,000g for 5 min, and 300 mL supernatant aliquot was used in the Pi assay. The reaction contained 700 mL assay solution (0.35% NH_4MoO_4 , 0.86 N H_2SO_4 , and 1.4% ascorbic acid) and 300 mL sample was incubated at 42°C for 30 min. Pi content was measured at 820 nm. For the total P assay, 30 mL of 10% $\text{Mg}(\text{NO}_3)_2$ in 95% ethanol was added to 100 mL of the aliquot. The samples were then dried and flamed to ash. After cooling, 300 mL of 0.5 N HCl was added to dissolve the samples at 65°C for 30 min. Total P content was measured as described for Pi by the addition of 700 mL of assay solution. Esterified phosphate was estimated as the difference between Total Pi and Free Pi. Phosphate concentrations were determined using a calibration curve based on known concentrations of inorganic phosphate standards and normalized by dry weight of biomass.

Protein extraction, digestion, and sample preparation for proteome analysis via mass spectrometry

The procedure is based on the single-pot solid-phase-enhanced sample preparations (SP4) protocol for proteomics experiments of Johnston et al. (2022). In brief, proteins were solubilized with sodium dodecyl sulfate (SDS) from lyophilized plant powder. Disulfide bridges were reduced with dithiothreitol (DTT) and subsequently alkylated with iodoacetamide (IAM). Glass beads were used to bind the denatured proteins. The beads were washed multiple times with 80 % ethanol to get rid of interfering components for the digestion. Finally, the proteins were digested overnight with trypsin (Mass Spectrometry Grade, Promega). The peptide-containing supernatants were collected and desalted on 50 mg Sep-Pak tC18 columns (Waters). The purified peptides were quantified with a quantitative Peptide Assay Kit (Thermo Scientific) and adjusted to 400 ng μl^{-1} in 0.1 % formic acid.

Quantitative Shotgun Proteomics by Ion Mobility Mass Spectrometry (IMS-MS/MS)

400 ng of peptides were injected via a nanoElute 2 UHPLC (Bruker Daltonik, Bremen, Germany) and separated on an analytical reversed-phase C18 column (Aurora Ultimate 25 cm x 75 μm , 1.6 μm , 120 Å; IonOpticks). Using MS grade water and a multi-staged gradient acetonitrile containing 0.1 % formic acid (0 min, 2 %; 54 min, 25 %; 60 min, 37 %; 62 min, 95 %; 70 min, 95 %), peptides were eluted and ionized by electrospray ionization with a

CaptiveSpray 2 ion source and a flow rate of 300 nl · min⁻¹. The ionized peptides were separated, fragmented and analyzed with a standard data-dependent acquisition parallel accumulation-serial fragmentation application method (DDA-PASEF) of the system with the following settings: Ion mobility window of 0.7-1.5 V s cm⁻², 4 PASEF ramps, target intensity 14,500 (threshold 1,200), and a cycle time of ~0.53 s. The analysis was performed on a timsTOF-HT mass spectrometer (Bruker Daltonik, Bremen, Germany).

Data processing and functional annotation

The ion mobility spectrometry (IMS-MS/MS) spectra from both experiments were analyzed with MaxQuant software (Cox and Mann 2008) using default search parameters and TAIR10 (Arabidopsis.org) as database for protein identification. The calculation of label-free quantification (LFQ) values and intensity-based absolute quantification (iBAQ) values were both enabled. Data evaluation was performed using Perseus software (Tyanova et al. 2016). Proteins were excluded from further analysis if they were not detected in at least n-1 biological replicates in at least one of the sample groups. Subsequently, missing values were replaced with randomly chosen low values from a normal distribution. Significant changes were calculated from the LFQ values using Student's t-test ($p = 0.05$).

Statistical analysis

The experiments were conducted using a completely randomized design with a minimum of four biological replicates per treatment. Phenotypic experiments were independently repeated at least twice to confirm reproducibility. Statistical analysis of growth and P content were first tested for normality prior to further analysis. Depending on the experimental design, differences among means were evaluated using either two-way factorial ANOVA, one-way ANOVA, or Student's *t*-test, with significance accepted at $P < 0.05$. For factors showing significant main or interaction effects in ANOVA, means were compared using Tukey's Honestly Significant Difference (HSD) post hoc test ($P < 0.05$). Statistical analyses were performed using R statistical software (www.r-project.org) with the ExpDes package. Graphs were generated in GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and RStudio (RStudio, Boston, MA, USA) using the ggplot2 package (RDC TEAM 2013).

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FIGURES

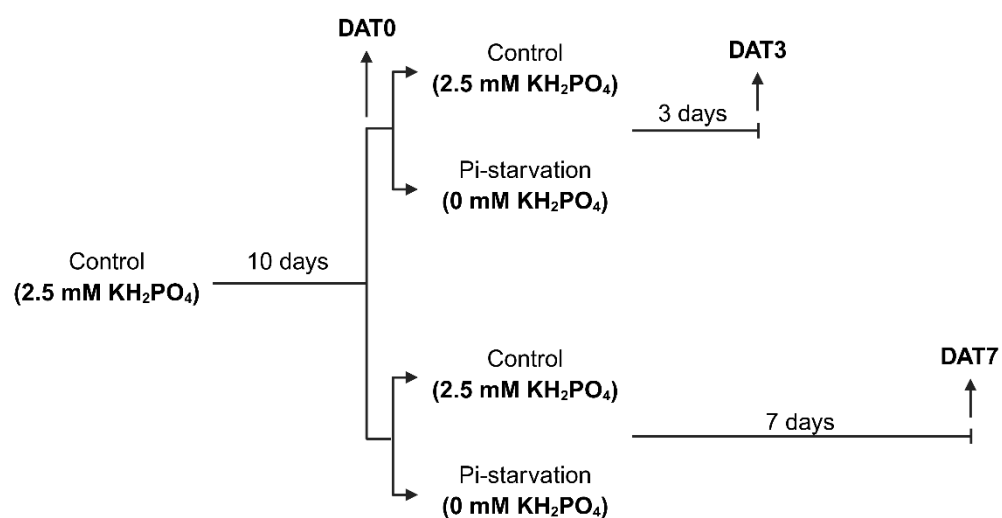


Figure 1. Experimental design for phosphate (Pi) starvation treatment. *Arabidopsis thaliana* seedlings were germinated and grown under control conditions (2.5 mM KH_2PO_4) for 10 days before being transferred (DAT0) to either control or Pi-starvation (0 mM KH_2PO_4) condition. Shoots and roots were harvested separately at 0, 3 and 7 days after transfer (DAT0, DAT3, and DAT7).

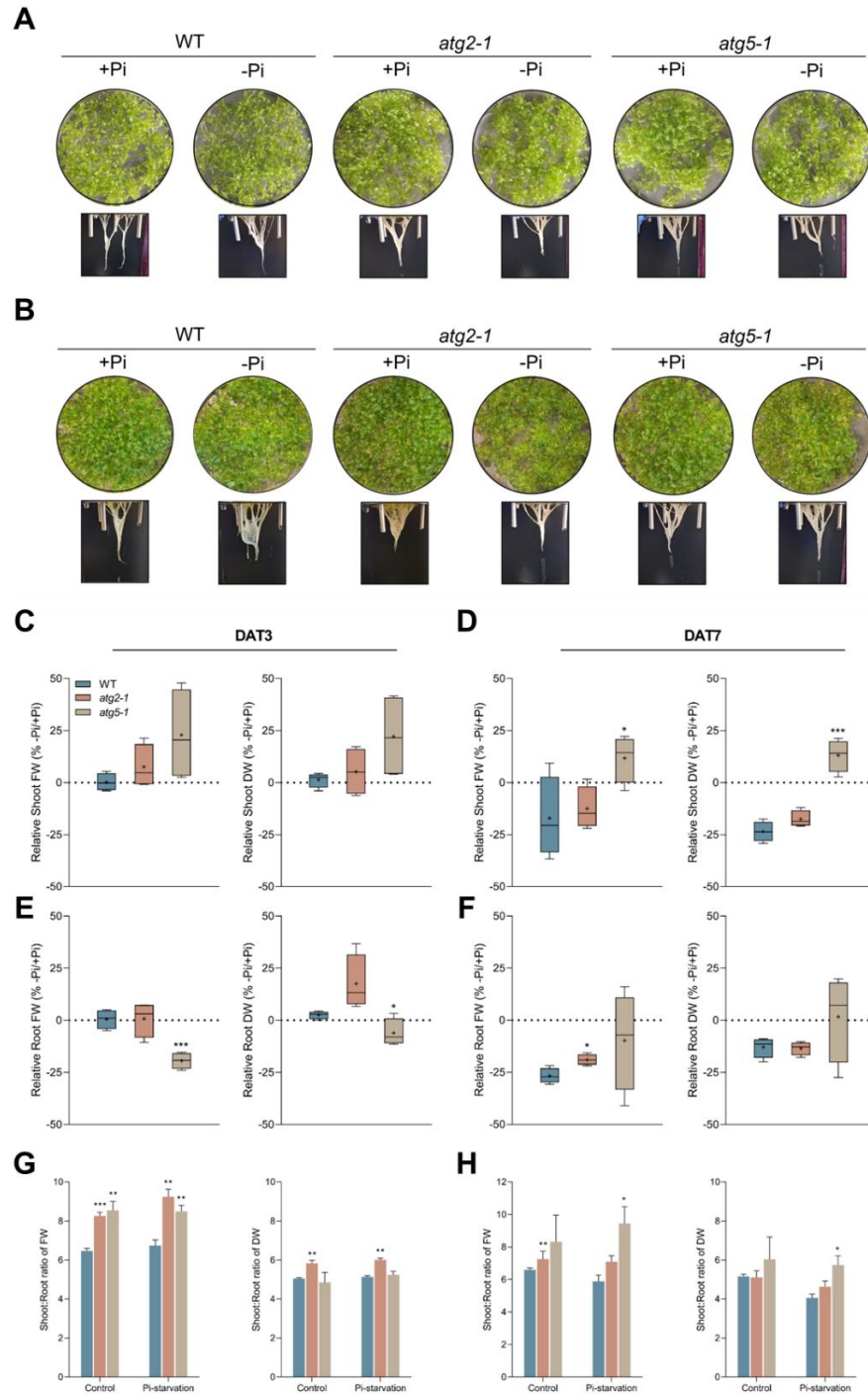


Figure 2. Autophagy deficiency affects the growth of *Arabidopsis thaliana* under Pi-starvation. (A-B) Representative images of *Arabidopsis atg* mutants under control (+Pi) and Pi-starvation (-Pi) conditions of (A) 3 days (DAT3) and (B) 7 days (DAT7). (C-F) Relative change in (C-D) shoot and (E-F) root fresh weight (FW) and dry weight (DW) after (C, E) 3 days and (D, F) 7 days of Pi-starvation. Data are expressed as the percentage difference between -Pi and +Pi conditions (% -Pi/+Pi), with the dotted line indicating no change relative to control. (G-H) Shoot-to-root biomass ratio based on FW and DW under +Pi and -Pi conditions at (G) DAT3 and (H) DAT7. Box plots show the median, interquartile range, and minimum/maximum values; crosses indicate means (+). Values presented are means $\pm$ SE ($n = 4$). Asterisks indicate significant difference based on two-tailed Student's *t*-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

