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**How does autophagy modulate heat stress and drought responses during
reproductive development?**

Julia de Paiva Gonçalves
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Thesis submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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To my mother for her unconditional love and support throughout this journey.
With love, I dedicate this.

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The Supreme Energy of the Universe that governs all things.

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ABSTRACT

GONÇALVES, Julia de Paiva, D.Sc., Universidade Federal de Viçosa, August, 2026.
How does autophagy modulate heat stress and drought responses during reproductive development?. Adviser: Wagner Luiz Araujo. Co-adviser: Jessica Aline Sousa Barros.

The increasing frequency and intensity of extreme climatic events, such as prolonged warming and drought, represent major threats to global agricultural productivity. Among the cellular mechanisms involved in plant adaptation to these stresses, autophagy plays a pivotal role by mediating the selective degradation and recycling of damaged proteins and organelles, thereby contributing to the maintenance of cellular and metabolic homeostasis. Although the importance of autophagy during vegetative growth has been extensively investigated, its role in coordinating physiological and metabolic responses during reproductive development remains poorly understood. In this study, we investigated how autophagy modulates plant responses to (i) prolonged warming and (ii) drought stress during the reproductive stage of *Arabidopsis thaliana*. To this end, wild-type (WT) plants and the autophagy deficient mutants *atg5-1* and *atg7-2* were exposed to prolonged warming and drought during reproductive development. In response to prolonged warming, autophagy was rapidly induced, reaching maximum activity on the third day of stress. Autophagy deficiency accelerated leaf senescence, disrupted redox homeostasis, promoted higher accumulation of the heat shock protein HSP101, caused profound alterations in primary metabolism, and impaired post-stress metabolic recovery. Moreover, the mutants exhibited reduced male fertility, decreased seed production, and altered seed reserve composition during seed filling. Under drought conditions, although all genotypes exhibited similar reductions in relative water content, the autophagy-deficient mutants displayed more severe leaf senescence, greater degradation of photosynthetic pigments, and pronounced alterations in carbohydrate, amino acid, and organic acid metabolism. These metabolic disturbances disrupted source–sink relationships, leading to reduced pollen viability, fewer siliques, and lower seed production. Despite the reduced reproductive output, the seeds that successfully developed maintained their physiological quality, exhibiting germination comparable to that of WT plants while displaying changes in reserve distribution and accumulation, suggesting preferential allocation of available resources to a smaller number of seeds. Collectively, these findings demonstrate that autophagy may play a central role in plant adaptation to prolonged warming and drought by maintaining redox

homeostasis, energy metabolism, and carbon and nitrogen remobilization. Furthermore, they reveal that autophagy could integrate leaf senescence, source–sink regulation, and reproductive development, thereby sustaining fertility and productivity under adverse environmental conditions. This work advances our understanding of the physiological and metabolic mechanisms through which autophagy enhances plant tolerance to abiotic stresses and provides a foundation for future strategies aimed at improving crop resilience under changing climatic conditions. Nevertheless, further studies are required to elucidate the molecular mechanisms underlying autophagy mediated stress adaptation and to determine how and to what extent, this metabolic and molecular reprogramming modulates plant responses to prolonged warming and drought during reproductive development.

Keywords: drought; metabolism; prolonged warming; reproductive development; senescence

RESUMO

GONÇALVES, Julia de Paiva, D.Sc., Universidade Federal de Viçosa, agosto de 2026. **Como a autofagia modula as respostas ao estresse térmico e à seca no desenvolvimento reprodutivo?**. Orientador: Wagner Luiz Araujo. Coorientadora: Jessica Aline Sousa Barros.

O aumento da frequência e da intensidade de eventos climáticos extremos, como ondas de calor e déficit hídrico, representa uma das principais ameaças à produtividade agrícola mundial. Entre os mecanismos celulares envolvidos na adaptação das plantas a esses estresses, a autofagia destaca-se por promover a degradação seletiva e a reciclagem de proteínas e organelas danificadas, contribuindo para a manutenção da homeostase metabólica. Embora diversos estudos tenham demonstrado a importância da autofagia durante a fase vegetativa, seu papel na coordenação das respostas fisiológicas e metabólicas durante o desenvolvimento reprodutivo permanece pouco compreendido. Neste trabalho investigou-se como a autofagia modula as respostas ao (i) aquecimento prolongado e ao (ii) déficit hídrico durante a fase reprodutiva de *Arabidopsis thaliana*. Para isso, plantas do tipo selvagem e os mutantes deficientes em autofagia *atg5-1* e *atg7-2* foram submetidos ao aquecimento prolongado e ao déficit hídrico durante o desenvolvimento reprodutivo. Em resposta ao aquecimento prolongado, a autofagia foi rapidamente induzida, apresentando pico de atividade no terceiro dia de estresse. A deficiência autofágica promoveu aceleração da senescência foliar, alterações na homeostase redox, maior acúmulo da proteína HSP101, modificações profundas no metabolismo primário e comprometimento da recuperação metabólica após o estresse. Além disso, os mutantes apresentaram alterações na fertilidade masculina, redução na produção de sementes e mudanças na composição das reservas acumuladas durante o enchimento das sementes. Sob déficit hídrico, embora todos os genótipos apresentassem redução semelhante no conteúdo relativo de água, os mutantes deficientes em autofagia exibiram senescência foliar mais intensa, maior degradação de pigmentos fotossintéticos e profundas alterações no metabolismo de carboidratos, aminoácidos e ácidos orgânicos. Essas alterações comprometeram a relação fonte-dreno, reduzindo a viabilidade do pólen, o número de siliquas e a produção total de sementes. Apesar da menor produção, as sementes remanescentes mantiveram sua qualidade fisiológica, apresentando germinação semelhante à do tipo selvagem e alterações na distribuição e no acúmulo de reservas, indicando um redirecionamento preferencial dos recursos para um número reduzido de sementes.

Coletivamente, os resultados demonstram que a autofagia pode exercer um papel central na adaptação das plantas ao aquecimento prolongado e ao déficit hídrico, atuando na manutenção da homeostase redox, do metabolismo energético e da remobilização de carbono e nitrogênio. Além disso, evidenciam que a autofagia pode integrar os processos de senescência foliar, metabolismo fonte-dreno e desenvolvimento reprodutivo, assegurando a fertilidade e a produtividade sob condições ambientais adversas. Este trabalho amplia o conhecimento sobre os mecanismos fisiológicos e metabólicos pelos quais a autofagia contribui para a tolerância a estresses abióticos, fornecendo bases para futuras estratégias de desenvolvimento de culturas mais resilientes às mudanças climáticas. Trabalhos futuros serão ainda necessários para esclarecer como e em que extensão essa aparente reprogramação metabólica e molecular modula as respostas vegetais ao calor e a seca durante o desenvolvimento reprodutivo.

Palavras-chave: calor prolongado; desenvolvimento reprodutivo ; metabolismo; seca; senescência

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General Introduction

Climate change is reshaping agricultural production worldwide by increasing the frequency, intensity and duration of extreme environmental events, particularly heat and drought (Food and Agriculture Organization, 2018). These abiotic stresses severely impair plant growth, metabolism and reproductive development, ultimately compromising crop productivity and threatening global food security (Jagadish et al., 2021; Seth & Sebastian, 2024). According to recent climate projections, global temperatures are expected to continue rising throughout the twenty-first century, while prolonged drought events are predicted to become increasingly frequent in many agricultural regions (IPCC, 2023). Understanding the mechanisms that enable plants to maintain growth and reproductive success under these adverse environmental conditions has therefore become a major challenge (Nair et al., 2022).

Plants have evolved a wide range of adaptive responses to cope with environmental stresses, including extensive metabolic reprogramming, redox regulation and nutrient remobilization (Wang et al., 2020). These responses are particularly critical during the reproductive stage, which represents one of the most stress sensitive phases of the plant life cycle (Lohani et al., 2020; Chaturvedi et al., 2021). Environmental disturbances occurring during flowering, pollination, fertilization and seed filling frequently result in pollen sterility, reduced fertilization efficiency, impaired seed development and substantial yield losses (Lohani et al., 2020; Chaturvedi et al., 2021; Seth & Sebastian, 2024). Although numerous studies have investigated stress responses during vegetative growth, considerably less attention has been devoted to understanding how plants coordinate physiological and metabolic adjustments to sustain reproductive development under prolonged environmental stress (Tang & Bassham, 2021; Yan et al., 2023; Qian et al., 2025).

Among the cellular mechanisms involved in stress adaptation, autophagy has emerged as one of the major pathways responsible for maintaining cellular homeostasis (Lv et al., 2026). Autophagy is an evolutionarily conserved degradation process that mediates the selective recycling of damaged proteins, protein aggregates and dysfunctional organelles through vacuolar degradation (Bassham et al., 2006; Marshall & Vierstra, 2018). Beyond its housekeeping role under optimal growth conditions, autophagy becomes strongly induced under nutrient limitation and abiotic stresses, contributing to protein quality control, oxidative damage mitigation, nutrient remobilization and metabolic homeostasis (Lv et al., 2026).

Consequently, autophagy has been recognized as a central regulator of plant survival under adverse environmental conditions (Avin-Wittenberg, 2018).

During heat shock, autophagy participates in the removal of heat damaged proteins and organelles, thereby preventing proteotoxicity and maintaining cellular integrity (Zhou et al., 2014; Yang et al., 2016; Sedaghatmehr et al., 2018). However, most of the current knowledge has been generated using short term stress treatments and vegetative tissues (Wang et al., 2020; Chen et al., 2026). Comparatively little is known about the role of autophagy during prolonged warming, post-stress recovery or reproductive development, despite reproduction being one of the developmental stages most severely affected by environmental stress (Dündar et al., 2019; Wang et al., 2020). Similarly, although autophagy has been implicated in drought tolerance (Wang et al., 2015; Bao, 2020), its contribution to coordinating source–sink relationships, nutrient remobilization, and reproductive performance under water deficit remains poorly understood (Tang & Bassham, 2021).

Thus, it was proposed that autophagy coordinates leaf senescence, redox balance, carbon and nitrogen remobilization, thereby maintaining pollen fertility, seed production and reproductive success under prolonged warming and drought conditions. Together, these findings provide a comprehensive understanding of how autophagy coordinates plant responses to two major climate related stresses and highlight a potential central role in maintaining reproductive resilience under changing environmental conditions.

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Chapter 1: Autophagy is required for reproductive development in response to heat stress?

Abstract

Prolonged warming has emerged as one of the major consequences of climate change, yet the physiological mechanisms that enable plants to maintain reproductive performance under sustained high temperatures remain poorly understood. Although autophagy is recognized as an important component of plant responses to acute heat stress, its role during prolonged warming, particularly throughout reproductive development, has received limited attention. Therefore, this study investigated the contribution of autophagy to the physiological, metabolic, and reproductive responses of *Arabidopsis thaliana* subjected to prolonged warming. Wild-type (WT) plants and the autophagy deficient mutants *atg5-1* and *atg7-2* were exposed to prolonged warming (28°C day/26°C night) during the reproductive stage, followed by a recovery period under optimal temperature conditions. Autophagic activity was rapidly induced by prolonged warming, reaching its maximum level on the third day of stress. In contrast, autophagy deficient mutants exhibited accelerated leaf senescence, altered pyridine nucleotide redox homeostasis, increased accumulation of the heat shock protein HSP101 and impaired metabolic recovery following stress. Furthermore, the absence of autophagy caused substantial changes in primary metabolism, including altered carbohydrate, amino acid and organic acid profiles, indicating disruption of carbon and energy homeostasis during prolonged warming. These metabolic disturbances were accompanied by reduced male fertility, lower seed production and alterations in seed reserve composition, whereas seed weight and germination capacity remained largely unaffected. Collectively, these findings demonstrate that autophagy can be essential for maintaining redox balance, metabolic homeostasis and reproductive performance during prolonged warming. By coordinating nutrient recycling, energy metabolism and reproductive development, autophagy can enhance plant resilience to high temperature conditions and contribute to reproductive success under climate warming scenarios.

Keywords: Metabolism; Prolonged Warming; Recovery Pos-stress; Redox Balance; Reproductive Performance.

Introduction

Global warming and climate change pose threats to food production needed to meet the demands of the growing human population, which is expected to exceed 9 billion by 2050 (Food and Agriculture Organization, 2018; Population Reference Bureau, 2020). It is projected that global annual average temperatures will increase by 0.3 to 4.8°C by 2100, with many regions likely to experience warming above the global average (IPCC, 2023). This rise in temperatures will alter plant phenology, physiological performance, and productivity (Jagadish et al., 2021). Consequently, a significant gap in supply and demand chains could arise, significantly impacting agro-economics (Jagadish et al., 2021; Nair et al., 2022).

High temperatures resulting from global warming can be classified into two categories: (i) heat shock, which consists of a sudden and intensive short-term heat wave (hours), and (ii) prolonged warming, characterized by a moderately high temperature increase lasting for days or weeks (Wang et al., 2020). Both cause developmental phase shortening and disruptions in carbon assimilation processes (transpiration, photosynthesis, and respiration) in plants (Djanaguiraman et al., 2018; Jagadish et al., 2021; Ullah et al., 2021). Plants respond to prolonged warming by reducing stomatal conductance and, consequently, CO₂ assimilation, increasing the glycolysis pathway while the tricarboxylic acid (TCA) cycle is inhibited and upregulating basic leucine zipper (bZIP) factors, which are endoplasmic reticulum stress sensors in plants that regulate stress signaling. Additionally, under these conditions, there is an increase in cellular hydrogen peroxide (H₂O₂) concentrations and antioxidant enzyme activity (Wang et al., 2020).

It is noteworthy that in response to oxidative stress, autophagy is one of the primary proteolytic systems in plants that degrade oxidized proteins and organelles (Kwon & Park, 2008; Liu et al., 2009; Pérez-Pérez et al., 2012). Autophagy, a major cellular degradation pathway, is involved in recycling cytoplasmic components into primary metabolites (Batoko et al., 2017). The autophagic process mediates the sequestration and transport of damaged organelles and proteins to the vacuole for degradation (Li et al., 2026). It is important to highlight that autophagy is not merely a generalized recycling system but can also target specific organelles, protein complexes, and aggregates (Üstün et al., 2017; Marshall & Vierstra, 2018), thus playing a crucial role in responses to various stresses.

In response to heat shock, an accumulation of autophagosomes occurs in tomato plants (*Solanum lycopersicum*), helping to mitigate damage. Tomato plants with virus-induced gene silencing (VIGS) of *ATG5* and *ATG7* exhibit greater sensitivity to heat stress compared to WT plants, manifested by increased wilting, electrolyte leakage, and reduced photosynthesis. Furthermore, *atg7* mutant plants showed an accumulation of insoluble protein aggregates marked by ubiquitin (Zhou et al., 2014). It has been postulated that heat stress induces autophagy by promoting endoplasmic reticulum (ER) stress (Yang et al., 2016). ER stress results from the accumulation of unfolded proteins, producing protein aggregates (Yeganeh et al., 2015). The treatment of *Arabidopsis* seedlings with tunicamycin (TM) and dithiothreitol (DTT), compounds that induce ER stress, led to the formation of autophagosomes. High temperatures cause protein misfolding, which is highly toxic and must be efficiently removed to reduce cellular proteotoxic stress if the restoration of native conformations is unsuccessful (Yang et al., 2016). Although autophagy is known to function in protein quality control by targeting misfolded and potentially toxic proteins for degradation in response to heat shock, its role and regulation in responses to prolonged warming remain incompletely elucidated.

Heat stress can affect plants at any stage of their development, but some growth stages are more sensitive to heat (Lohani et al., 2020). During these stages, such as flowering, heat stress induces strong negative impacts, affecting grain size and filling (Seth & Sebastian, 2024). When the grain maturation process occurs under warming conditions, there is a restriction in the translocation of resources to the growing grains, resulting in a reduction in the grain filling period, which culminates in a decrease in the accumulation of starch and protein in soybeans (Lohani et al., 2020). This phenomenon is attributed to the decrease in the activity of enzymes involved in the mobilization of reserves from the stem (Ullah et al., 2021). It is also worth noting that increased temperatures cause damage to gametes. Male gametophytes (pollens) are more sensitive to heat stress throughout their development, affecting pollen quantity and morphology, cell wall architecture, and, mainly, pollen metabolism (Chaturvedi et al., 2021). In response to increased temperature, autophagy acts on tapetum degeneration by PCD and proper pollen development. It is also essential for male gamete fertility, playing a role in pollen grain germination (Zhou et al., 2021). It has been shown that *atg* mutants exhibit visibly impaired pollen development and anther dehiscence, leading to male sterility induced by increased temperature (Dündar et al., 2019).

Taken together, the above evidence suggests an important role for autophagy in heat stress responses (Ogata et al., 2006; Zhou et al., 2014; Yeganeh et al., 2015; Dündar et al., 2019). However, knowledge about the significance of autophagy in modulating responses to prolonged warming during the reproductive phase is still quite incipient and fragmented. Thus, the role of autophagy in controlling damage caused by exposure to high temperatures during the reproductive phase was investigated, based on the characterization of the physiological and metabolic changes caused by the absence of the autophagic process in plants subjected to prolonged warming.

Methodology

Growth conditions and temperature treatment

A. thaliana wild-type (Col-0) and mutant lines *atg5-1* (SAIL_129B079; Thompson et al., 2005), *atg7-2* (GK-655B06; Hofius et al., 2009) and ATG8-GFP (GFP-ATG8g-HA; Huang et al., 2019) were used to study the autophagic process. Seeds were disinfected in 1 mL of 70% (v/v) ethanol for 1 minute and then in 2.5% (v/v) sodium hypochlorite for 15 minutes. All sodium hypochlorite residue was removed by washing (6x) with sterile water. After disinfection, the seeds underwent the vernalization process at 4°C for 48 h and were distributed in sterile Petri dishes containing ½ MS culture medium (Murashige & Skoog, 1962). The plates were then kept in a growth chamber for 10 days under the same conditions described above.

After transplantation, WT *Arabidopsis thaliana* plants (Colombia-0) and mutants for the autophagic process (*atg5-1* and *atg7-2*) were grown in a growth chamber with a neutral photoperiod (12 h light: 12 h dark), temperature of 22 °C during the light period and 20 °C during the dark period for four weeks. Relative humidity was maintained at 60% and irradiance at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Treatment of prolonged warming

Prolonged warming treatment was initiated, consisting of maintaining the pots at 28°C during the day and 26°C at night, for 7 days after the emergence of the floral peduncle in the reproductive phase (Olmo et al., 2019). Daytime and nighttime temperatures were maintained at 22/20°C as a controlled temperature. The physiological and biochemical analyses described below were performed on plants at five weeks of age at 0, 3 and 7 days of stress and 3 days of recovery time following prolonged warming.

Assessment of autophagic flux

Transgenic plants expressing an ATG8-GFP fusion were used to monitor autophagic flux (Chen et al., 2019). ATG8-GFP plants were grown under control (22°C/20°C) and heat stress (28°C/26°C) conditions for 7 days in the reproductive phases. To evaluate the induction of autophagy during prolonged warming, collections were performed during zero, three and seven days of stress and the three days of recovery. Plant samples kept in the dark for 7 days were used as positive control. Proteins were extracted from three leaf discs of fresh leaf material with extraction buffer 50 mM sodium phosphate pH 7.0; (with 0.1% SDS; 10 mM EDTA; 0.1% Triton; 0.1 mM PMSF; 1:50 Pi-protease inhibitor) and centrifuged at 12,000 rpm at 4°C for 30 min, collecting the supernatant in a new tube. After removal, the proteins were quantified (Bradford, 1976), standardized and denatured for 10 min at 95°C for separation via 12% SDS-PAGE. The electrophoretic profile was visualized by treating the gel with Coomassie Brilliant Blue reagent. Another aliquot of the extract was used for a new separation on 12% SDS-PAGE, followed by transfer of the proteins to a nitrocellulose membrane. The transfer efficiency was evaluated by treating the membrane with Ponceau reagent. For immunological detection of GFP and GFP-ATG8a, as soon as the samples were distributed to the membrane, it was immersed in a primary antibody solution (two hours) for GFP protein (anti-GFP ThermoFischer Scientific, #A-6455, 1:7.500) and subsequently in the secondary antibody (Invitrogen, #A-16110, 1:10.000) by the broad-spectrum alkaline phosphatase occurrence detection method (1 hour and a half). The last revelation was performed by (Chemidoc MP Imaging System), with visualization by colorimetric occurrence with NBT. The signal intensity was quantified by ImageJ, and the induction of autophagy was calculated by the ratio between the GFP-ATG8a and GFP proteins. All experiments were repeated with at least three biological replicates. The bands revealed on the membrane were quantified by ImageJ.

Confocal microscopy

Transgenic plants expressing an ATG8-GFP fusion were used to monitor autophagosome formation (Chen et al., 2019). ATG8-GFP plants were grown under control (22°C/20°C) and heat stress (28°C/26°C) conditions for 7 days in reproductive phases. Leaf cells after 3 and 7 days of prolonged warming and 3 days of recovery were observed using a Zeiss LSM510 META laser scanning confocal microscope. Plant samples kept in the dark for 7 days were used as positive control.

Generation of Arabidopsis lines expressing redox-sensitive fluorescent sensor protein

Mutant lines, as well as the WT, were transformed to express the fluorescent sensor proteins Peredox-mCherry (Steinbeck et al., 2020) and Grx1-roGFP2 (Ugalde et al., 2022) in the cytosol. The constructs used for each transformation were generated by PCR amplification of the coding sequence of the fluorescent protein fused to targeting sequences directing them to the cytosol, chloroplast, mitochondria, and peroxisome. Plant transformation was performed by the floral dip method, a widely used technique for *Arabidopsis thaliana* transformation (Clough & Bent, 1998). Transformed lines were selected based on antibiotic resistance and the fluorescence intensity of the redox sensor proteins. After selection on culture medium, these lines were propagated to obtain homozygous lines. At each step of transformation and selection, fluorescence of the redox sensor proteins was used as a control. Fluorescence excitation and image acquisition were performed using confocal microscopy.

Assessment of cytosolic redox status using fluorescent sensor proteins

To assess alterations in the cytosolic redox status, the reduced nicotinamide adenine dinucleotide / oxidized nicotinamide adenine dinucleotide (NADH/NAD⁺) and oxidized glutathione / reduced glutathione (GSSG/GSH) ratios were monitored using the biosensors Peredox-mCherry and Grx1-roGFP2, respectively. Leaf discs from *Arabidopsis thaliana* WT plants and the *atg5-1* and *atg7-2* mutants, grown under control conditions (22°C/20°C) or heat stress conditions (28°C/26°C) for three and seven days, followed by three days of recovery, were transferred to the wells of a 96-well plate containing 200 µL of imaging buffer (10 mM MES, 5 mM KCl, 10 mM CaCl₂, 10 mM MgCl₂, pH 5.8). Fluorescence spectra were recorded using a microplate reader (Clariostar®, BMG) with excitation wavelengths ranging from 400 to 540 nm and emission collected between 520 and 615 nm for Peredox-mCherry, and excitation wavelengths ranging from 386 to 495 nm with emission collected between 530 and 540 nm for Grx1-roGFP2. Fluorescence signals were normalized to the fluorescence intensity of the sensor tSapphire at 400–520 nm for Peredox-mCherry and to the isosbestic point (IP) of roGFP2 at 425 nm for Grx1-roGFP2, respectively (Steinbeck et al., 2020; Bohle et al., 2024).

Determination of pyridine nucleotide content

NAD(H) and NADP(H) contents were determined as previously described by Schippers et al. (2008). Approximately 5 mg of lyophilized material was used for the analysis. Reduced and oxidized forms were distinguished by their preferential degradation under acidic or basic conditions, respectively. Absorbance was measured at 570 nm using an ELISA plate reader. Nucleotide concentrations were determined by regression analysis based on a standard curve and subsequently normalized to dry mass.

Analysis of the heat-responsive proteins HSP101 by Western blot

The differential accumulation of heat-responsive proteins HSP101 was evaluated in *Arabidopsis thaliana* plants exposed to prolonged warming conditions. WT and mutant plants *atg* were grown under control (22°C/20°C) and heat stress conditions (28°C/26°C) for 7 days in the reproductive phases. To evaluate the induction of autophagy during prolonged warming, collections were performed during zero, three and seven days of stress and the three days of recovery. Proteins were extracted from three leaf discs of fresh leaf material with extraction buffer 50 mM sodium phosphate pH 7.0; (with 0.1% SDS; 10 mM EDTA; 0.1% Triton; 0.1 mM PMSF; 1:50 Pi-protease inhibitor) and centrifuged at 12,000 rpm at 4°C for 30 min, collecting the supernatant in a new tube (Faurobert et al., 2007). After removal, the proteins were quantified (Bradford, 1976), standardized and denatured for 10 min at 95°C for separation via 12% SDS-PAGE. The electrophoretic profile was visualized by treating the gel with Coomassie Brilliant Blue reagent. Another aliquot of the extract was used for a new separation on 12% SDS-PAGE, followed by transfer of the proteins to a nitrocellulose membrane. The transfer efficiency was evaluated by treating the membrane with Ponceau reagent. For immunological detection of HSP101 as soon as the samples were distributed to the membrane, it was immersed in a primary antibody solution (two hours) for HSP101 proteins (anti-HSP101 Agrisera, AS07 253, 1:7,500) and subsequently in the secondary antibody (BIO-RAD, 1706518, 1:5,000) by the broad-spectrum alkaline phosphatase occurrence detection method (1 hour and a half). The last revelation was performed by (Chemidoc MP Imaging System), with visualization by colorimetric occurrence with NBT. The signal intensity was quantified by ImageJ, and the accumulation of this protein was calculated. All experiments were repeated with at least three biological replicates.

Gene expression

Quantitative real-time PCR (RT-qPCR) analysis was performed with total RNA isolated from whole rosettes. RNA extraction was performed with TRizol® (Ambion, Life Technology) following the manufacturer's manual. RNA integrity was verified on a 1% (w/v) agarose gel, and the concentration was measured before and after DNase I digestion using a Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com/>). DNase I digestion (Ambion; <http://www.ambion.com/>) was performed according to the manufacturer's instructions. cDNA was synthesized from 1 µg of total RNA using High-Capacity reverse transcriptase (ThermoFisher; <http://www.thermofisher.com/>) according to the manufacturer's instructions. Quantitative PCR was performed using the Power Syber Green master mix kit. The samples were amplified according to the program: 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 56 °C for 15 s and 72 °C for 15 s. To confirm the presence of a single amplification product, specific for each reaction, a denaturation curve was performed immediately after amplification. The readings were performed on the Applied Biosystem StepOnePlus™ Software v2.0 equipment. The expression levels were determined by the comparative CT (Threshold Cycle) method, according to Schmittgen and Livak (2008). *EF1α* and *Actin* were used as reference genes and the evaluated genes and their respective primers are described in Table S1.