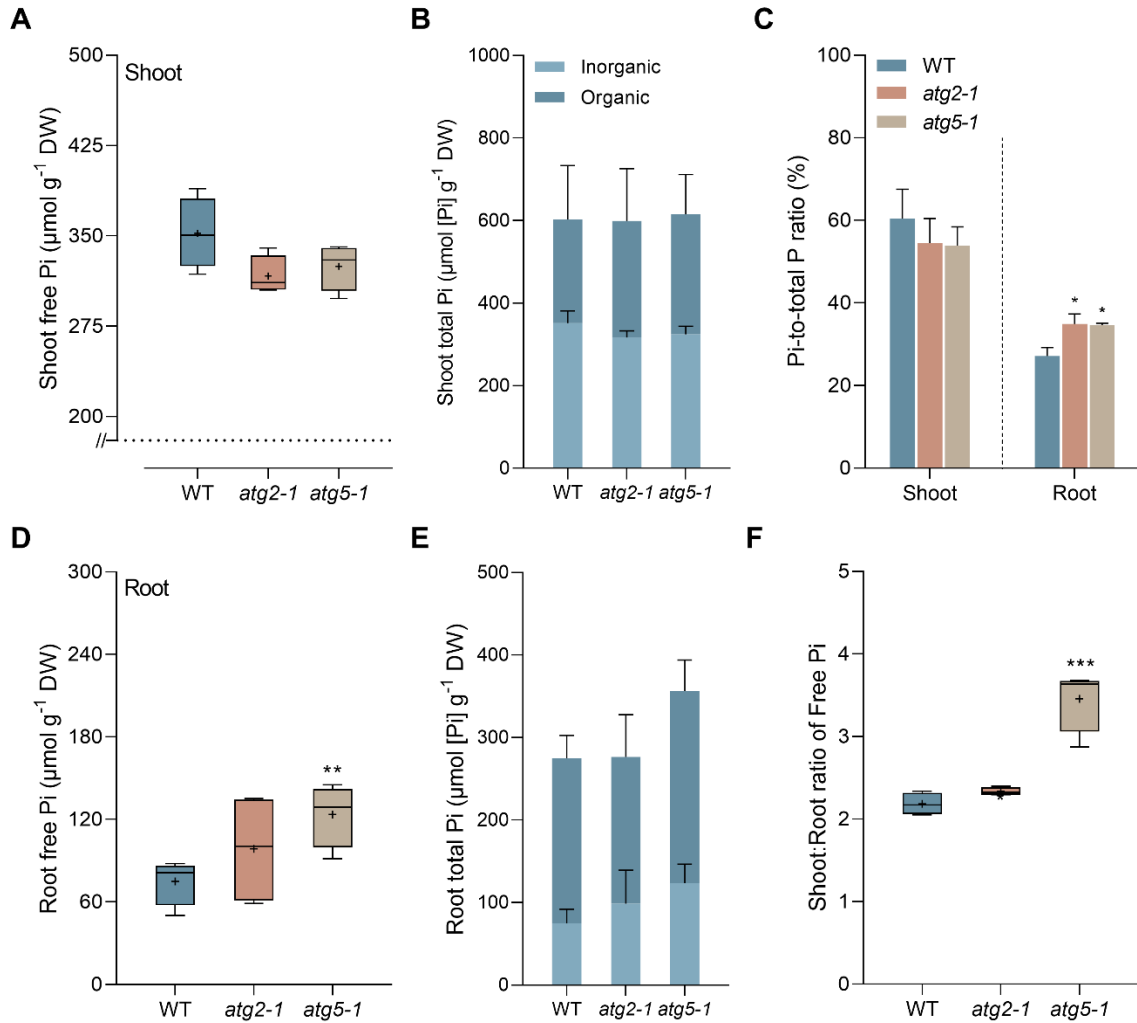


Figure 3. Baseline phosphate (Pi) content and partitioning in *Arabidopsis thaliana* prior to starvation. (A) Free inorganic phosphate content in shoots and roots. (B) Total phosphorus (P) content in shoots and roots, partitioned into inorganic and organic fractions. (C-D) Pi partitioning expressed as the ratio of (C) shoot Pi-to-total plant Pi and (D) root Pi-to-total plant Pi. Box plots show the median, interquartile range, and minimum/maximum values; crosses indicate means (+). Values presented are means $\pm$ SE ($n = 4$). Asterisks indicate significant difference based on two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

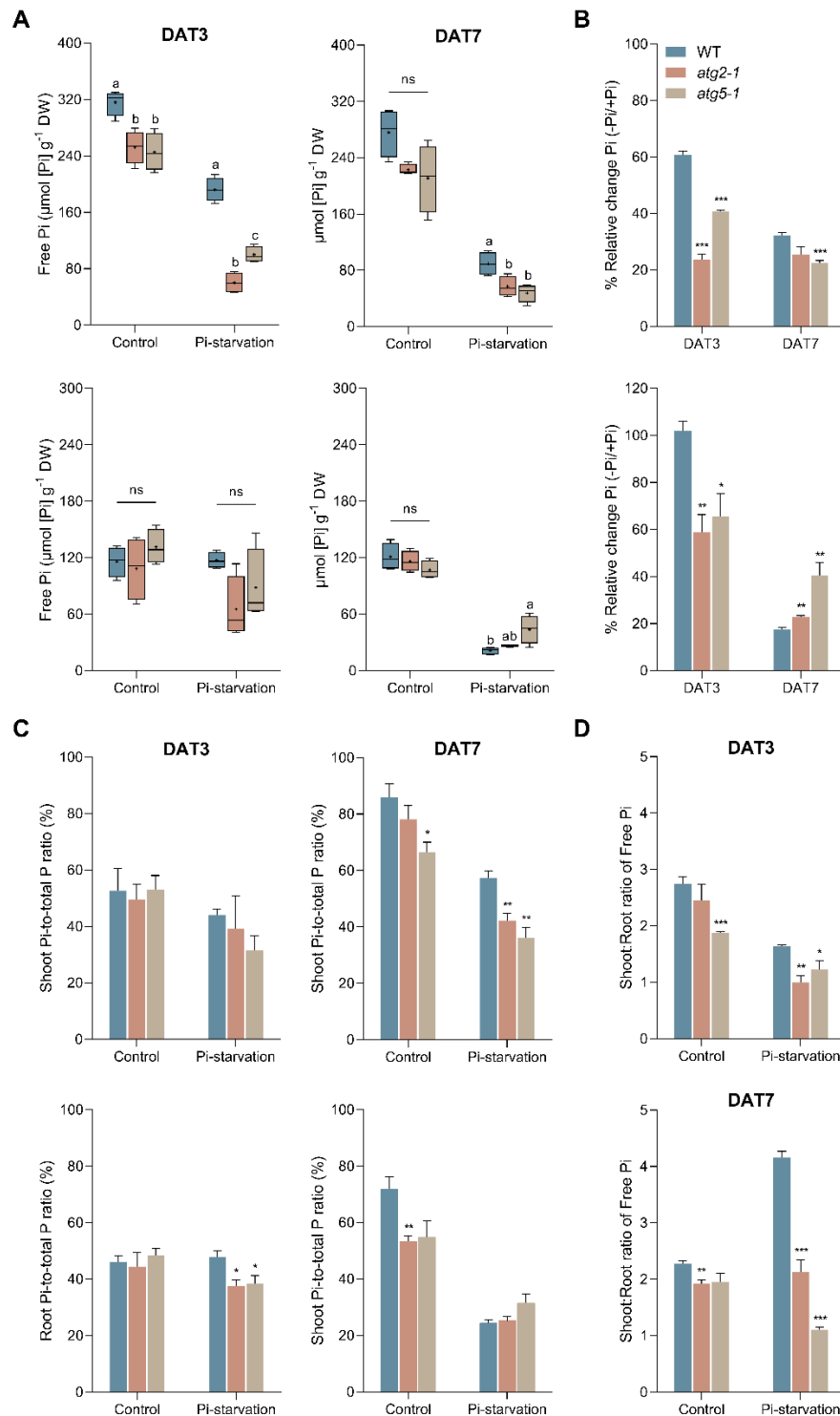


Figure 4. Autophagy deficiency affects phosphate (Pi) homeostasis under Pi-starvation. (A) Free Pi content in shoots (top) and roots (bottom) of *Arabidopsis thaliana* wild type (WT) and autophagy-deficient mutants (*atg2-1*, and *atg5-1*) under control and Pi-starvation conditions for 3 (DAT3) and 7 days (DAT7) after transfer; (B) Relative change in free Pi content expressed as the percentage of Pi-starvation relative to control (-Pi/+Pi); (C) Ratio of inorganic to total P (free Pi:total P) in shoots (top) and roots (bottom); (D) Shoot-to-root ratio of free Pi. Values presented are means $\pm$ SE (n = 4 biological replicates). Asterisks indicate significant difference based on two-tailed Student's t-test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