Determination of metabolites in rosettes

The rosette discs were collected and homogenized and subjected to methanolic extraction. Briefly, 700 µL of methanol was added to the sample followed by shaking (750 rpm) at 80°C for 20 minutes. Afterwards, the samples were centrifuged, and the supernatant was separated from the precipitate. To the supernatant were added 375 µL of chloroform (99.9%) and 750 µL of milli-Q water. The samples were centrifuged and the aqueous phase was separated for future analyses. In the soluble fraction, the contents of chlorophyll (Sulpice et al., 2009), glucose, fructose and sucrose (Ferne et al., 2001), total soluble amino acids (Cross et al., 2006), malate and fumarate (Nunes-Nesi et al., 2007) were determined. In the insoluble fraction the starch (Ferne et al., 2001) and protein (Bradford, 1976) contents. For GC-MS analysis the extraction was performed using 700 µL of methanol and shaking (750 rpm) at 70°C for 15 min, and 30 µL of ribitol (0.1 mg mL⁻¹) was added as an internal standard. The followed extraction and derivatization procedure was performed exactly as described (Daloso et al., 2015). Peaks were annotated

manually, and ion intensity was determined by the aid of TagFinder software (Luedemann et al., 2012), using a reference library from the Golm Metabolome Database (Kopka et al., 2005) and following the recommended reporting format (Fernie et al., 2011).

Evaluation of oxygen consumption rates

Potential oxygen (O₂) consumption rates on days zero, three and seven of prolonged warming and after three days of recovery were assessed using a Clark-type oxygen electrode, following the methodology described by Jiang et al. (2019). Before measuring respiratory rates, the fresh weight of the leaves was determined, and they were then incubated in a buffer containing 50 mM HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)), 10 mM MES (2-N-morpholinoethanesulfonic acid), pH 6.6, and 0.2 mM CaCl₂. To prevent light interference, the measurements were conducted in the dark for 20 minutes. The O₂ consumption rate was calculated assuming that the O₂ concentration in the air-saturated buffer was 260 µM at 23°C.

Count of flowers, siliquas, branches, seeds per siliqua and siliqua length

The number of flowers, siliquas, and branches per plant were analyzed. Siliquas from WT and mutant plants were collected from five plants per genotype and clarified in 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS) solution (Yoo et al., 2012). The clarified siliquas were used for siliqua length and number of seeds per siliqua that were analyzed under a dissecting microscope (Stemi 2000-C, Zeiss) with an adapter (SOLIGOR tube for Canon A650 IS B52 Wide) to a 12.1-megapixel CANON camera (Power Shot A650 IS).

Pollen grain viability

Pollen viability was performed on WT and *atg* mutant plants by collecting flowers from five plants per genotype. The flowers were then dismembered by removing the petals and exposing the anthers to remove the pollen by mechanical force with the aid of a magnifying glass. The pollen was then stained with Alexander's histochemical reagent, which stains viable and nonviable pollen differently. The photos were taken and measured by microscope (Zeiss Vision Imager.A2 Axion 10 photo microscope). They were subsequently evaluated using the ImageJ program.

Seed count and weight per plant

The weight of a thousand seeds and the weight of all seeds produced by the same plant were quantified. The biometric data obtained from the photographic records were evaluated using the ImageJ program.

Seed germination test

Seeds of Col-0 plants and *atg* mutants from both the control and heat treatments were properly disinfested and germinated in MS medium for 60 hours. The seeds then underwent a stratification process for 48 hours at 4°C in the dark.

Subsequently, the plates were transferred to a growth chamber regulated at 22 ± 2°C. Evaluations were performed at intervals of 0, 12, 24, 36, 48 and 60 hours. Germination percentage and rate, and germination speed index were evaluated. Five replicates with 50 seeds of each genotype were included in the analysis and the variables will be calculated according to Bruginski et al. (2009).

Determination of seed metabolites

Seeds of Col-0 plants and *atg* mutants from both the control and heat treatments were collected and embedded in historesin (Historesin Leica Microsystems, Heidelberg, Germany) according to the manufacturer's recommendations. Cross sections (5 µm thick) were obtained using an automatic rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA). Histochemical tests for the detection of seed storage carbohydrates, proteins, and lipids were performed using Lugol's solution (Johansen, 1940), Coomassie Brilliant Blue (Fisher, 1968), and Sudan Red B (Brundrett et al., 1991), respectively. For reserve quantification, the seeds were homogenized and subjected to methanolic extraction. In summary, 700 µL of methanol were added to the sample followed by agitation (750 rpm) at 80°C for 20 minutes. Afterwards, the samples were centrifuged, and the supernatant was separated from the precipitate. To the supernatant were added 375 µL of chloroform (99.9%) and 750 µL of milli-Q water. The samples were centrifuged and the aqueous phase was separated for future analyses. In the soluble fraction, the contents of sucrose (Fernie et al., 2001), total soluble amino acids (Cross et al., 2006), malate and fumarate (Nunes-Nesi et al., 2007) were determined. In the insoluble fraction the starch (Fernie et al., 2001) and protein (Bradford, 1976) contents. Seeds oil was extracted with hexane, and the recovered lipid fraction was subsequently weighed to determine seed oil content (Bligh and Dyer, 1959).

Experimental design and statistical analysis

The experimental design was completely randomized, consisting of two levels: control study (22°C/20°C) and heat stress (28°C/26°C), with five replicates. Each experimental unit consisted of ten plants.

The normality and homoscedasticity of the data were analyzed by the Shapiro-Wilk and Bartlett tests, respectively, both at 0.05 probability. Then, the data was subjected to analysis of variance (ANOVA) using the F test ($P \leq 0.05$). When significant, the characteristics were subjected to the Tukey test ($P < 0.05$). All statistical analysis of the data was performed using protocols developed in the R software (R Development Core Team, 2019).

Results

Autophagic mutants appear to senesce faster in response to warming

To evaluate the impact of autophagy deficiency on the rosette and inflorescence under prolonged warming conditions and subsequent recovery, we compared autophagy deficient mutants (*atg5-1* and *atg7-2*) with WT plants grown at 28°C during the day and 26°C at night for seven days (Olmo et al., 2019) (Figure 1A), followed by a three-day recovery period (Figure 1B). Visual analysis suggests an acceleration of leaf senescence in autophagy-deficient plants when exposed to prolonged warming. At the same time, there also appears to be an acceleration of inflorescence development in plants subjected to prolonged warming conditions.

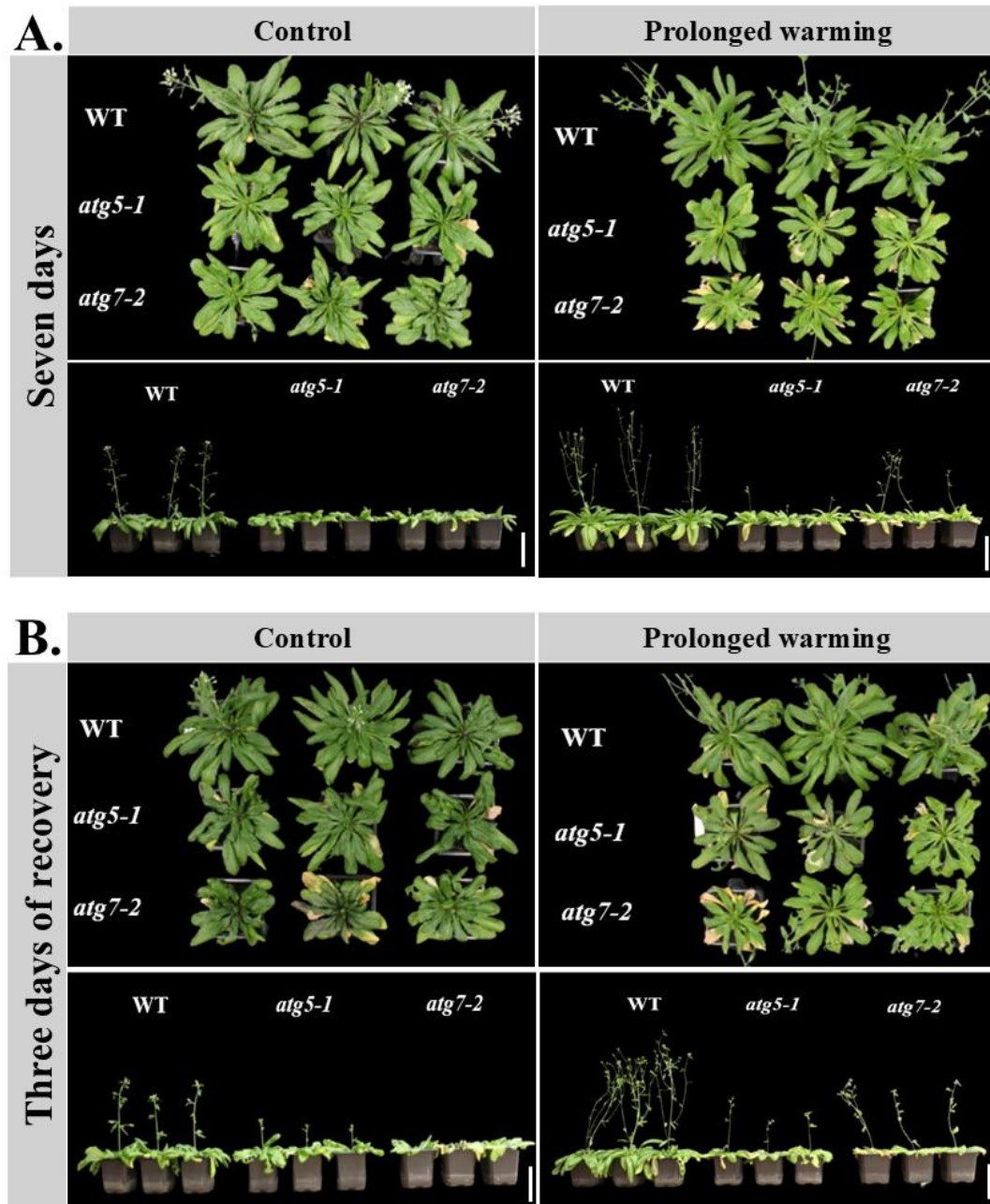


Figure 1: The absence of autophagy accelerates senescence and enhances inflorescence development under prolonged warming. Phenotypic characterization of autophagy-deficient (*atg*) mutant plants under prolonged warming conditions and recovery. WT and *atg* mutant (*atg5-1* and *atg7-2*) *Arabidopsis thaliana* plants with impaired autophagic flux were compared (A) after seven days of prolonged warming and (B) after three days of recovery. The white scale bar represents 5 cm.

The peak of autophagy occurs on the third day of prolonged warming

To determine the timing of autophagic flux induction over seven days of prolonged warming (28°C/26°C), followed by three days of recovery, transgenic plants expressing the ATG8–GFP fusion protein were used. Initially, total protein extraction was performed, followed by detection of GFP using a specific antibody (Figure 2C). This approach allowed the identification of both free GFP and the fused ATG8–GFP form, enabling the calculation of the GFP/ATG8–GFP ratio and, consequently, the assessment of autophagic flux (Figure 2B). Interestingly, autophagic activity peaked on the third day of prolonged warming, followed by a slight decrease, while remaining elevated until the seventh day of exposure. During the recovery period, autophagic flux returned to basal levels, comparable to those observed at day zero, prior to stress application. Plants subjected to seven days of darkness were used as positive control, as this condition is a well-established inducer of autophagy. For additional confirmation, samples from day three and day seven of prolonged warming, three days of recovery, and seven days of darkness were selected for GFP fluorescence analysis by confocal microscopy (Figure 2D). Although visual detection is more challenging, increased fluorescence intensity was observed on the third day of stress compared to the seventh day and the recovery period, both in the adaxial epidermis and mesophyll, corroborating the protein-based analysis results.

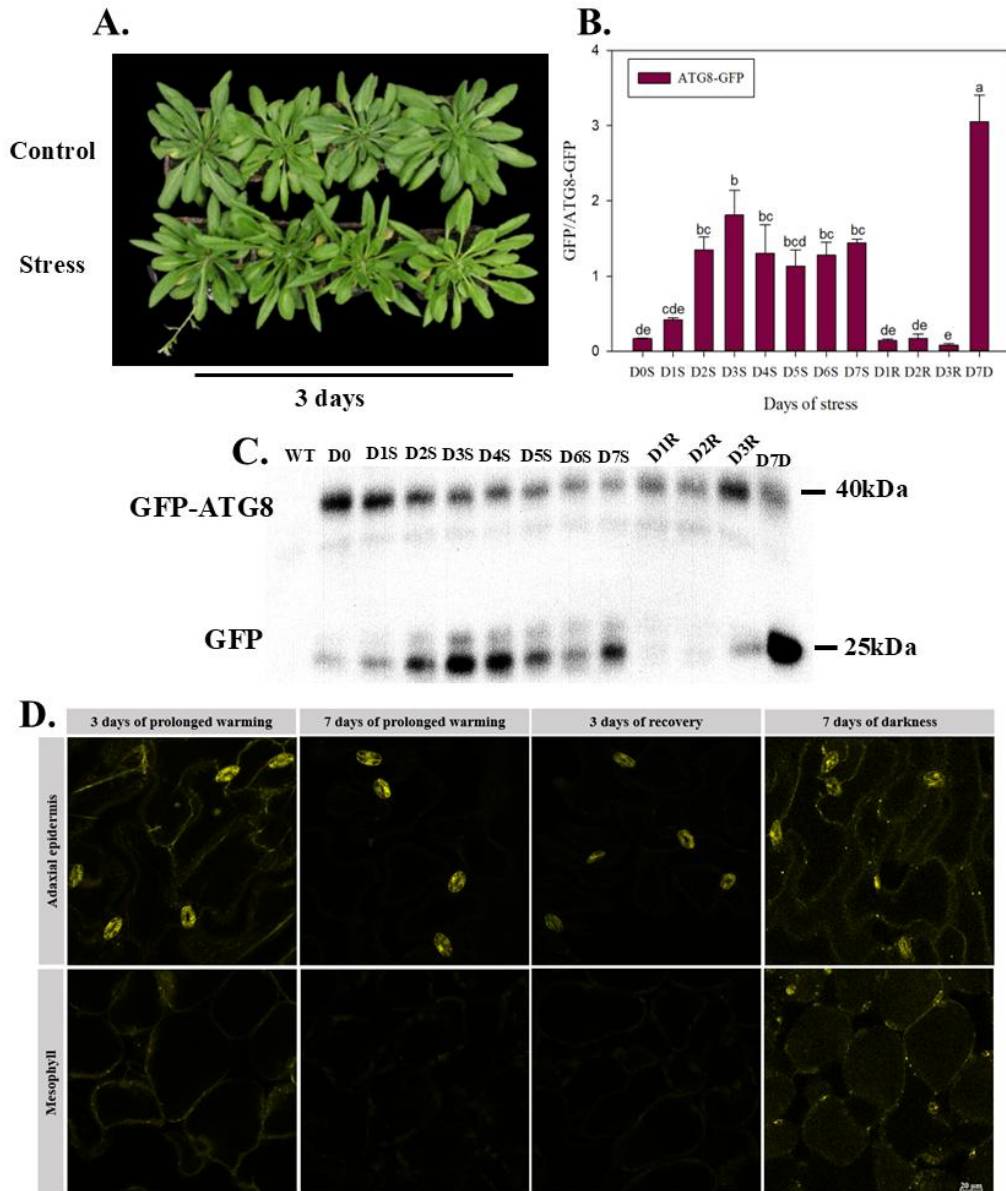


Figure 2: The peak of autophagic flux occurs on the third day of prolonged warming and decreases during recovery in *Arabidopsis thaliana*. (A) Plant phenotype on the third day of stress. (B) Quantification of autophagic flux in transgenic plants expressing ATG8–GFP subjected to prolonged warming (28°C/26°C) for seven days, followed by three days of recovery. Total protein extracts were analyzed by Western blot using an anti-GFP antibody, allowing the detection of both free GFP and ATG8–GFP. Autophagic activity was estimated based on the GFP/ATG8–GFP ratio. Plants subjected to seven days of darkness were used as a positive control for autophagy induction. Abbreviations: D0S: day zero of stress; D1S: day one of stress; D2S: day two of stress; D3S: day three of stress; D4S: day four of stress; D5S: day five of stress; D6S: day six of stress; D7S: day seven of stress; D1R: day one of recovery; D2R: day two of recovery; D3R: day three of recovery; D7D: day seven of darkness. (C) Representative Western blot membrane showing the detection of ATG8–GFP and free GFP in transgenic *Arabidopsis thaliana* plants expressing ATG8–GFP subjected to prolonged warming (28°C/26°C) for seven days, followed by three days of recovery. Plants exposed to seven days of darkness were used as a positive control for autophagy induction. (D) Confocal microscopy images showing GFP fluorescence in leaf tissues on day 3 and day 7 of heat stress, after 3 days of recovery, and after 7 days of darkness. Representative images of the adaxial epidermis and mesophyll are shown. Increased GFP fluorescence on day 3 indicates the peak of autophagic activity during prolonged warming. Scale bars: 20 μ m.

Autophagy mutants differentially modulate gene expression during three days of prolonged warming and induce the accumulation of the HSP101 protein

Genes involved in both glycolysis, TCA cycle and stress response was evaluated in WT, *atg5-1*, and *atg7-2* plants subjected to 22°C and 28°C using quantitative gene expression assay. Among the genes associated with primary metabolism, *FBA1* expression, gene encoding fructose-1,6-bisphosphate aldolase (FBA) involved in glycolysis, showed no significant differences among genotypes or temperatures, indicating transcriptional stability of this gene under the evaluated conditions (Figure 3B). In contrast, *FUM1*, which encodes fumarase and is involved in the TCA cycle, displayed higher expression in WT plants at 22°C, whereas autophagy deficient mutants showed a significant reduction in transcript levels, especially *atg7-2*. In turn, under 28°C, *FUM1* expression remained low in all genotypes (Figure 3C). Similarly, *PMDH1* expression, which encodes peroxisomal malate dehydrogenase (MDH), was reduced in the mutants compared to WT at 22°C, with an additional decrease observed under warming conditions, particularly in *atg5-1* and *atg7-2* (Figure 3D). For *GAP*, which encodes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved in glycolysis and the TCA cycle, higher expression was detected in WT plants at 22 °C, whereas the mutants showed significantly reduced transcript accumulation, a trend that persisted at 28°C (Figure 3E). These results suggest impaired regulation of genes associated with energy metabolism in autophagy-deficient plants.

Among the stress responsive regulatory genes, members of the basic leucine zipper (bZIP) transcription factor family that regulates the expression of other genes involved in development, metabolism and responses to environmental stresses showed changes in response to genotype and temperature. *bZIP9* gene showed increased expression in *atg5-1* at both temperatures, suggesting differential activation of this pathway in the mutants (Figure 3F). In contrast, *bZIP24* exhibited higher expression in *atg5-1* at 22°C, whereas under 28 °C transcript levels were higher in WT plants and reduced in *atg7-2* (Figure 3G). The *E3* gene, which encodes E3 ubiquitin ligases involved in the protein ubiquitination system, was induced in *atg7-2* at 22°C, while at 28 °C its expression was reduced in both autophagy mutants (Figure 3H).

Genes related to heat response and autophagy displayed marked induction patterns in the mutants. *WRKY75*, a member of the WRKY transcription factor family that plays a major role in regulating the expression of genes involved in stress responses,

metabolism, root development and nutrient homeostasis, showed a pronounced increase in transcript accumulation in both *atg5-1* and *atg7-2* at both temperatures (Figure 3I). The selective autophagy marker gene *NBR1* was strongly induced under warming conditions, particularly in *atg7-2*, indicating accumulation associated with autophagy deficiency (Figure 3J). Similarly, *HSP17.6*, which encodes a small heat shock protein (sHSP), was highly induced in *atg7-2* at 22°C and remained elevated at 28°C, whereas WT plants exhibited low expression at both temperatures (Figure 3K). Finally, *HSFA2*, member of the Heat Shock Factor (HSF) transcription factor family, showed higher expression in WT plants under both temperature conditions, while the mutants displayed significantly reduced transcript levels, suggesting that autophagy deficiency compromises the activation of this important heat responsive transcription factor (Figure 3L).

In general, these results demonstrate that autophagy deficiency profoundly alters the transcriptional regulation of genes associated with primary metabolism and heat stress responses, indicating that ATG genes play an important role in maintaining metabolic homeostasis and adaptation to elevated temperatures.

The accumulation of the heat shock protein HSP101 was evaluated in WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*) throughout heat stress and the three-day recovery period (Figure 3A). At day zero of stress (D0), HSP101 levels were low in all genotypes, although the autophagy mutants showed significantly higher values than WT. After three days of heat stress (D3), a significant increase in HSP101 levels was observed in all genotypes, with greater accumulation in *atg5-1* and *atg7-2* mutants compared to WT (Figure 3A). This pattern became even more pronounced after seven days of heat stress (D7), when the autophagy-deficient mutant *atg7-2* exhibited significantly higher HSP101 levels (Figure 3B). After three days of recovery (D3R), HSP101 levels were partially reduced in both mutants compared to D7. However, they remained significantly higher than those observed in WT. Partially reduced in both mutants compared to D7. However, they remained significantly higher than those observed in WT. However, they remained significantly higher than those observed in WT.

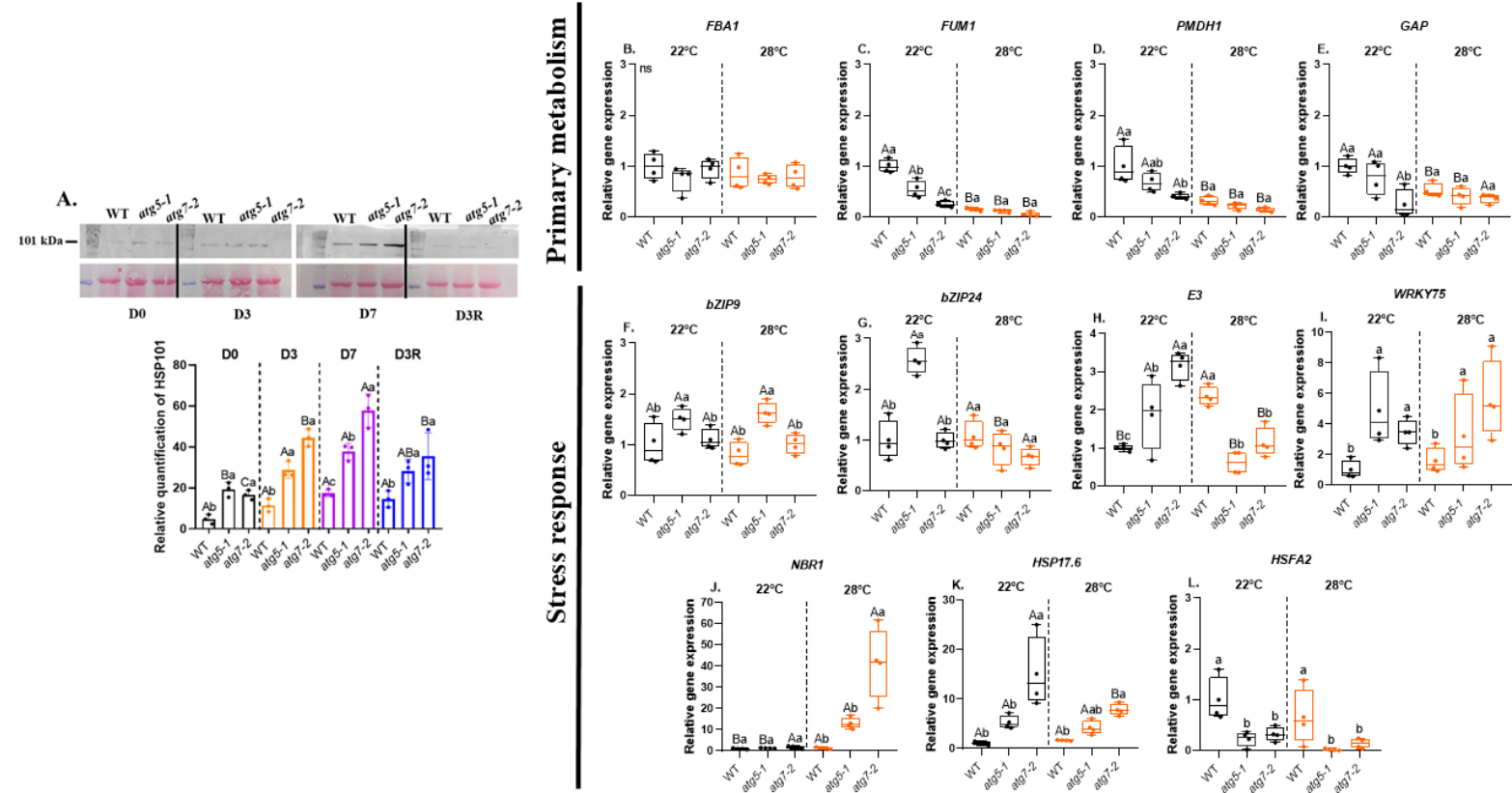


Figure 3. Absence of autophagy results in HSP101 protein accumulation and alters the transcription of genes involved in primary metabolism and stress responses under three days prolonged warming. The detection of the HSP101 protein was performed in *Arabidopsis thaliana* plants subjected to prolonged warming (28 °C/26 °C) for zero (D0), three (D3), and seven (D7) days, followed by a three-day recovery period (D3R). Total protein extracts were analyzed by Western blot using an anti-HSP101, specific antibody, allowing the detection and quantification of this protein. **(A)** The figure shows a representative Western blot membrane illustrating HSP101 detection and Relative quantification of HSP101 protein. Ponceau staining confirmed the size of the total proteins among the samples. Genes of primary metabolism **(B)** *FBA1*, **(C)** *FUM1*, **(D)** *PMDH* and **(E)** *GAP* and stress response **(F)** *bZIP9*, **(G)** *bZIP24*, **(H)** *E3*, **(I)** *WRKY75*, **(J)** *NBR1*, **(K)** *HSP17.6* and **(L)** *HSFA2* were evaluated in *A. thaliana* plants in response to three days of prolonged warming. WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until the emergence of the floral peduncle and subsequently exposed to elevated temperatures, with 28 °C during the daytime and 26 °C during the nighttime. The effect of the post-stress recovery period was also evaluated. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes under different temperature treatments, whereas lowercase letters indicate comparisons among genotypes within the same warming condition. Data are presented as mean $\pm$ standard error (SE) from three biological replicates per genotype.

Prolonged warming affects the cytosolic redox status in plants exposed to sustained prolonged warming

To investigate changes in cytosolic redox status induced by prolonged warming in WT plants and autophagy-deficient mutants, the NADH/NAD⁺ and GSSG/GSH ratios were monitored using the Peredox-mCherry and Grx1-roGFP2 biosensors, respectively (Figure 4). Overall, autophagy deficiency did not significantly impact the balance between oxidized and reduced glutathione, a parameter often associated with oxidative stress in plants. However, a significant increase in oxidized glutathione (GSSG) was observed in *atg7-2* plants on the third day of heat exposure, an effect not detected in *atg5-1* or WT plants (Figure 4A). In contrast, on the seventh day of prolonged warming, only WT plants showed a significant increase in GSSG compared to the control (Figure 4B). During the recovery phase, all genotypes exhibited elevated GSSG levels, suggesting that oxidative stress-related responses persist during this period, regardless of genotype (Figure 4C). Regarding the NADH/NAD⁺ redox pair, consistent differences were observed between WT and the mutants under both control and prolonged warming conditions. However, the lack of significant variation in this ratio between treatments indicates that the experimental conditions were not sufficient to induce a global perturbation in NAD-dependent redox status (Figure 4D-F). On the other hand, the consistently higher ratio observed in the mutants suggests a more oxidized cellular state compared to WT, possibly resulting from intrinsic metabolic alterations associated with the genotype.