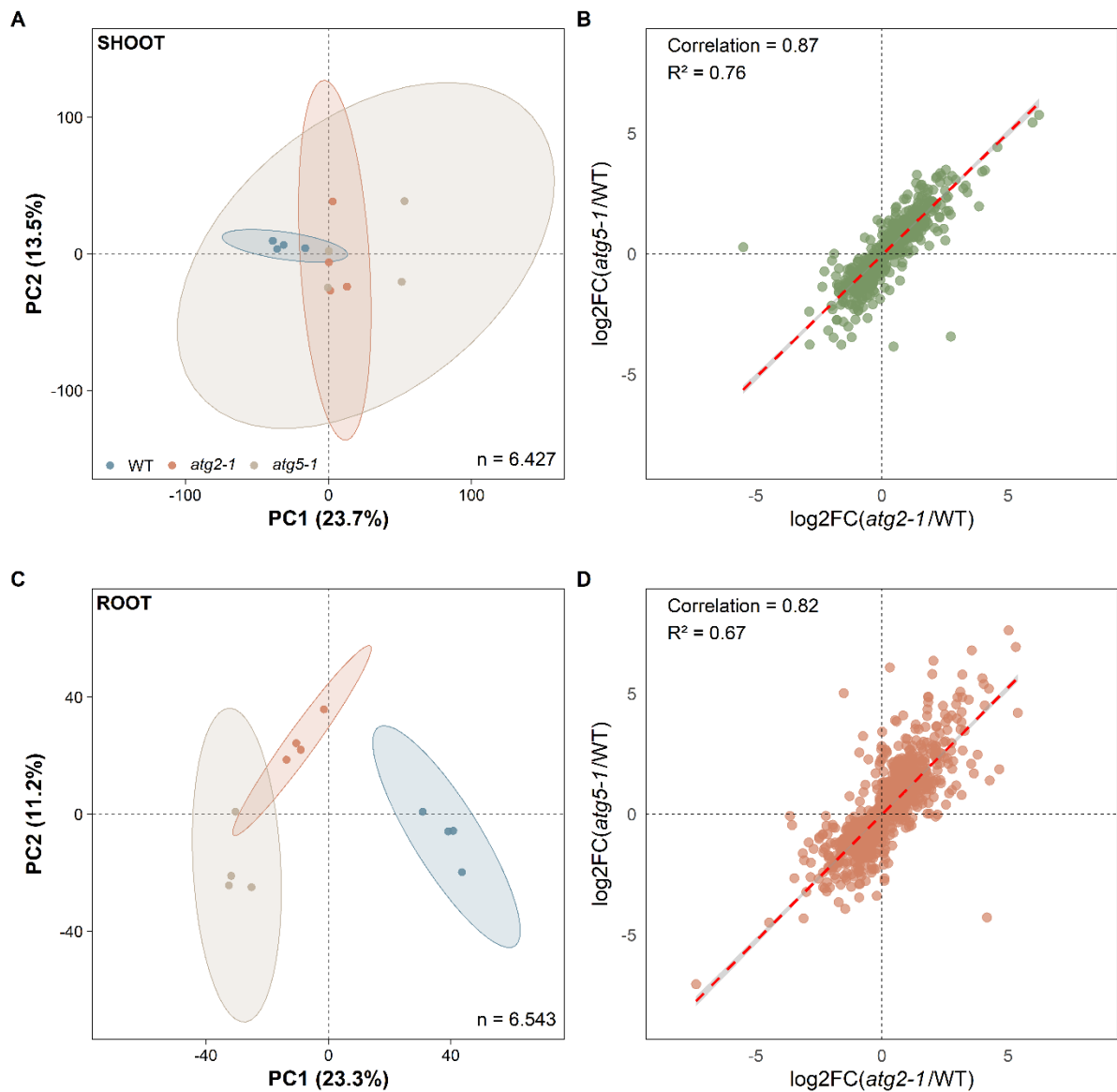


Figure 5. Autophagy deficiency alters the basal proteome of *Arabidopsis thaliana* under phosphate-replete conditions. Proteomic analyses were performed on shoots and roots of 10-day-old seedlings grown under Pi-sufficient conditions. (A, C) Principal component analysis (PCA) of protein abundance in (A) shoots and (C) roots. Ellipses represent 95% confidence intervals. (B, D) Pairwise comparison of $\log_2$ fold changes ($\log_2FC$) in protein abundance between *atg2-1/WT* and *atg5-1/WT* in (B) shoots and (D) roots, with Pearson correlation coefficients and coefficients of determination (R^2) indicated. The number of quantified proteins is shown in each PCA plot. The dashed lines show a $\text{corr} = 1$. All data are based on five biological replicates. All plots were generated using the ggplot2 package in R.

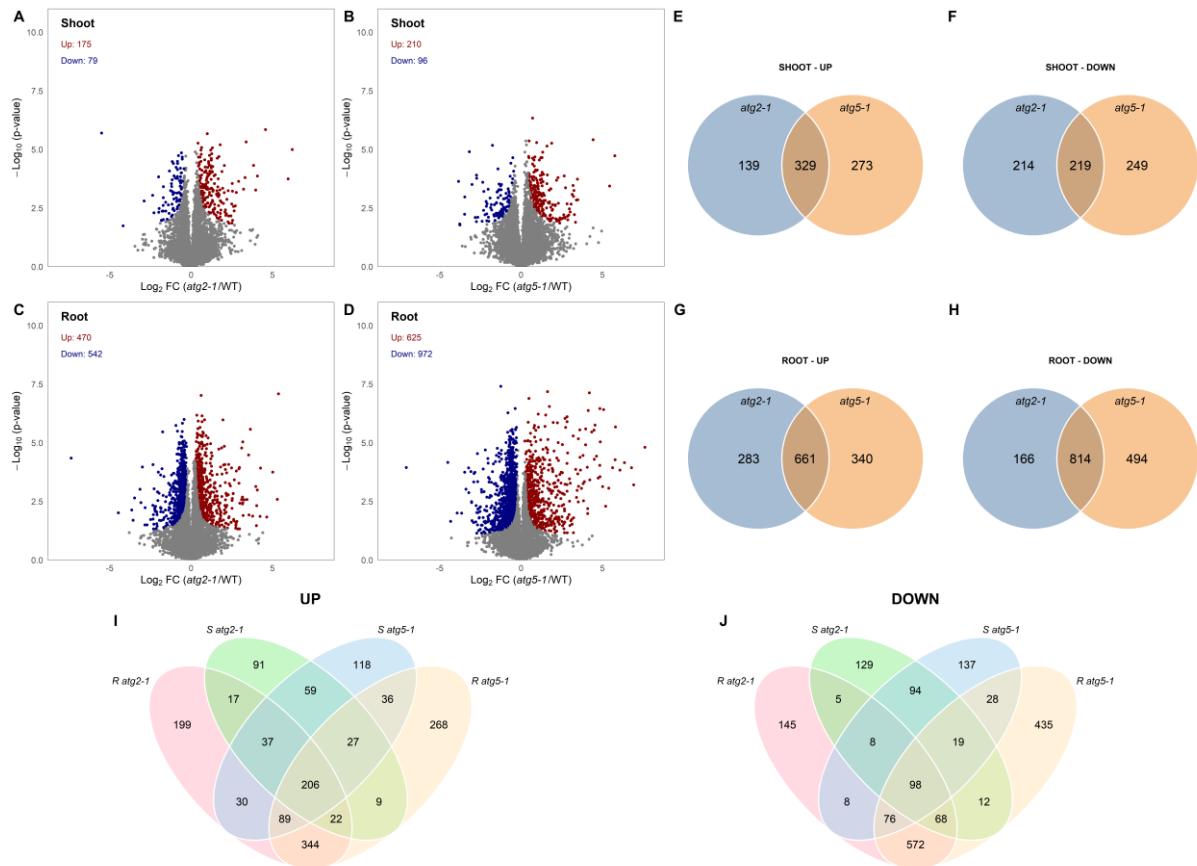
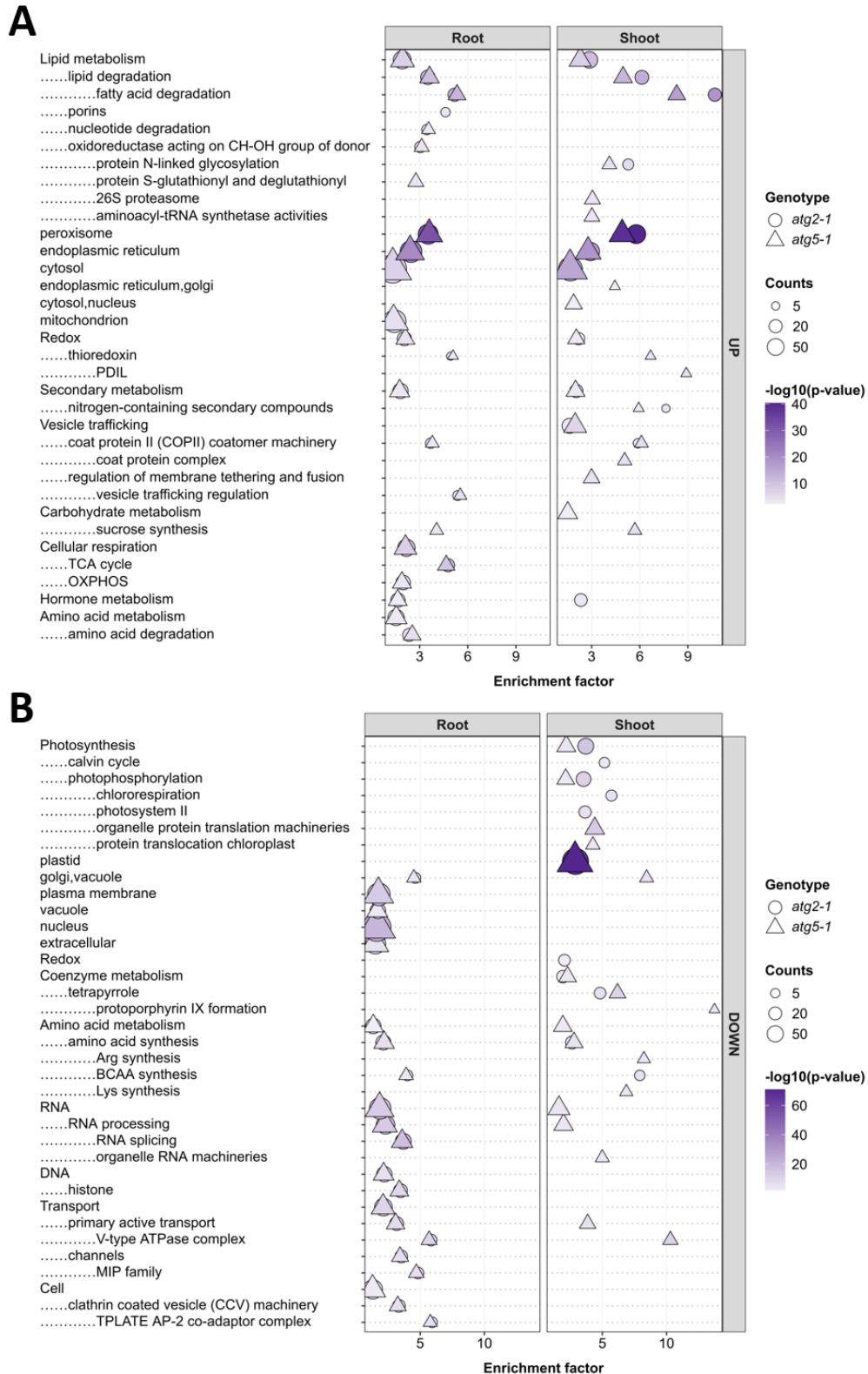