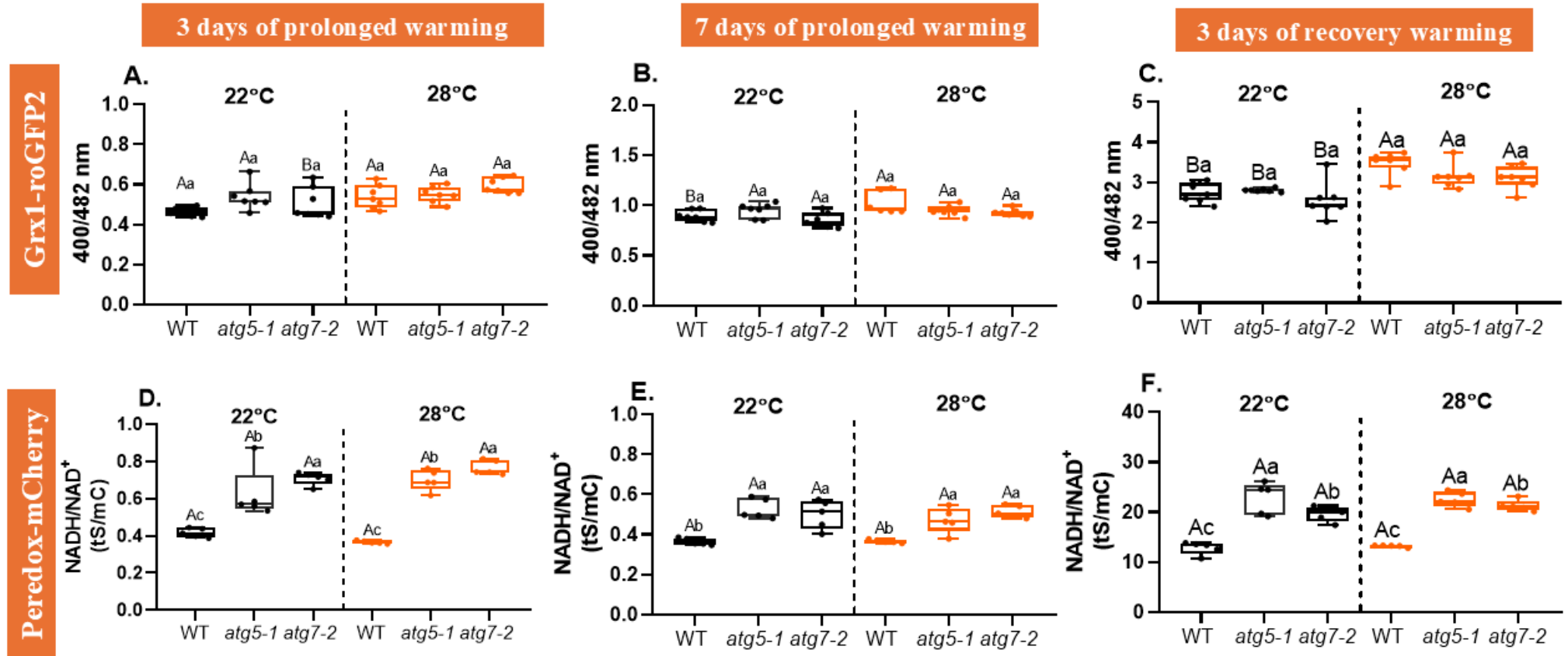


Figure 4: Cytosolic redox status changes induced by prolonged warming in WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*). (A, B e C) The GSSG/GSH and (D, E e F) NADH/NAD⁺ ratios were monitored using Col-0 (WT) and *atg* mutants (*atg5-1* and *atg7-2*) transformed with the Grx1-roGFP2 and Peredox-mCherry biosensors, respectively, under control conditions, during prolonged warming (3 and 7 days), and after the recovery period. Autofluorescence from the corresponding Col-0 controls without a sensor was used for background subtraction. Reduction-oxidation-sensitive green fluorescent protein 2 (roGFP2): Ex = 400 ± 5 and 482 ± 16 nm / Em = 520 ± 10 nm; and tSapphire for mCherry: Ex = 400 ± 10 and 520 ± 10 nm / Em = 520 ± 10 and 615 ± 18 nm, respectively. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data are presented as means ± standard error (SE) of eight biological replicates per genotype.

Autophagy-deficient mutants exhibit altered pyridine nucleotide redox status during warming and recovery

The NADH/NAD⁺ and NADPH/NADP⁺ ratios were evaluated in WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) under prolonged warming (28°C) and recovery conditions. After three days of prolonged warming, the *atg5-1* mutant exhibited a significant increase in the NADH/NAD⁺ ratio compared with WT and *atg7-2* plants under heat conditions, whereas no major differences were observed among genotypes at 22°C (Figure 5A). After seven days of warming, both *atg* mutants maintained higher NADH/NAD⁺ ratios than WT plants at 28°C, with *atg5-1* showing the highest values (Figure 5B). During the recovery period, *atg* mutants displayed increased NADH/NAD⁺ ratios (Figure 5C).

Similarly, the NADPH/NADP⁺ ratio was affected by prolonged warming and recovery. After three days of warming, WT plants showed higher NADPH/NADP⁺ ratios at 22°C, while at 28°C the *atg7-2* mutant presented an upward trend in the ratio among the evaluated genotypes (Figure 5D). After seven days of prolonged warming, *atg5-1* maintained significantly elevated NADPH/NADP⁺ ratios under prolonged warming compared with WT and *atg7-2* plants (Figure 5E). During recovery, the NADPH/NADP⁺ ratio decreased in all genotypes, but the accumulation was markedly greater in *atg5-1* plants exposed to 28°C (Figure 5F).

Overall, the absence of autophagy altered cellular redox homeostasis during prolonged warming and recovery, promoting higher NADH/NAD⁺ and NADPH/NADP⁺ ratios, especially in the *atg5-1* mutant.

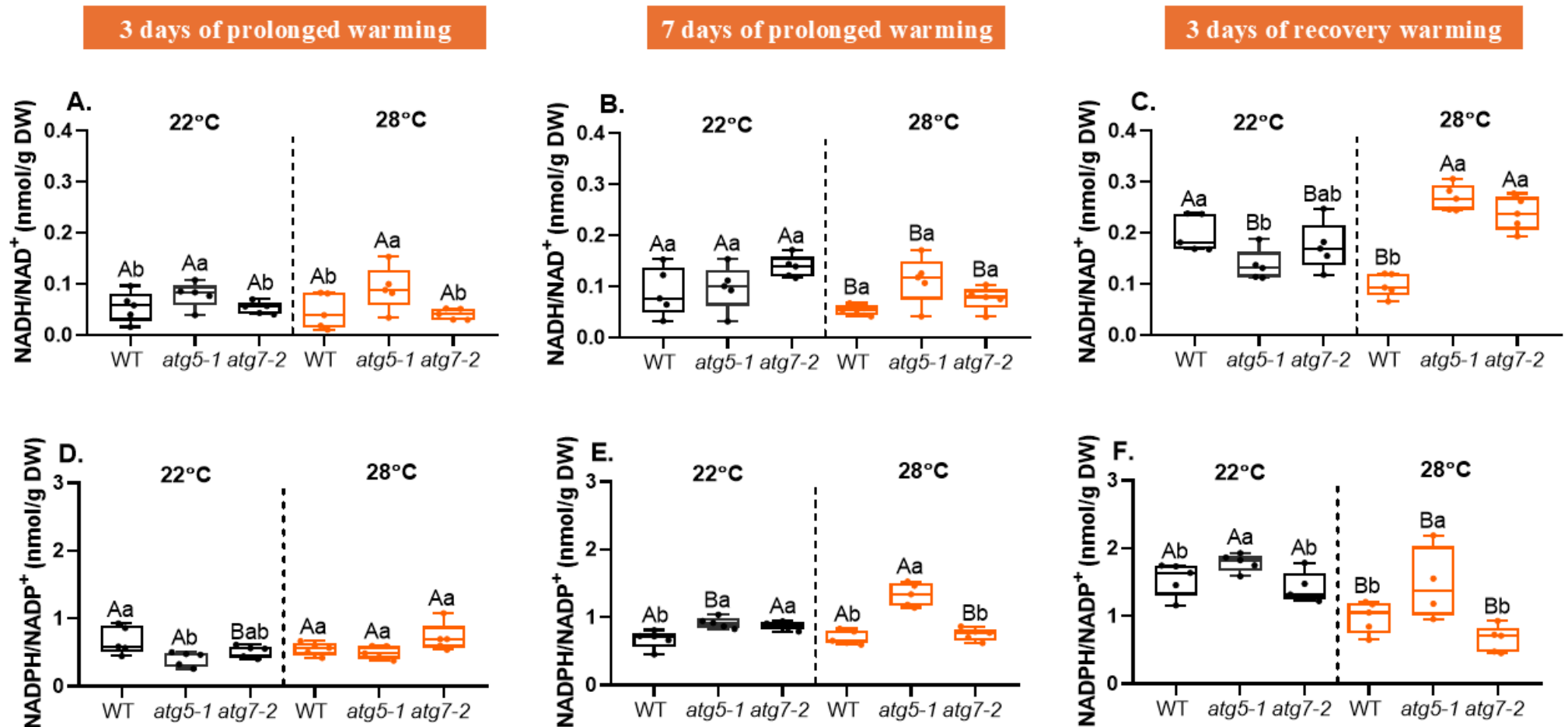


Figure 5: Pyridine nucleotide ratios (NAD(H)/NADP(H)) changes induced by prolonged warming stress in WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*). (A, B e C) The NADH/NAD⁺ and (D, E e F) NADPH/NADP⁺ ratios were monitored using Col-0 (WT) and *atg* mutants (*atg5-1* and *atg7-2*), under control conditions, during prolonged warming (3 and 7 days), and after the recovery period. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data are presented as means ± standard error (SE) of eight biological replicates per genotype.

The absence of autophagy leads to alterations in the metabolic profile

GC-MS based metabolic profiling revealed dynamic and time-dependent metabolic reprogramming in WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*) under prolonged warming and subsequent recovery. At day zero (Figure 6A), hierarchical clustering analysis already indicated genotype-dependent differences, with *atg* mutants displaying distinct metabolic profiles compared to WT, particularly in sugars, organic acids, and amino acids, suggesting basal metabolic alterations associated with autophagy deficiency.

After three days of prolonged warming (Figure 6B), a clear shift in metabolic profiles was observed, with clustering primarily driven by temperature. WT plants exhibited increased accumulation of several soluble sugars and stress-related metabolites, whereas *atg* mutants showed a differential and less coordinated response, with altered levels of organic acids and amino acids.

By day seven of prolonged warming (Figure 6C), metabolic reprogramming became more pronounced. A strong separation between control and heat-treated samples was observed, with significant accumulation of sugars such as raffinose, galactinol, and other osmoprotective compounds, particularly in WT plants. In contrast, autophagy deficient mutants displayed altered accumulation patterns of tricarboxylic acid (TCA) cycle intermediates and amino acids, indicating a disruption in central carbon metabolism and energy balance under prolonged stress conditions.

During the recovery phase (three days), metabolic profiles shifted again, reflecting post-stress adjustment (Figure 6D). WT plants showed partial reversion toward control-like metabolic states, whereas *atg* mutants maintained distinct metabolic signatures. Notably, mutants exhibited increased accumulation of several amino acids and organic acids, along with persistent alterations in sugar metabolism, indicating impaired metabolic recovery and a sustained stress imprint.

Taken together, these results demonstrate that autophagy plays a critical role in maintaining metabolic homeostasis during prolonged warming and is particularly important for efficient metabolic reprogramming during the recovery phase. The absence of autophagy leads to genotype specific and time dependent metabolic imbalances affecting key pathways related to energy metabolism, osmoprotection and stress adaptation.