Figure 6. Autophagy deficiency reshapes the proteome of *Arabidopsis thaliana* proteome under phosphate-replete conditions. Proteome analyses were performed on shoots and roots of 10-day-old plants grown under Pi-sufficient conditions. (A–D) Volcano plots illustrating differentially abundant proteins in shoots (A, B) and roots (C, D) of *atg2-1* (A, C) and *atg5-1* (B, D) relative to WT. The x-axis indicates $\log_2$ fold change ($\log_2$ FC), and the y-axis shows $-\log_{10}(\text{p-value})$. Proteins with significantly increased abundance are shown in red, decreased abundance in blue, and non-significant proteins in gray. (E–H) Venn diagrams illustrating overlap of significantly upregulated (E, G) and downregulated (F, H) proteins between *atg2-1* and *atg5-1* in shoots (E, F) and roots (G, H). (I, J) Four-way Venn diagrams showing shared and genotype- or tissue-specific up-regulated (I) and downregulated (J) proteins across shoots (S) and roots (R) of *atg2-1* and *atg5-1* under Pi-starvation. All data are based on four biological replicates. All plots were generated using the ggplot2 package in RStudio.



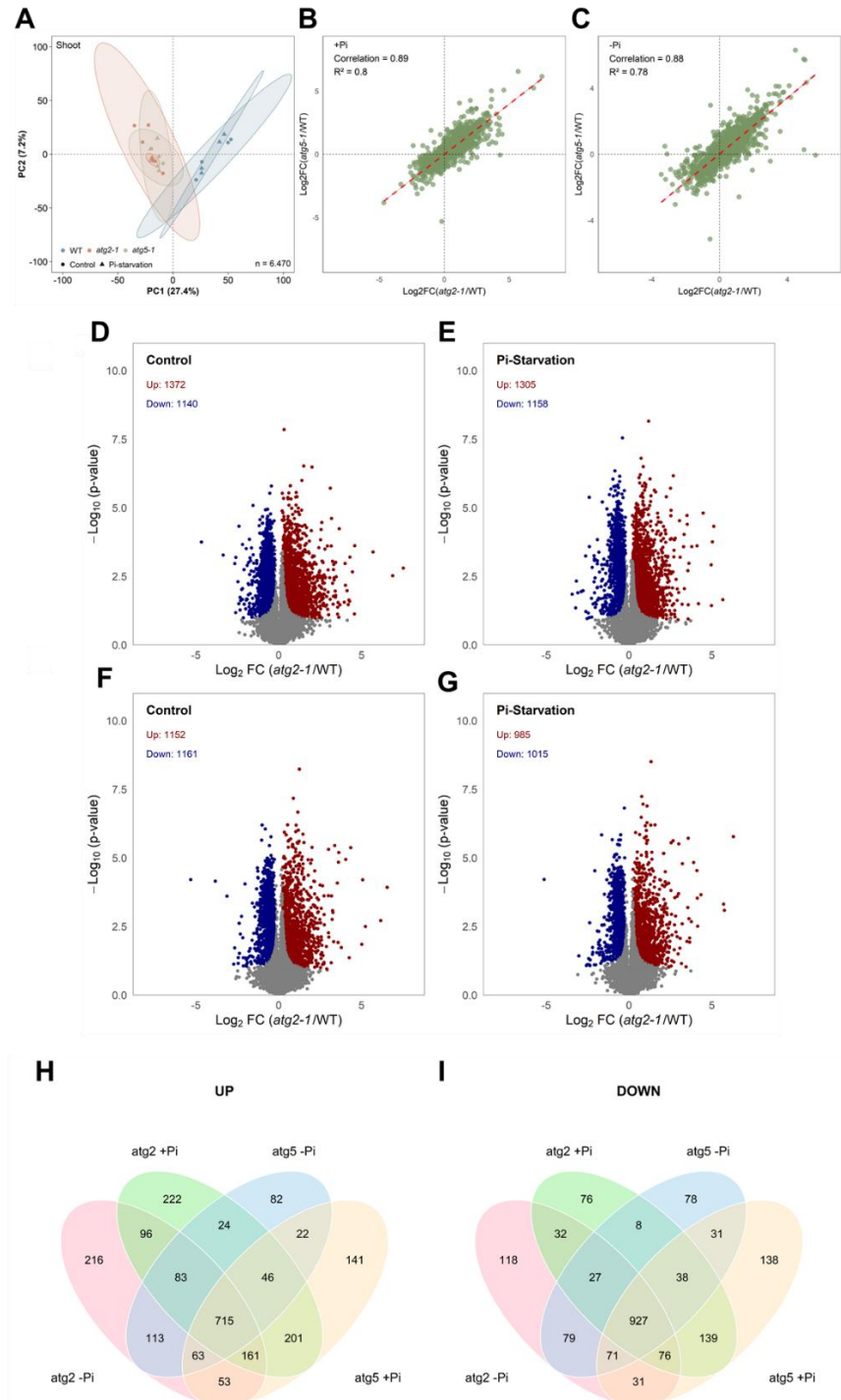


Figure 8. Autophagy deficiency affects the proteome of shoots *Arabidopsis thaliana* in early Pi limitation response. Proteome analysis was carried out in shoots of *Arabidopsis* seedlings of wild type (WT) and autophagy-deficient mutants (*atg2-1*, and *atg5-1*) under control and Pi-starvation conditions (DAT3). (A) Principal component analysis (PCA) of proteome samples. Ellipses represent 95% confidence intervals. The number of quantified proteins is shown in each PCA plot. (B, C) Pairwise comparison of log₂ fold changes (log₂FC) in protein abundance between *atg2-1*/WT and *atg5-1*/WT in shoots under (B) control and (C) Pi-starvation, with Pearson correlation coefficients and coefficients of determination (R²) indicated. The dashed lines show a corr = 1. (D–G) Volcano plots illustrating differentially abundant proteins in shoots of *atg2-1* under (D) control and (E) Pi-starvation, and *atg5-1* under (D) control and (E) Pi-starvation, both relative to WT. The x-axis indicates log₂ fold change (log₂ FC), and the y-axis shows -log₁₀(p-value). Proteins with significantly increased abundance are shown in red, decreased abundance in blue, and non-significant proteins in gray. (H, I) Four-way Venn diagrams show shared and genotype- or condition-specific up-regulated (I) and down-regulated (J) proteins across *atg2-1* and *atg5-1* under control and Pi-starvation. All data are based on four biological replicates. All plots were generated using the ggplot2 package in R.