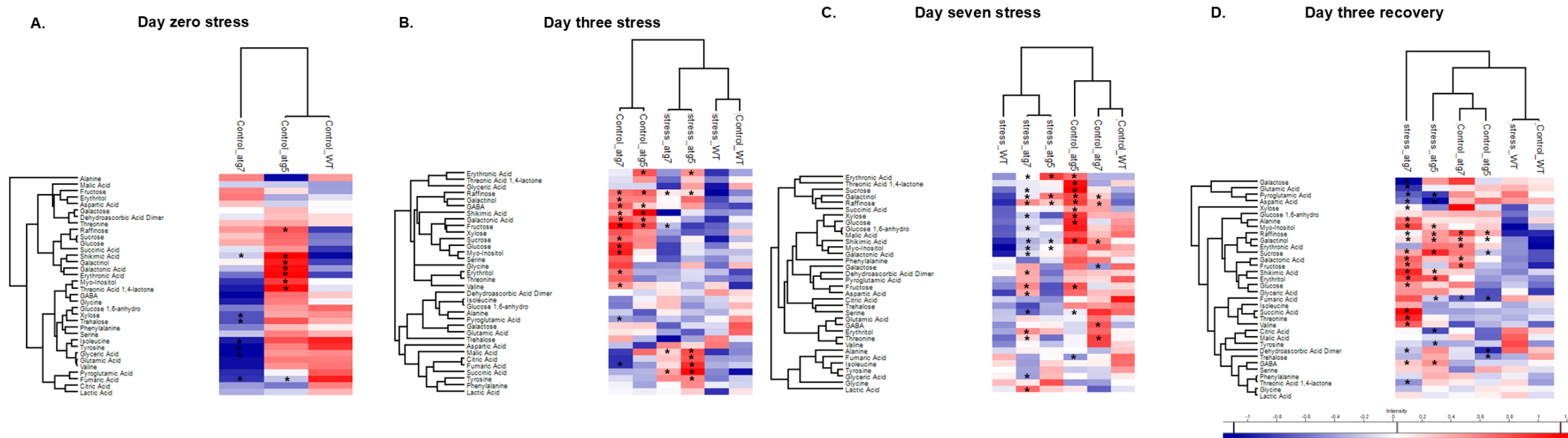


Figure 6: Prolonged warming alters genotype-dependent clustering patterns. Hierarchical clustering heatmap of metabolite levels at (A) day zero (control conditions), (B) three days, (C) seven days and (D) three days of recovery of prolonged warming. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were analyzed in leaves. The color code of the heat map is given at log₂ following the scale above the diagram. Metabolite abundance was normalized (z-score) and is represented as a color scale, where red indicates higher levels and blue indicate lower levels relative to the mean $\pm$ se ($n = 5$). Dendrograms represent hierarchical clustering of metabolites based on similarity in accumulation patterns. Asterisks (*) indicate that the values from mutant lines were determined by Student's *t* test to be significantly different ($P < 0.05$) from the WT within each condition.

Autophagy is essential for maintaining reproductive development and seed yield

Under control conditions (22°C), WT plants exhibited better reproductive performance compared to autophagy-deficient mutants (*atg5-1* and *atg7-2*), as evidenced by greater plant height, number of branches, open flowers and floral buds (Figure 7B-E). Under prolonged heat conditions (28°C), this superior performance of WT relative to the mutants became even more pronounced, with a significant increase compared to control conditions. In contrast, autophagy-deficient mutants did not show a proportional increase in these parameters under heat, maintaining significantly lower values than WT, which displayed a positive response to elevated temperature. Thus, autophagy deficiency appears to impair plant reproductive performance, particularly under prolonged warming conditions.

Analysis of pollen viability revealed significant effects of both genotype and temperature (Figure 7F). Under control conditions (22°C), WT plants exhibited the highest percentage of viable pollen grains, while autophagy deficient mutants showed reduced viability. In particular, the *atg7-2* mutant showed a markedly lower percentage of viable pollen compared to WT, while *atg5-1* showed intermediate values. Exposure to elevated temperatures (28°C) resulted in a significant reduction in pollen viability in all genotypes. WT plants showed a clear decrease in viable pollen under heat stress compared to 22°C. A similar trend was observed for the *atg7-2* mutant, which maintained the lowest viability among the genotypes evaluated. In contrast, *atg5-1* showed a comparatively smaller reduction under elevated temperature, although its pollen viability did not differ significantly from that of WT at 28°C.

Under control conditions (22°C), WT plants exhibited a higher number and greater size of siliques compared to autophagy-deficient mutants (*atg5-1* and *atg7-2*), indicating a stronger impairment of reproductive development in the absence of autophagy. Prolonged warming (28°C) affected the genotypes differently. Prolonged warming reduced the number of seeds per silique, particularly in the autophagy-deficient mutants (Figure 7I). WT plants showed a significant increase in the number of siliques compared to control conditions, whereas both autophagy deficient mutants did not exhibit a similar increase, maintaining significantly lower values than WT (Figure 7G). Regarding silique size, heat promoted a significant reduction in WT plants compared to control conditions (Figure 7H). However, even under 28 °C, *atg5-1* and *atg7-2* mutants exhibited

significantly shorter siliques than WT, suggesting that autophagy is important for maintaining reproductive success, particularly under prolonged warming conditions.

Taken together, these results indicate that autophagy contributes to maintaining pollen viability under optimal temperature conditions and that elevated temperature negatively impacts reproductive performance, with autophagy-deficient lines exhibiting intrinsically reduced basal viability.

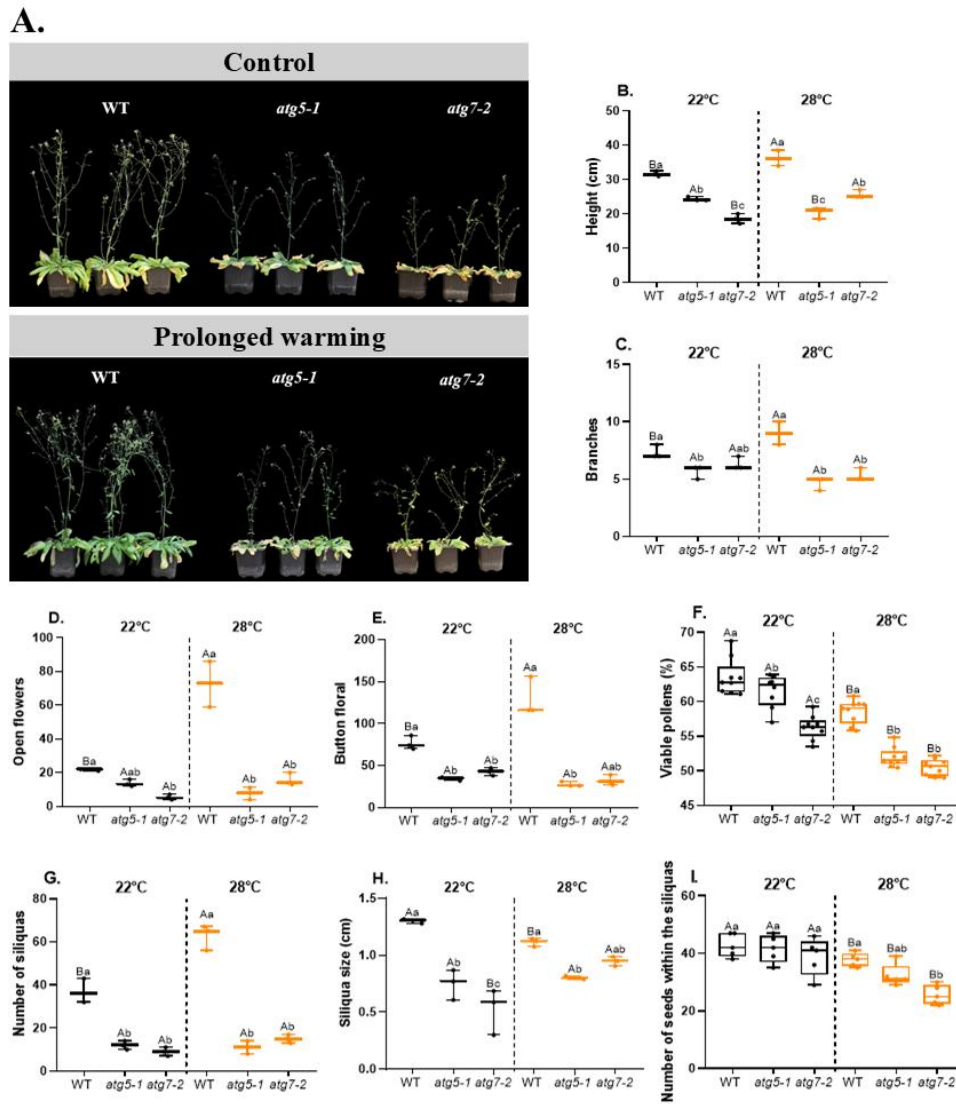


Figure 7: Prolonged warming promotes improved reproductive performance in WT *Arabidopsis thaliana* plants. (A) Phenotypic characterization of reproductive aspects of autophagy-deficient mutant plants (*atg*) after prolonged warming conditions. WT and *atg* mutants (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were compared with seven weeks old after enduring seven days of prolonged warming. The white scale bar represents 5 cm. The graphs show (B) plant height (cm), (C) number of branches, (D) number of open flowers, (E) number of flower buds, (F) viable pollens (%), (G) number of siliques, (H) silique size (cm), number of siliques and (I) number of seeds within the siliques under control (22°C) and prolonged warming (28°C) conditions of autophagy-deficient mutant plants (*atg5-1* and *atg7-2*) compared to the WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$).

Uppercase letters denote comparisons among genotypes under different temperature treatments, whereas lowercase letters indicate comparisons among genotypes within the same warming condition. Data are presented as mean $\pm$ standard error (SE) from five biological replicates per genotype.

Under control conditions (22°C), no significant differences were observed in the seed weight among WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*), indicating similar reproductive output under optimal growth conditions (Figure 8B). In contrast, prolonged warming stress (28°C) significantly affected reproductive performance in a genotype dependent manner. While WT showed only a slight reduction under warming conditions, WT and *atg5-1* exhibited a pronounced decrease in seed number, with *atg7-2* being the most affected genotype (Figure 8C). Seed germination was not altered in response to prolonged warming in any of the genotypes (Figure 8D-E). Altogether, these findings indicate that autophagy plays a crucial role in sustaining reproductive success under elevated temperatures. The absence of autophagy compromises seed production under heat stress, suggesting that this process is essential for maintaining reproductive fitness under adverse environmental conditions.

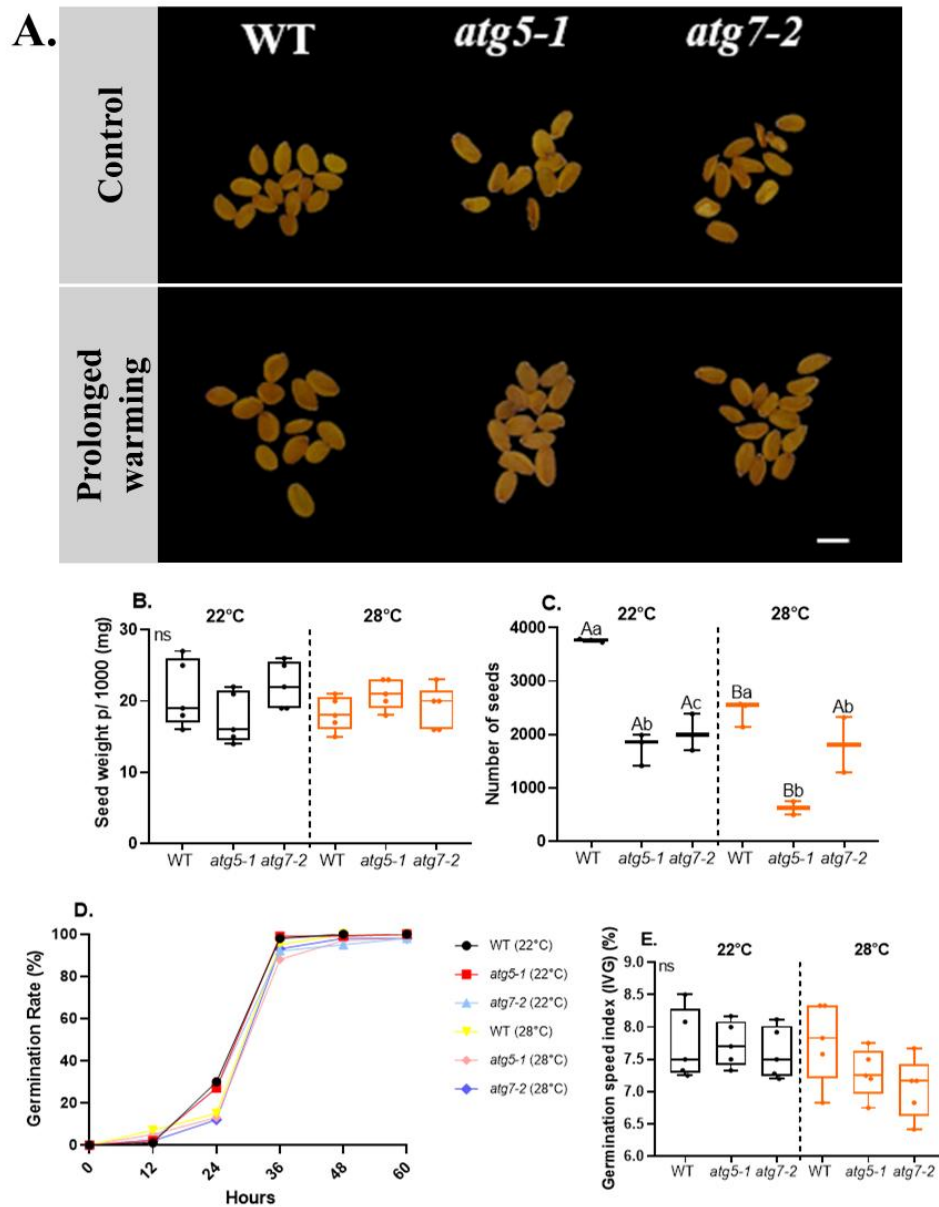


Figure 8: Prolonged warming does not affect seed germination or seed weight among the different genotypes. WT and *atg* mutant (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were evaluated after 26 days of prolonged warming. The white scale bar represents 1 mm. The graphs show (B) seed weight p/ 1000, (C) number of seeds, (D) germination rate and (E) germination speed index (IVG) under control (22°C) and prolonged warming (28°C) conditions of autophagy-deficient mutant plants (*atg5-1* and *atg7-2*) compared to the WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes under different temperature treatments, whereas lowercase letters indicate comparisons among genotypes within the same warming condition. Data are presented as mean $\pm$ standard error (SE) from five biological replicates, each consisting of five plants per genotype. Abbreviation: ns: non-significant interaction.