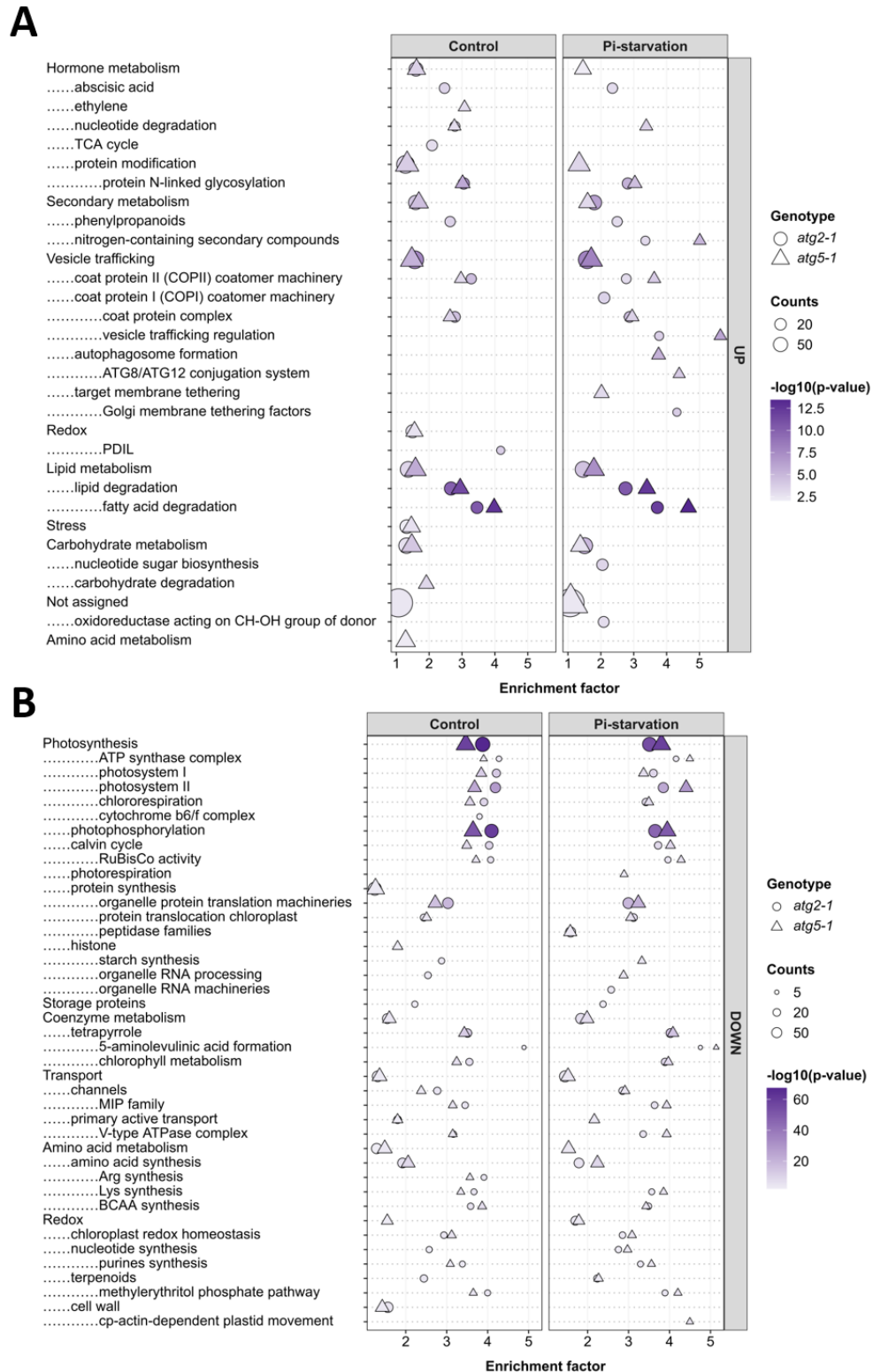


Figure 9. Autophagy deficiency affects the proteome of shoots *Arabidopsis thaliana* in early Pi limitation response. Functional categories enriched among proteins with increased (A) or decreased (B) abundance in the shoots of *atg2-1* and *atg5-1* relative to WT under phosphate-sufficient (Control) and phosphate-starved (Pi-starvation) conditions at DAT3. Enrichment analyses were performed using significantly differentially abundant proteins identified by quantitative proteomics. The x-axis represents the enrichment factor, while symbol size indicates the number of proteins assigned to each category. Color intensity corresponds to enrichment significance ($-\log_{10} P\text{-value}$). Circles represent *atg2-1* and triangles represent *atg5-1*. All the plots were generated using ggplot2 package in R Studio.

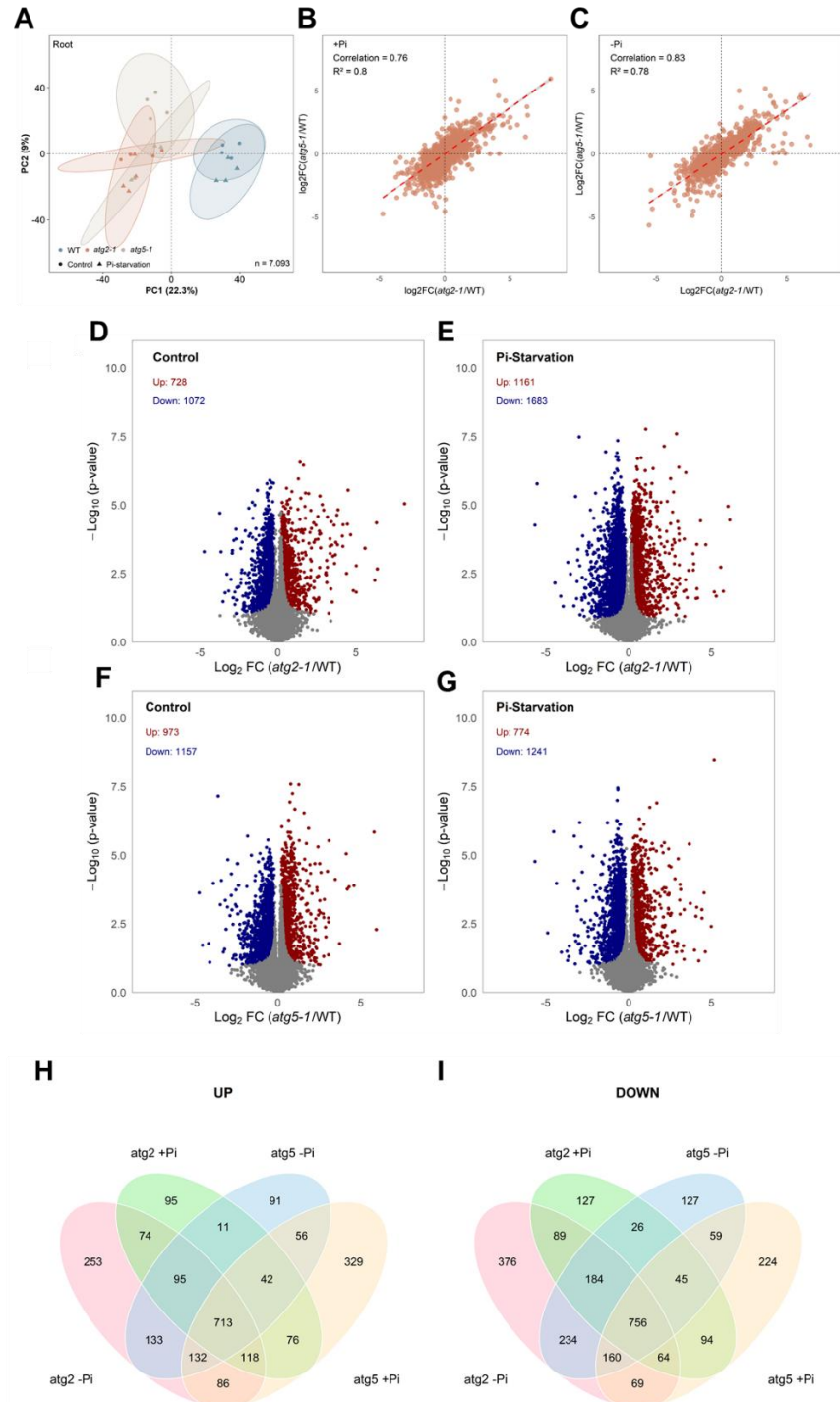


Figure 10. Autophagy deficiency affects the proteome of roots *Arabidopsis thaliana* in early Pi limitation response. Proteome analysis was carried out in roots of *Arabidopsis* seedlings of wild type (WT) and autophagy-deficient mutants (*atg2-1*, and *atg5-1*) under control and Pi-starvation conditions (DAT3). (A) Principal component analysis (PCA) of proteome samples. Ellipses represent 95% confidence intervals. The number of quantified proteins is shown in each PCA plot. (B, C) Pairwise comparison of $\log_2$ fold changes ($\log_2$ FC) in protein abundance between *atg2-1*/WT and *atg5-1*/WT in shoots under (B) control and (C) Pi-starvation, with Pearson correlation coefficients and coefficients of determination (R^2) indicated. The dashed lines show a $\text{corr} = 1$. (D–G) Volcano plots illustrating differentially abundant proteins in shoots of *atg2-1* under (D) control and (E) Pi-starvation, and *atg5-1* under (D) control and (E) Pi-starvation, both relative to WT. The x-axis indicates $\log_2$ fold change ($\log_2$ FC), and the y-axis shows $-\log_{10}(\text{p-value})$. Proteins with significantly increased abundance are shown in red, decreased abundance in blue, and non-significant proteins in gray. (H, I) Four-way Venn diagrams show shared and genotype- or condition-specific up-regulated (I) and down-regulated (J) proteins across *atg2-1* and *atg5-1* under control and Pi-starvation. All data are based on four biological replicates. All plots were generated using the ggplot2 package in R.

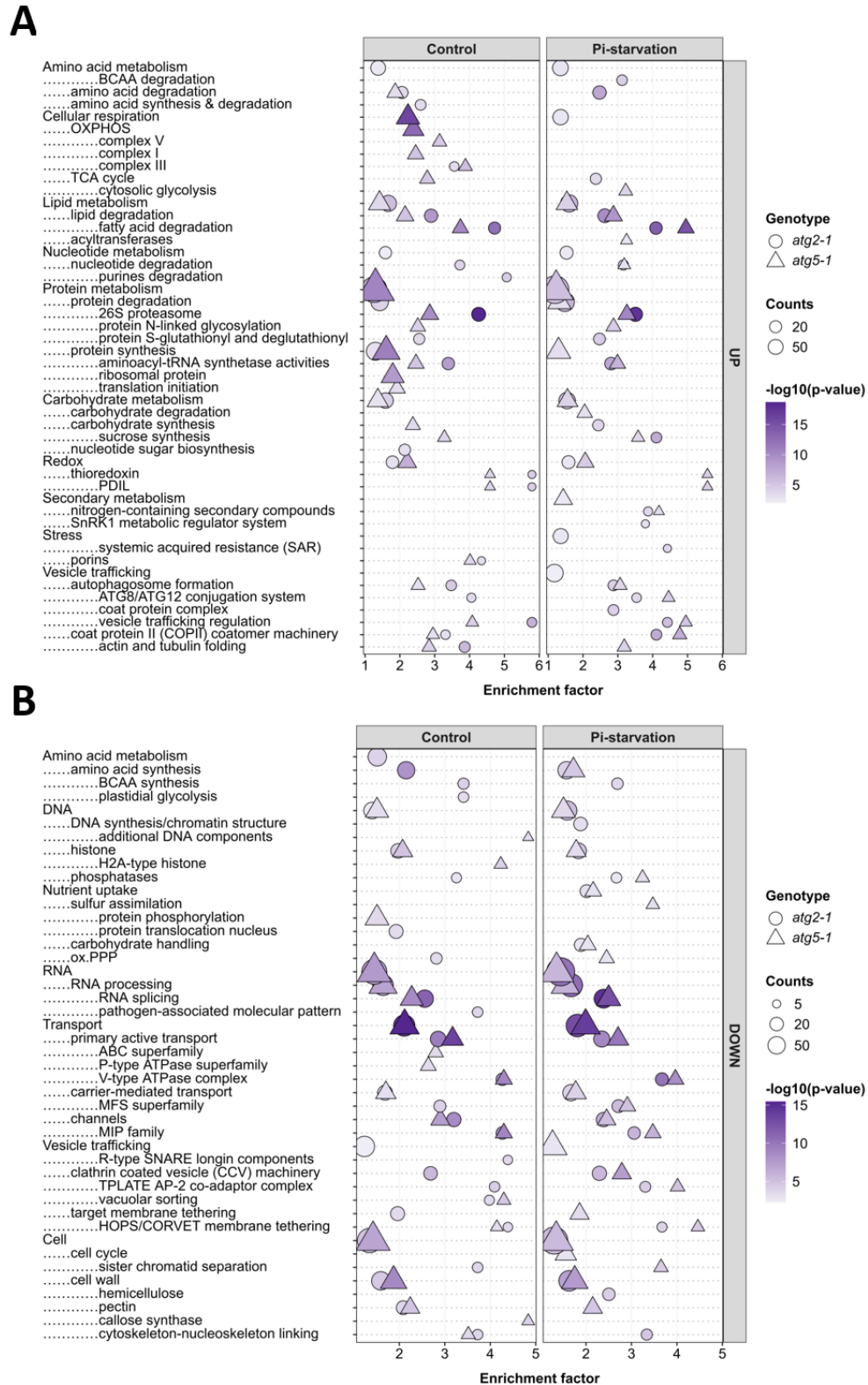


Figure 11. Autophagy deficiency affects the proteome of roots *Arabidopsis thaliana* in early Pi limitation response. Functional categories enriched among proteins with increased (A) or decreased (B) abundance in the roots of *atg2-1* and *atg5-1* relative to WT under phosphate-sufficient (Control) and phosphate-starved (Pi-starvation) conditions at DAT3. Enrichment analyses were performed using significantly differentially abundant proteins identified by quantitative proteomics. The x-axis represents the enrichment factor, while symbol size indicates the number of proteins assigned to each category. Color intensity corresponds to enrichment significance ($-\log_{10} P$ -value). Circles represent *atg2-1* and triangles represent *atg5-1*. All the plots were generated using ggplot2 package in R Studio.

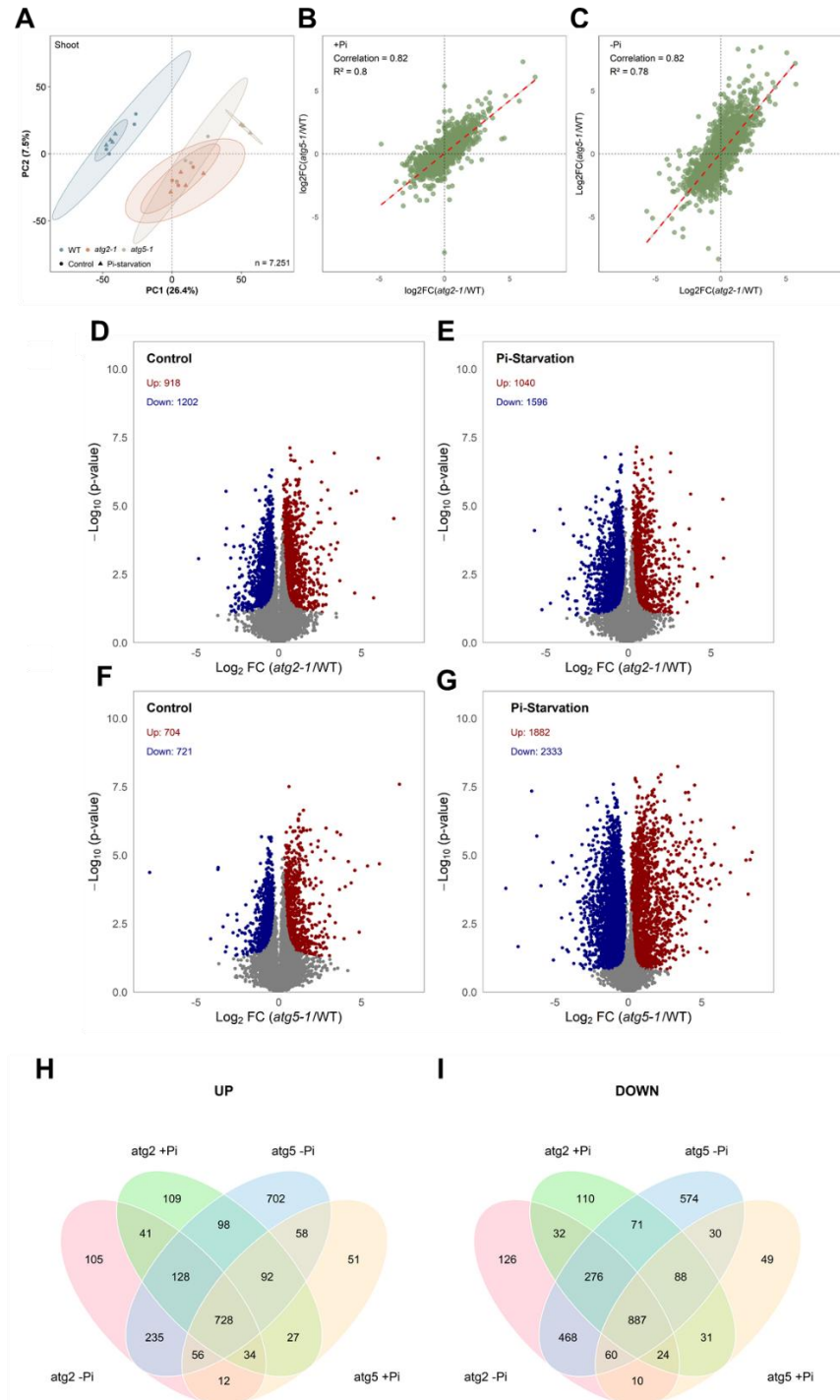


Figure 12. Autophagy deficiency affects the proteome of *Arabidopsis thaliana* shoots under prolonged Pi-starvation. Proteome analysis was carried out in shoots of *Arabidopsis* seedlings of wild type (WT) and autophagy-deficient mutants (*atg2-1*, and *atg5-1*) under control and Pi-starvation conditions (DAT7). (A) Principal component analysis (PCA) of proteome samples. Ellipses represent 95% confidence intervals. The number of quantified proteins is shown in each PCA plot. (B, C) Pairwise comparison of $\log_2$ fold changes ($\log_2$ FC) in protein abundance between *atg2-1*/WT and *atg5-1*/WT in shoots under (B) control and (C) Pi-starvation, with Pearson correlation coefficients and coefficients of determination (R^2) indicated. The dashed lines show a $\text{corr} = 1$. (D–G) Volcano plots illustrating differentially abundant proteins in shoots of *atg2-1* under (D) control and (E) Pi-starvation, and *atg5-1* under (D) control and (E) Pi-starvation, both relative to WT. The x-axis indicates $\log_2$ fold change ($\log_2$ FC), and the y-axis shows $-\log_{10}(\text{p-value})$. Proteins with significantly increased abundance are shown in red, decreased abundance in blue, and non-significant proteins in gray. (H, I) Four-way Venn diagrams show shared and genotype- or condition-specific up-regulated (I) and down-regulated (J) proteins across *atg2-1* and *atg5-1* under control and Pi-starvation. All data are based on four biological replicates. All plots were generated using the ggplot2 package in R.