Histochemical and quantitative analyses were performed to investigate changes in the accumulation of lipid, protein and starch reserves in seeds of WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) of *Arabidopsis thaliana* under prolonged warming conditions (Figure 9). Quantitative analyses corroborated the histochemical observations of seed reserve accumulation under prolonged warming conditions (Figure 9B–H). Seed lipid content did not show significant differences among genotypes or temperature conditions, indicating that prolonged warming had little effect on total lipid accumulation in seeds (Figure 9B). Although histochemical staining suggested variations in lipid distribution patterns, especially in autophagy-deficient mutants, these changes were not reflected in total oil quantification.

In contrast, significant differences were observed in seed protein content (Figure 9C). Under control conditions (22°C), *atg5-1* and *atg7-2* seeds exhibited higher protein accumulation compared with WT plants. However, prolonged warming (28°C) reduced protein content in all genotypes, with the strongest decrease observed in WT and *atg5-1* seeds. Despite this reduction, *atg7-2* seeds maintained comparatively higher protein levels under warming conditions, consistent with the histochemical staining pattern.

Starch quantification also supported the histochemical results (Figure 9D). Under control conditions, *atg5-1* seeds accumulated significantly higher starch levels than WT and *atg7-2* plants. Following prolonged warming, starch content increased in *atg7-2* seeds, whereas *atg5-1* plants showed a decrease. Consequently, under heat conditions, *atg5-1* seeds maintained the highest starch accumulation among the evaluated genotypes.

Sucrose content differed among genotypes under control conditions, with *atg5-1* plants showing the highest accumulation, whereas *atg7-2* exhibited the lowest levels (Figure 9H). Under prolonged warming, sucrose levels increased in *atg7-2* plants compared with their respective controls, while WT and *atg5-1* plants showed a reduction relative to control conditions. Consequently, no significant differences were observed among genotypes under prolonged warming.

Amino acid contents were also affected by prolonged warming (Figure 9E). Under control conditions, *atg7-2* plants accumulated higher amino acid levels than WT and *atg5-1* plants. Exposure to prolonged warming promoted a significant increase in amino acid content in all genotypes, resulting in similar accumulation patterns under stress conditions.

Figure 9. Histochemical and quantitative analyses of seed storage reserves demonstrate that prolonged warming affects reserve contents in autophagy-deficient mutants (*atg5-1* and *atg7-2*). **(A)** Histochemical detection of lipids, protein and starch reserves in seeds from plants grown under control (22°C) and prolonged warming (28°C) conditions. Lipids were stained with Sudan Red B, proteins with Coomassie Brilliant Blue and starch with Lugol's solution. Scale bar = 100µm. Quantification of **(B)** seed lipids, **(C)** protein, **(D)** starch, **(E)** amino acids, **(F)** malate, **(G)** fumarate and **(H)** sucrose content in seeds of WT, *atg5-1* and *atg7-2* plants under control and prolonged warming conditions. Different uppercase letters indicate significant differences

between temperatures within the same genotype, whereas different lowercase letters indicate significant differences among genotypes within the same temperature condition ($P < 0.05$). Data are presented as mean $\pm$ standard error (SE) from five biological replicates per genotype. Abbreviation: ns: non-significant interaction; DW: dry weight.

Discussion

Our understanding of how prolonged warming modulates reproductive responses in plants remains fragmented. Indeed, most studies have focused on autophagy in responses to acute heat shock during the vegetative stage (Zhou et al., 2014; Sedaghatmehr et al., 2018; Wang et al., 2020; Chen et al., 2026). Therefore, the present study aimed to investigate the role of autophagy in response to prolonged warming during reproductive development. Autophagy plays an important role in plant responses to various abiotic stresses, including the recycling of damaged or unwanted proteins and organelles, nutrient remobilization and the mitigation of oxidative stress, ultimately promoting plant survival (Avin-Wittenberg, 2018). Some functions of autophagy have already been described in reproductive tissues under elevated temperatures, such as its involvement in tapetum degeneration through programmed cell death (PCD) and in supporting proper pollen development without causing male sterility (Dündar et al., 2019). Based on these observations, we hypothesized that autophagy is a fundamental component of plant responses to prolonged warming during the reproductive phase. Consistent with this hypothesis, our results demonstrate that autophagy emerges as an essential component of reproductive thermotolerance in *Arabidopsis thaliana*, acting as a key process coordinating metabolism, cellular protection and reproductive development under elevated temperatures.

Prolonged Warming Accelerates Inflorescence Development and Exacerbates Leaf Senescence in Autophagy-Deficient Mutants

Following floral stem emergence, plants exposed to seven days of prolonged warming exhibited accelerated inflorescence development and enhanced leaf senescence, particularly in the autophagy deficient mutants (Figure 1 and Figure 7). In *Arabidopsis thaliana*, even a modest increase of approximately 2°C above the optimal growth temperature (22-23°C) is sufficient to trigger developmental changes, including accelerated flowering (Lippmann et al., 2019; Kan et al., 2023). This thermomorphogenic response, which occurs under moderately elevated temperatures (up to 30°C in *Arabidopsis*), is primarily regulated by phytochrome interacting factors (PIFs) and

phytohormone signaling pathways (Quint et al., 2016; Kan et al., 2023). However, as temperature increases and exposure becomes prolonged, plant performance progressively declines, resulting in reduced growth, accelerated leaf senescence, impaired reproductive development and ultimately growth arrest (Seth & Sebastian, 2024).

Autophagy Deficiency Triggers Proteotoxic Stress and Compensatory Heat Shock Responses Under Prolonged Warming

The temporal pattern of autophagic activity observed during prolonged warming suggests that autophagy is dynamically regulated throughout the acclimation process (Figure 2). The peak observed after three days of heat exposure indicates that this mechanism is strongly induced early in response to elevated temperature. This response may reduce the progression of cellular damage throughout the treatment period, limiting the need for prolonged activation of auxiliary protective systems (Thirumalaikumar et al., 2021). In contrast, autophagy deficiency compromises this capacity for cellular adjustment, resulting in a greater reliance on molecular chaperones after extended periods of heat exposure (Sedaghatmehr et al., 2018). The importance of this process is further supported by the accumulation of HSP101 proteins observed in autophagy-deficient mutants after three and seven days of heat stress (Figure 3A). HSP101 is a key molecular chaperone for plant thermotolerance, acting in the resolubilization of protein aggregates formed under high temperature conditions (Tripathi et al., 2026). The increased abundance of HSP101 in the *atg* mutants combined with the increased expression of *NBR1* (Figure 3J) and *HSP17.6* (Figure 3K) under heat stress (28°C), suggests that the absence of autophagy leads to a greater demand for protein quality control mechanisms as a compensatory response, likely due to the accumulation of damaged proteins that cannot be efficiently removed through the autophagic pathway (Sedaghatmehr et al., 2018). The strong induction of *NBR1*, particularly in *atg7-2* under heat stress (Figure 3J), provides direct evidence for the activation of mechanisms involved in the recognition and targeting of damaged proteins for selective degradation (Zhou et al., 2014). As *NBR1* functions as a selective autophagy receptor, its accumulation in autophagy-deficient mutants is often interpreted because of disrupted autophagic flux (Zhang & Chen, 2020). Whereas *NBR1* reflects the cellular perception of proteotoxic damage, the increased abundance of *HSP17.6* and HSP101 indicates a greater accumulation of denatured proteins and protein aggregates that cannot be efficiently removed through the autophagic pathway (Sedaghatmehr & Balazadeh, 2024).

Gene expression analysis revealed that autophagy deficiency promotes significant alterations in genes associated with the heat stress response (Figure 3). The transcription factors *bZIP9* and *bZIP24* exhibited genotype dependent expression patterns (Figure 3F and 3G). Members of the *bZIP* family play important roles in the integration of stress responses, particularly through their involvement in the response to misfolded proteins and the maintenance of cellular proteostasis (Wang et al., 2020). The reduction in their expression under heat stress conditions suggests that autophagy deficiency may impair the transcriptional coordination required for metabolic adaptation to elevated temperatures. The results obtained for *WRKY75* further support this interpretation. *WRKY75* was strongly induced in the mutants, especially under prolonged warming exposure (Figure 3I). This transcription factor has been associated with the regulation of autophagy, senescence, nutrient metabolism and responses to environmental stresses (Üstün et al., 2018; Wang et al., 2023; Ma et al., 2025). Its induction may represent a compensatory attempt to activate alternative adaptive pathways in response to autophagy deficiency.

In response to heat stress, plants have evolved multiple adaptive mechanisms, including the induction of heat shock transcription factors (HSFs) and heat shock proteins (HSPs) (Qian et al., 2025). *HSFA2*, one of the major transcriptional regulators of heat stress memory and thermotolerance in *Arabidopsis*, exhibited reduced expression levels in the mutants compared with the WT (Figure 3L). This result suggests that autophagy deficiency may compromise the proper activation of transcriptional programs associated with long term warming acclimation (Zhang et al., 2021). Given that *HSFA2* regulates numerous genes involved in cellular protection, its reduced expression may help explain the greater dependence of the mutants on compensatory mechanisms observed at other physiological and metabolic levels (Kan et al., 2023).

Autophagy Deficiency Disrupts Pyridine Nucleotide Homeostasis and Alters Respiratory Dynamics Under Prolonged Warming

Autophagy deficiency significantly affects redox and energy homeostasis during prolonged warming exposure. Data obtained using the Peredox-mCherry biosensor (Figure 4D-F), together with biochemical quantification, revealed an increase in the NADH/NAD⁺ ratio (Figure 5A–C) in autophagy-deficient mutants at different time points throughout the treatment. The maintenance of the balance between the oxidized and reduced forms of pyridine nucleotides is essential for processes such as mitochondrial

respiration, energy metabolism, antioxidant defense, and cellular biosynthesis (Noctor et al., 2007). In this context, the absence of autophagy likely imposes metabolic constraints that ultimately alter NAD(H) and NADP(H) dynamics. Particularly noteworthy was the marked increase in NADPH/NADP⁺ ratio levels observed in the *atg5-1* mutant after seven days of heat treatment and during the recovery period (Figure 5E and 5F). As NADP(H) is directly involved in antioxidant metabolism by providing reduced power to glutathione and thioredoxin dependent systems, this accumulation may reflect a compensatory response to the increased demand for cellular redox control (Foyer & Noctor, 2011). This result suggests that autophagy deficiency promotes metabolic reprogramming aimed at maintaining oxidative homeostasis during prolonged stress exposure (Signorelli et al., 2019). Interestingly, the alterations observed in NAD(H) and NADP(H) pools were more pronounced than those detected by the Grx1-roGFP2 biosensor during the heat treatment period, which remained relatively stable (Figure 4A–C). This apparent uncoupling suggests that compensatory mechanisms were able to preserve the overall glutathione redox balance despite substantial alterations in pyridine nucleotide metabolism (Foyer & Noctor, 2011).

Autophagy contributes to sustaining the high energetic demands associated with the initial acclimation to prolonged warming. Assessments of mitochondrial respiration revealed that the absence of autophagy alters metabolic dynamics in response to heat (Figure S2). After three days of heat treatment, a time point that coincided with the peak of autophagic activity, WT plants exhibited a marked increase in mitochondrial respiration at 28°C, whereas the mutants displayed significantly lower respiratory responses (Figure S2B). Considering that heat stress increases cellular energy demand due to the activation of repair mechanisms, metabolic remodeling, and protein quality control systems, the pronounced reduction in sucrose and starch levels, particularly in the WT (Figure S4), suggests that autophagy promotes an intense mobilization of carbon reserves to support the energetic demands imposed by stress (Kan et al., 2023). The higher respiratory activity observed in the *atg* mutants during the post-stress recovery period may reflect a compensatory effort to provide the energy and reducing power required for the removal of damage accumulated during heat stress (Figure S2D) (Sedaghatmehr & Balazadeh, 2024).

Autophagy Deficiency Disrupts Primary Metabolomic Reprogramming, TCA Cycle Flexibility, and Post-Heat Stress Recovery

Metabolomic analysis revealed that the absence of autophagy promotes extensive alterations in primary metabolism during prolonged warming exposure and particularly, during recovery from heat stress. Hierarchical clustering analyses demonstrated a clear separation among genotypes and treatments, indicating that autophagy deficiency profoundly affects the metabolic reorganization required for acclimation to prolonged warming. Even in the absence of warming stress (day zero), the *atg5* mutant already exhibited increased levels of soluble sugars (including raffinose and galactinol) and organic acids relative to the WT, whereas the *atg7* mutant displayed a marked depletion of amino acids (including tyrosine, valine, glycine, and glutamic acid) and tricarboxylic acid (TCA) cycle intermediates, such as fumaric acid (Figure 6A), suggesting that autophagy exerts constitutive effects on cellular metabolism. This observation is consistent with the well-established role of autophagy in the constitutive recycling of proteins and organelles, contributing to the balance between synthesis, degradation and metabolite availability (Masclaux-Daubresse et al., 2014; Avin-Wittenberg, 2018; Wang et al., 2026).