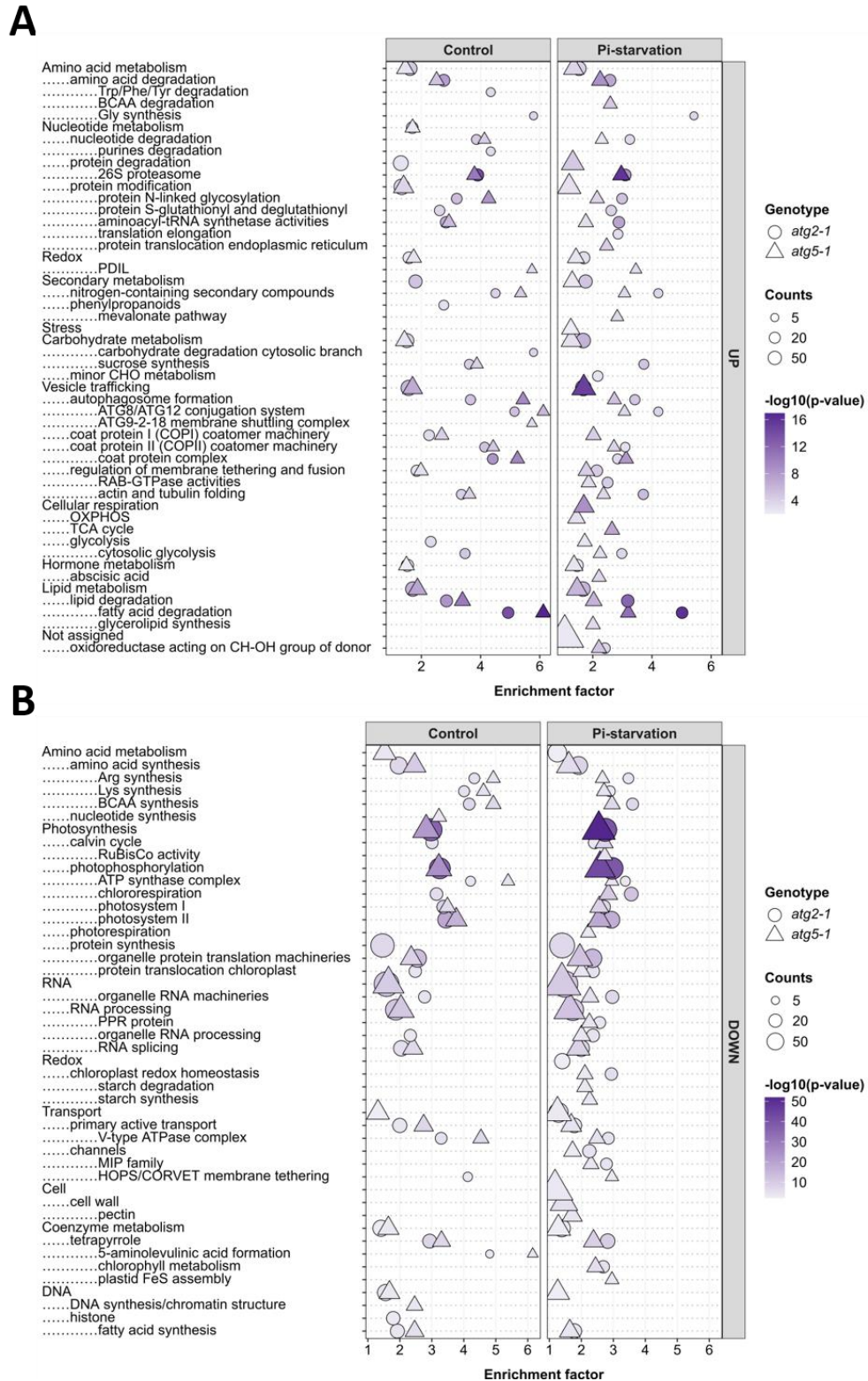


Figure 13. Autophagy deficiency affects the proteome of *Arabidopsis thaliana* shoots under prolonged Pi-starvation. Functional categories enriched among proteins with increased (A) or decreased (B) abundance in the shoots of *atg2-1* and *atg5-1* relative to WT under phosphate-sufficient (Control) and phosphate-starved (Pi-starvation) conditions at DAT7. Enrichment analyses were performed using significantly differentially abundant proteins identified by quantitative proteomics. The x-axis represents the enrichment factor, while symbol size indicates the number of proteins assigned to each category. Color intensity corresponds to enrichment significance ($-\log_{10} P$ -value). Circles represent *atg2-1* and triangles represent *atg5-1*. All the plots were generated using ggplot2 package in R Studio.

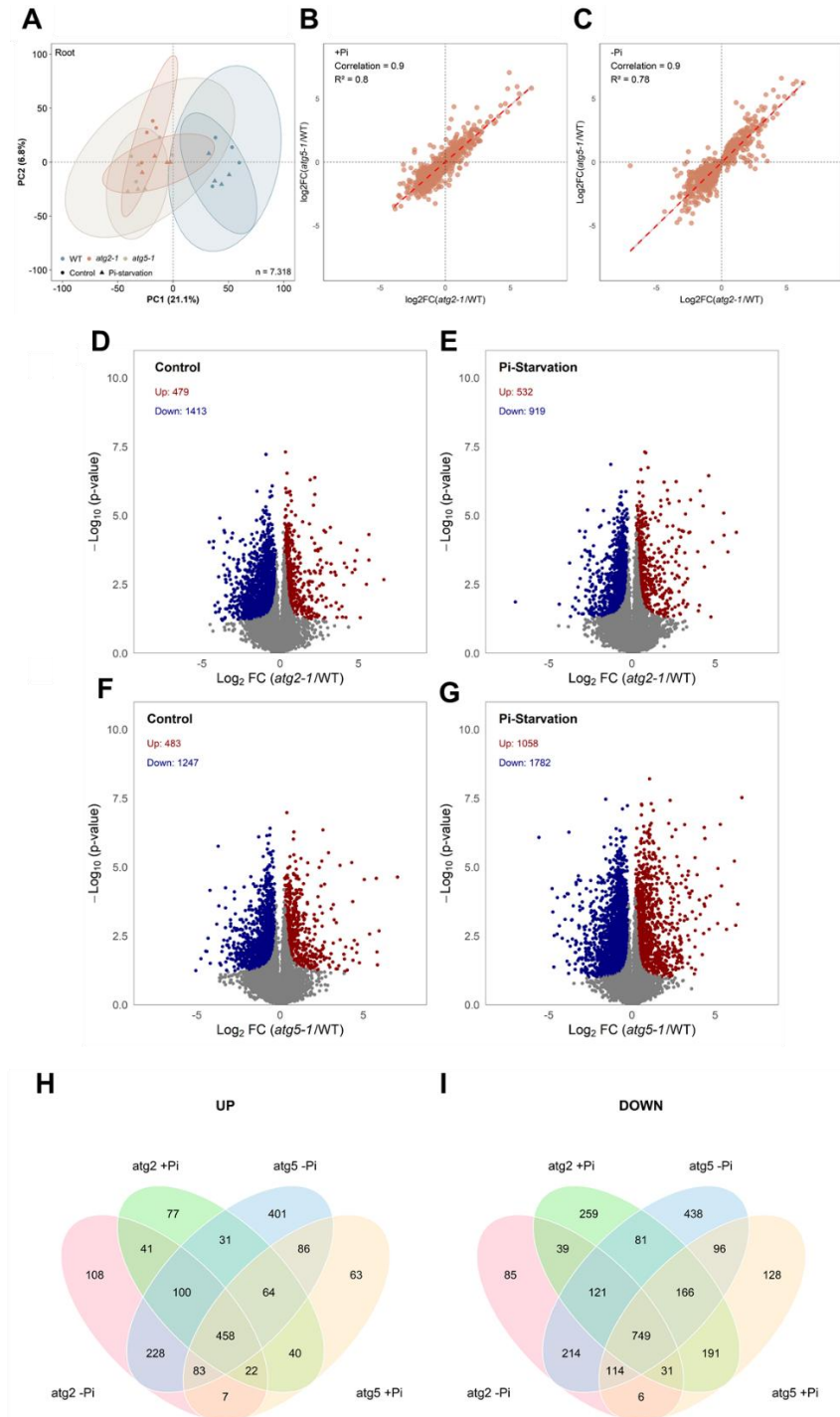


Figure 14. Autophagy deficiency affects the proteome of roots *Arabidopsis thaliana* under prolonged Pi-starvation. Proteome analysis was carried out in roots of *Arabidopsis* seedlings of wild type (WT) and autophagy-deficient mutants (*atg2-1*, and *atg5-1*) under control and Pi-starvation conditions (DAT7). (A) Principal component analysis (PCA) of proteome samples. Ellipses represent 95% confidence intervals. The number of quantified proteins is shown in each PCA plot. (B, C) Pairwise comparison of $\log_2$ fold changes ($\log_2$ FC) in protein abundance between *atg2-1*/WT and *atg5-1*/WT in shoots under (B) control and (C) Pi-starvation, with Pearson correlation coefficients and coefficients of determination (R^2) indicated. The dashed lines show a $\text{corr} = 1$. (D–G) Volcano plots illustrating differentially abundant proteins in shoots of *atg2-1* under (D) control and (E) Pi-starvation, and *atg5-1* under (F) control and (G) Pi-starvation, both relative to WT. The x-axis indicates $\log_2$ fold change ($\log_2$ FC), and the y-axis shows $-\log_{10}$ (p-value). Proteins with significantly increased abundance are shown in red, decreased abundance in blue, and non-significant proteins in gray. (H, I) Four-way Venn diagrams show shared and genotype- or condition-specific up-regulated (I) and downregulated (J) proteins across *atg2-1* and *atg5-1* under control and Pi-starvation. All data are based on four biological replicates. All plots were generated using the ggplot2 package in R.

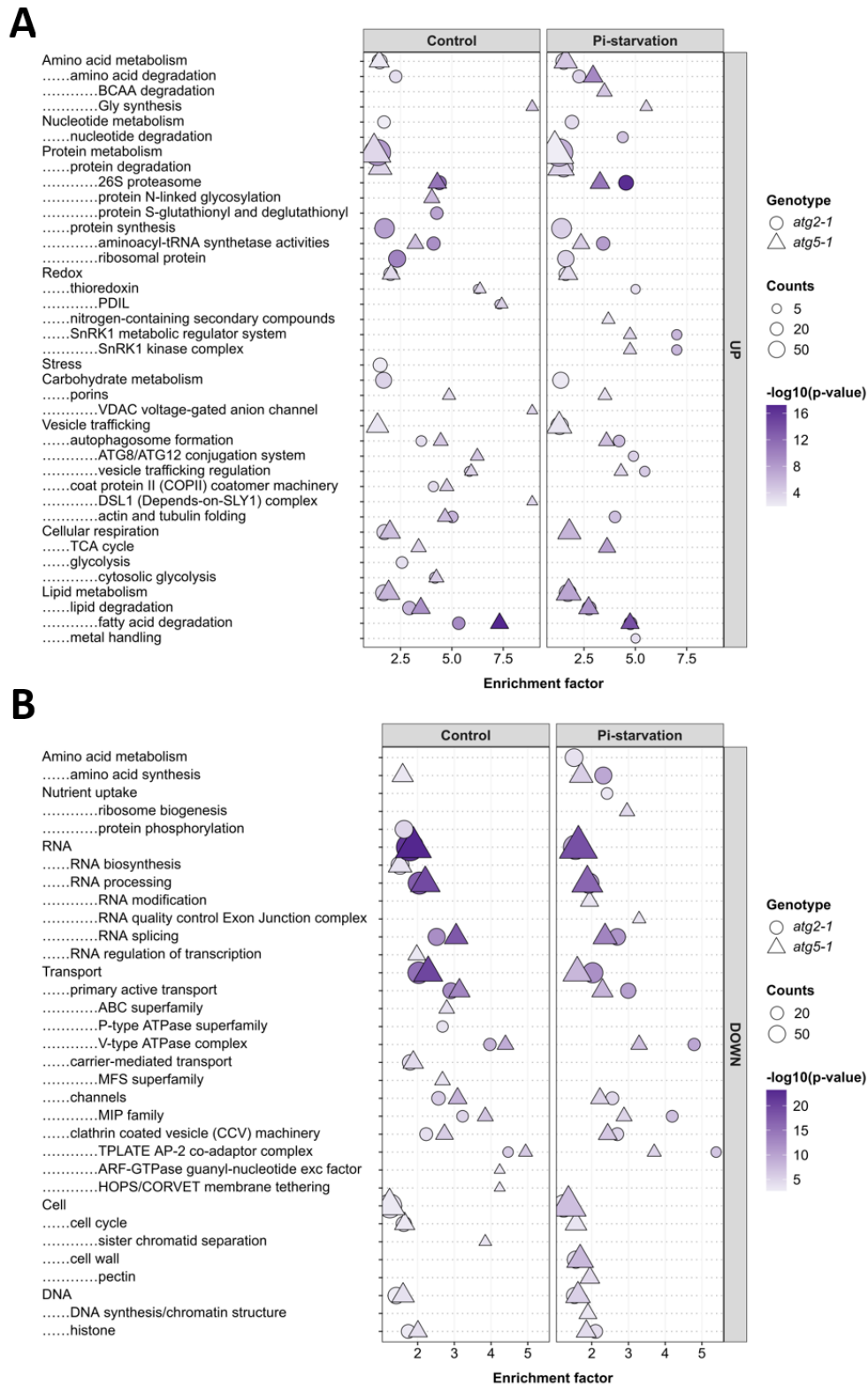


Figure 15. Autophagy deficiency affects the proteome of *Arabidopsis thaliana* roots under prolonged Pi-starvation. Functional categories enriched among proteins with increased (A) or decreased (B) abundance in the roots of *atg2-1* and *atg5-1* relative to WT under phosphate-sufficient (Control) and phosphate-starved (Pi-starvation) conditions at DAT7. Enrichment analyses were performed using significantly differentially abundant proteins identified by quantitative proteomics. The x-axis represents the enrichment factor, while symbol size indicates the number of proteins assigned to each category. Color intensity corresponds to enrichment significance ($-\log_{10} P$ -value). Circles represent *atg2-1* and triangles represent *atg5-1*. All the plots were generated using ggplot2 package in R Studio.

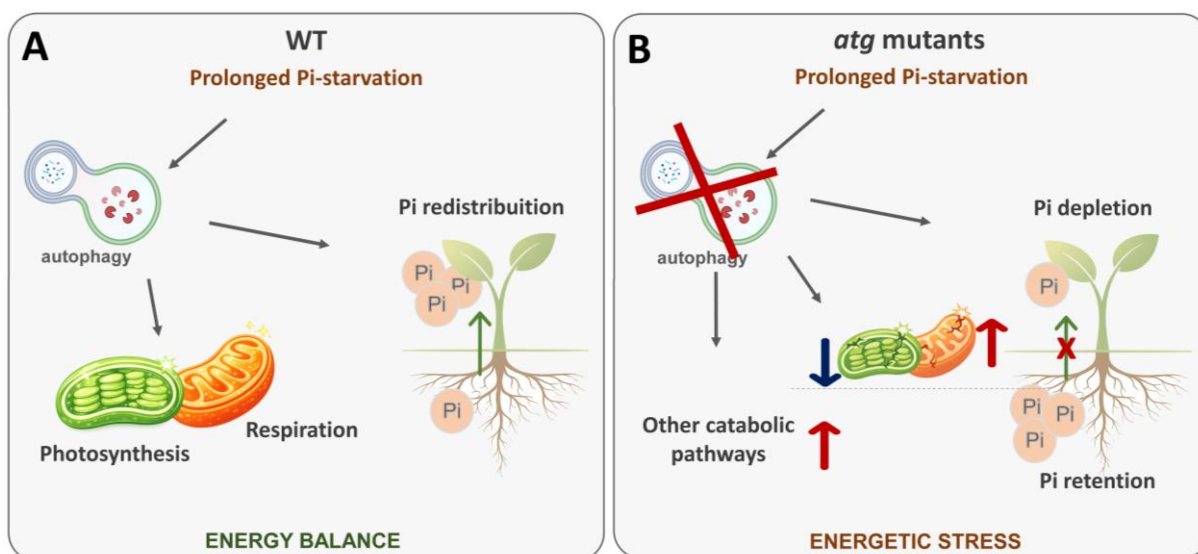


Figure 16. Autophagy coordinates phosphate allocation and energy metabolism during prolonged phosphate starvation. (A) In wild-type (WT) plants, autophagy supports intracellular phosphate (Pi) recycling and promotes root-to-shoot Pi redistribution during prolonged Pi-starvation. This coordination helps maintain shoot Pi availability, sustaining photosynthetic and respiratory functions and preserving whole-plant energy balance. (B) In autophagy-deficient mutants (*atg*), disruption of autophagic recycling impairs systemic Pi redistribution, resulting in Pi retention in roots and depletion of shoot Pi pools. Reduced shoot Pi availability is associated with impaired plastidial function and enhanced reliance on mitochondrial respiration and alternative catabolic pathways to sustain energy production. Together, these responses indicate a shift from metabolic homeostasis toward energetic stress. The model positions autophagy as a whole-plant buffering system that integrates Pi homeostasis, organellar function, and metabolic flexibility during prolonged Pi limitation. Blue arrows indicate reduced plastidial/photosynthetic metabolism, whereas red arrows indicate increased reliance on mitochondrial respiration and alternative catabolic pathways.

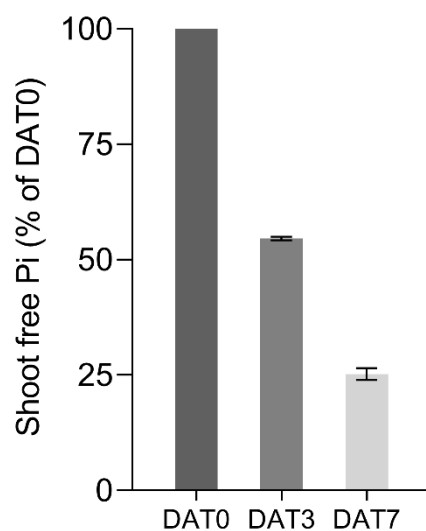
SUPPLEMENTAL DATA

Figure S1. Definition of early and late phases of phosphate starvation based on shoot Pi depletion dynamics in wild-type plants. Free inorganic phosphate (Pi) content in shoots of wild-type (WT) seedlings grown under Pi-starvation was measured at DAT0, DAT3, and DAT7 and expressed as a percentage of DAT0. Values presented are means $\pm$ SE of 4 biological replicates.

GENERAL CONCLUSIONS

This thesis demonstrates that autophagy is a central component of plant metabolic resilience under abiotic stress. Although traditionally viewed as a degradative recycling pathway, the results presented here indicate that autophagy functions as an integrative homeostatic system that coordinates proteome maintenance, organelle quality control, nutrient redistribution, and energy metabolism. Across both prolonged warming and Pi-starvation, autophagy consistently enabled metabolic flexibility, allowing plants to adjust resource allocation and maintain energetic balance under conditions that constrain growth and cellular function.

A central theme emerging from this thesis is that autophagy sustains metabolic flexibility during prolonged stress. Environmental challenges such as elevated temperature and Pi limitation impose substantial energetic constraints that require extensive metabolic reprogramming. Under prolonged warming, autophagy-deficient plants exhibited impaired thermomorphogenic growth, reduced photosynthetic performance, excessive respiratory and transpiratory costs, and disruption of metabolic coordination. Similarly, under Pi-starvation, autophagy deficiency compromised respiratory acclimation, accelerated depletion of intracellular Pi pools, and promoted premature metabolic transitions associated with energy stress. Together, these findings indicate that autophagy is not simply activated in response to metabolic perturbation but actively contributes to the reorganization of cellular metabolism required for successful acclimation.

This thesis further highlights the importance of autophagy in preserving organelle function under stress. The review chapter synthesized growing evidence that selective autophagy contributes to heat adaptation through the maintenance of proteostasis, organelle quality control, thermomemory, and post-stress recovery. The experimental chapters extended these concepts by demonstrating that autophagy supports chloroplast integrity during prolonged warming and promotes mitochondrial acclimation during Pi-starvation. In both cases, autophagy deficiency resulted in extensive proteome remodeling and disruption of key metabolic processes. These findings support a model in which autophagy safeguards organelle functionality by preventing the accumulation of damaged or maladaptive cellular components while facilitating the reconfiguration of organellar proteomes during stress acclimation.

Another insight is that autophagy operates as a regulator of nutrient economy across multiple organizational scales. Beyond its established role in intracellular recycling, autophagy influenced Pi partitioning between tissues, affected Pi transporter abundance and vesicle trafficking pathways, and contributed to the maintenance of shoot Pi availability during nutrient

limitation. The observation that autophagy-deficient plants accumulated Pi in roots while simultaneously experiencing reduced shoot Pi availability suggests that autophagy contributes not only to nutrient recycling but also to nutrient allocation. These findings expand the current understanding of autophagy from a cell-autonomous recycling process to a broader regulator of whole-plant nutrient homeostasis.

Collectively, the results support a conceptual framework in which autophagy functions as a multi-scale homeostatic system linking intracellular recycling, organelle maintenance, proteome remodeling, and systemic nutrient allocation. Through these interconnected functions, autophagy buffers plants against energetic imbalances generated by environmental stress. Rather than acting solely as an emergency survival mechanism, autophagy emerges as a proactive regulator of metabolic efficiency that preserves cellular functionality, delays metabolic deterioration, and facilitates long-term acclimation.

Importantly, this work also identifies several outstanding questions that help to direct future research on autophagy and plant stress physiology. How do plants establish cargo selectivity to differentially target mitochondria, chloroplasts, endoplasmic reticulum, and other cellular structures under distinct environmental conditions? Through which mechanisms does autophagy preserve mitochondrial respiratory competence during Pi-starvation? How does autophagy mechanistically preserve mitochondrial respiratory competence under Pi limitation: by sustaining NAD(H)/NADP(H) balance, maintaining electron transport chain integrity, or preventing maladaptive retrograde signaling triggered from damaged organelles? How does autophagy interact with membrane trafficking pathways, Pi transport systems, and energy-sensing networks to coordinate nutrient allocation at the whole-plant level? What signals trigger the premature repression of photosynthesis and photorespiration observed in autophagy-deficient plants under Pi-starvation? Finally, how are autophagic responses integrated when plants experience multiple environmental constraints simultaneously, as commonly occurs under natural and agricultural conditions?

Addressing these questions will be essential for fully understanding how autophagy shapes plant metabolic resilience in increasingly challenging environments. More broadly, this thesis positions autophagy as an integrator of energy metabolism and stress adaptation, providing support for future studies aimed at improving plant performance under conditions of climate warming and nutrient limitation.