After three days of heat treatment (Figure 6B), a time point that coincided with the peak of autophagic activity (Figure 2B), a pronounced metabolic reorganization was observed in the *atg* mutants. Among the most evident alterations was the change in the metabolism of sugars and amino acids. The reorganization of these metabolites suggests profound changes in nitrogen metabolism and in the use of amino acids as alternative sources of carbon and energy (Araújo et al., 2011). Although total amino acid levels did not show marked changes during heat exposure (Figure S5B), the mutants displayed increased protein content during the recovery period (Figure S5E). This result may reflect the accumulation of stress associated proteins or proteins that would normally be removed through autophagy (Sedaghatmehr & Balazadeh, 2024). Changes in organic acid levels further indicate modifications in respiratory metabolism. The *atg* mutants exhibited lower malate levels during heat treatment, whereas the WT maintained higher concentrations (Figure S5C). Considering that malate is an important intermediate of the tricarboxylic acid (TCA) cycle, its reduction suggests limitations in the respiratory metabolism of the mutants (Araújo et al., 2012). This interpretation is reinforced by the lower capacity of *atg5-1* to increase respiration after three days of heat exposure and by the reduced

expression of the genes *FUM1* and *PMDH1*, both directly involved in mitochondrial metabolism (Figure 3C and 3D). Interestingly, although the expression of respiratory metabolism related genes was reduced under stress conditions, the WT exhibited an increase in respiratory rate after three days of heat treatment (Figure S2B). This apparent discrepancy suggests that the regulation of respiration during warming acclimation does not depend exclusively on the transcriptional upregulation of TCA cycle genes, but also on post transcriptional mechanisms and the availability of metabolic substrates (Araújo et al., 2012). Collectively, these findings suggest that autophagy contributes to maintaining the metabolic flexibility of the tricarboxylic acid (TCA) cycle, thereby supporting the respiratory flux required during the early stages of heat stress.

Metabolic differences remained evident after seven days of prolonged warming, particularly in the autophagy deficient mutants. According to the PCA analysis, the *atg5-1* mutant exhibited the greatest separation from the WT along the first principal component, indicating a substantially distinct metabolic reorganization (Figure S8C). Whereas WT plants accumulated osmoprotectants and protective sugars (e.g., raffinose, galactinol, and sucrose) to mitigate heat induced damage (Luo et al., 2020), the *atg* mutants exhibited a widespread depletion of these soluble compounds (Figure 6C). Furthermore, the marked reduction in tricarboxylic acid (TCA) cycle intermediates (citric, fumaric, malic, and succinic acids), together with metabolites associated with the γ -aminobutyric acid (GABA) pathway and the shikimate pathway in the mutants, suggests a severe impairment of respiratory energy metabolism and amino acid recycling (Masclaux-Daubresse et al., 2014; Avin-Wittenberg et al., 2015). Although respiratory rates became similar among the genotypes at this stage (Figure S2C), the metabolomic profiles indicate that the mechanisms underlying this physiological state likely differ between WT and mutant plants. Thus, the apparent functional recovery of respiration may mask a substantially different metabolic reorganization in the absence of autophagy (Avin-Wittenberg et al., 2015).

The post-stress recovery period (Figure 6D) displayed a clear separation among genotypes and extensive reprogramming of sugars, amino acids, and organic acids. The *atg* mutants exhibited an impaired ability to re-establish metabolic homeostasis. Whereas heat stressed WT plants clustered closely with their respective control conditions, indicating metabolic resilience, heat-stressed *atg* mutants remained metabolically divergent and accumulated disproportionate levels of specific sugars and organic acids,

including galactose, sucrose, glucose and fumaric acid. This pattern is characteristic of a metabolic emergency response, reflecting defective cellular recycling and an inability to restore post-stress homeostasis (Sedaghatpanah et al., 2022; Thirumalaikumar et al., 2018). Collectively, these findings indicate that autophagic flux represents a central hub for orchestrating metabolomic reprogramming under heat stress and for ensuring the metabolic memory required for plant recovery. These findings suggest that the loss of autophagy compromises the capacity for metabolic adjustment during heat stress, resulting in a more intense and persistent reprogramming of the central processes involved in energy metabolism (Sedaghatmehr & Balazadeh, 2024).

The maintenance of photosynthetic pigments during the first three days of heat exposure suggests that the temperature regime employed did not cause severe damage to the photosynthetic apparatus (Figures S1 and S3). However, during the recovery period, the mutants exhibited lower chlorophyll and carotenoid contents compared with the WT (Figures S3H and S3J). These results suggest that, although autophagy deficient plants can survive prolonged warming exposure, they display a reduced capacity to fully restore photosynthetic performance following stress (Izumi et al., 2017). This limitation may contribute to the lower capacity for replenishing carbon reserves observed in the mutants.

Energetic Constraints and Proteotoxic Stress Impair Pollen Viability in atg Mutants Under Heat Stress

Adaptive responses divert valuable metabolic and energetic resources that could otherwise be allocated to optimizing productivity (Sedaghatmehr & Balazadeh, 2024). High temperatures have been shown to disrupt essential reproductive processes, including flowering, pollen development, pollination and seed formation (Qian et al., 2025). Autophagy plays a fundamental role in maintaining fertility and productivity in *Arabidopsis* subjected to elevated temperatures (Dündar et al., 2019). During prolonged warming exposure, WT plants consistently outperformed the *atg* mutants across all reproductive parameters evaluated, indicating that the absence of autophagy compromises both the development of reproductive structures and the subsequent processes of fertilization and seed filling (Lippmann et al., 2019). Although the autophagy-deficient genotypes already exhibited delayed reproductive development under control conditions, the effects of heat became significantly more pronounced in the mutants, highlighting the importance of autophagy in sustaining reproduction under conditions of high metabolic demand. The delay in floral stem emergence observed in the *atg5-1* and *atg7-2* mutants

suggests that autophagy contributes to the proper transition from vegetative growth to reproductive development (Figure S6) (Masclaux-Daubresse et al., 2017). This finding is consistent with the metabolomic profiles obtained even before stress imposition, in which the mutants displayed metabolic signatures distinct from those of the WT (Figure 6A). Because floral induction and reproductive growth depend on a precise coordination between energy availability and metabolic signaling, the alterations observed in sugars, amino acids and organic acids likely contribute to the phenological delay observed in the autophagy deficient genotypes (Cho et al., 2016).

The lower pollen viability observed in the mutants under prolonged warming may be directly linked to the alterations detected in energy metabolism and redox homeostasis (Figure 7F). Pollen development is one of the most energy demanding processes in the plant life cycle and depends heavily on mitochondrial activity, carbohydrate supply, and the maintenance of oxidative balance (Qian et al., 2025). In this context, the changes observed in NAD(H), NADP(H), malate and fumarate levels suggest that autophagy deficiency compromises the ability to sustain the respiratory metabolism required for proper male gametophyte development (Geisler et al., 2012; Geng et al., 2018). The alterations in tricarboxylic acid cycle intermediates and the lower respiratory capacity observed in the mutants during the early stages of heat exposure, further supports the hypothesis that metabolic limitations contribute to reduced fertility (Müller & Rieu, 2016). The increased expression of *NBR1*, *HSP17.6*, and *WRKY75* (Figure 3I-K), along with the accumulation of HSP101 in the mutants (Figure 3A), suggests a greater proteotoxic burden during prolonged warming. Reproductive tissues, particularly anthers, microspores and developing pollen grains, are highly sensitive to the accumulation of heat-induced denatured proteins (Fragkostefanakis et al., 2016; Resentini et al., 2023). Therefore, the inability to efficiently remove damaged proteins through autophagy may directly impair pollen viability and fertilization efficiency.

Autophagy Deficiency May Trade Seed Quantity for Preserved Seed Quality to Maintain Germination Capacity Under Heat Stress

The alterations observed in carbon metabolism also showed a strong relationship with reproductive performance (Cho et al., 2016). During heat exposure, WT plants exhibited intense mobilization of starch and sucrose reserves (Figures S4C and S4D), coinciding with the increase in mitochondrial respiration (Figure S2) and the peak of autophagic activity observed after three days of treatment (Figure 2). This pattern

suggests that autophagy contributes to the availability of energetic substrates required to simultaneously sustain heat acclimation and reproductive development (Signorelli et al., 2019). In contrast, the mutants displayed alterations in glucose and sucrose contents, indicating lower efficiency in carbon mobilization and redistribution (Figure S4). Considering that pollen development, pollen tube growth, and seed filling are highly dependent on carbohydrate supply, these metabolic limitations likely contribute to the reduction in seed number observed in the mutants (Masclaux-Daubresse et al., 2017; Erlichman et al., 2023). Early seed development is considered a particularly vulnerable stage to heat stress (Seth & Sebastian, 2024). Although individual seed weight and germination rate were not significantly affected by heat treatment (Figure 8B and 8E), a marked reduction in the total number of seeds produced by the mutants was observed (Figure 8C). This result suggests that the primary impact of autophagy deficiency does not occur during seed maturation or affect the quality of the seeds that are successfully formed, but rather during earlier reproductive stages, including floral development, pollen viability, fertilization and silique formation.

Heat stress can affect seed nutritional quality by altering protein composition, reducing starch accumulation, and causing a marked decrease in seed oil content in oilseed crops (Seth & Sebastian, 2024). The changes observed in the distribution of proteins and starch within the seeds suggest that autophagy deficiency influences the allocation and storage of reserves during seed development under prolonged warming (Figure 9). These differences may reflect alterations in nutrient remobilization from maternal tissues, a process in which autophagy plays an important role during periods of high metabolic demand (Erlichman et al., 2023). Considering the reduction in the total number of seeds produced, it is possible that the available resources were distributed among a smaller number of reproductive sinks, thereby favoring the maintenance or even the increase of reserve content per seed (Bennett et al., 2012). After seven days of heat exposure and during the recovery period, the mutants exhibited extensive metabolic remodeling, increased respiratory activity, alterations in NAD(H)/NADP(H) pools, greater cellular oxidation, and the accumulation of proteins associated with stress responses. These findings suggest that, in the absence of autophagy, a substantial proportion of metabolic and energetic resources is redirected toward cellular repair processes and the maintenance of homeostasis, thereby reducing the availability of

carbon, nitrogen and energy for reproductive development (Masclaux-Daubresse et al., 2017).

Taken together, the results support a model in which autophagy acts as a central mechanism integrating proteostasis, energy metabolism and reproductive development during prolonged warming. Early activation of autophagy promotes the maintenance of mitochondrial function, redox homeostasis and the availability of metabolic resources, thereby allowing reproductive development to proceed even under adverse conditions (Signorelli et al., 2019). In contrast, autophagy deficiency results in the progressive accumulation of cellular damage, persistent metabolic reprogramming and increased energetic costs associated with acclimation, ultimately leading to reduced fertility, lower seed production and impaired reproductive success under prolonged warming.

Conclusions

This study demonstrates that autophagy plays a fundamental role in the acclimation of *Arabidopsis thaliana* to prolonged warming during the reproductive stage. The early induction of autophagy, observed after three days of prolonged warming, allowed the maintenance of respiratory activity and the stability of redox homeostasis, indicating that this process triggers an important mechanism of metabolic adaptation to warming stress. Autophagy deficiency promoted alterations in primary metabolism, including changes in sugar, amino acid and organic acid profiles, accompanied by modifications in NADH/NAD⁺ pools, global metabolic reprogramming and a greater reliance on alternative protein quality control mechanisms. The increased expression of *NBR1*, *WRKY75*, and *HSP17.6*, together with the accumulation of HSP101 protein, suggests that the absence of autophagy results in a greater proteotoxic burden and increased energetic demand for maintaining cellular homeostasis. During the recovery period, autophagy-deficient mutants exhibited extensive metabolic reorganization, increased respiratory activity and persistent redox alterations, indicating that the lack of autophagy increases the metabolic costs associated with re-establishing homeostasis following stress. In contrast, WT plants were able to recover sugar levels, carbon reserves and photosynthetic pigments more efficiently. These physiological and metabolic alterations were directly reflected in reproductive performance. Autophagy deficiency affected floral development, reduced pollen viability, decreased the number of seeds per siliqua and significantly reduced the final productivity of plants exposed to prolonged

warming. Thus, the results indicate that autophagy not only protects cells against heat induced damage but also preserves the availability of metabolic resources required to sustain reproduction under elevated temperatures.

Supplementary materials

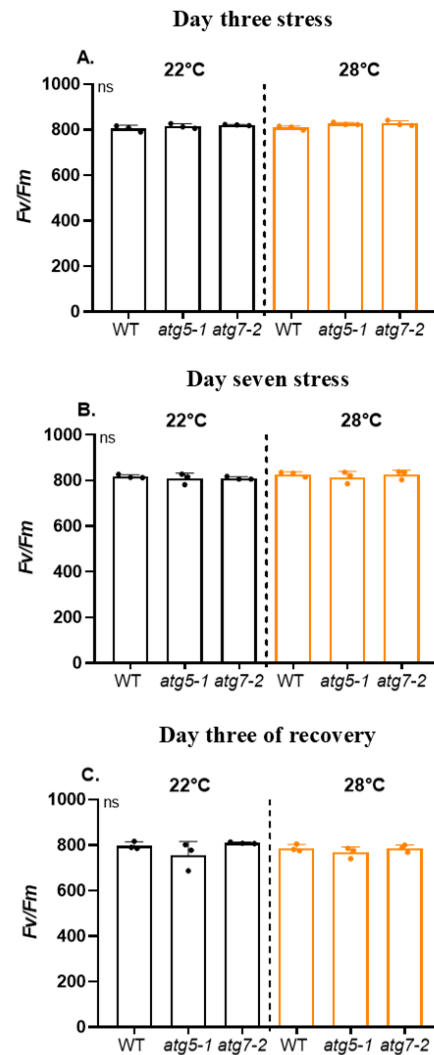


Figure S1. Maximum quantum efficiency of PSII (F_v/F_m) in *A. thaliana* plants in response to prolonged warming. (A e B) WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until bolting and subsequently subjected to prolonged heat stress (28°C day / 26°C night) for three and seven days. (C) A three-day post-stress recovery period was also analyzed. Bars represent mean $\pm$ SE. No significant differences were observed among genotypes within each temperature and time point (ns).

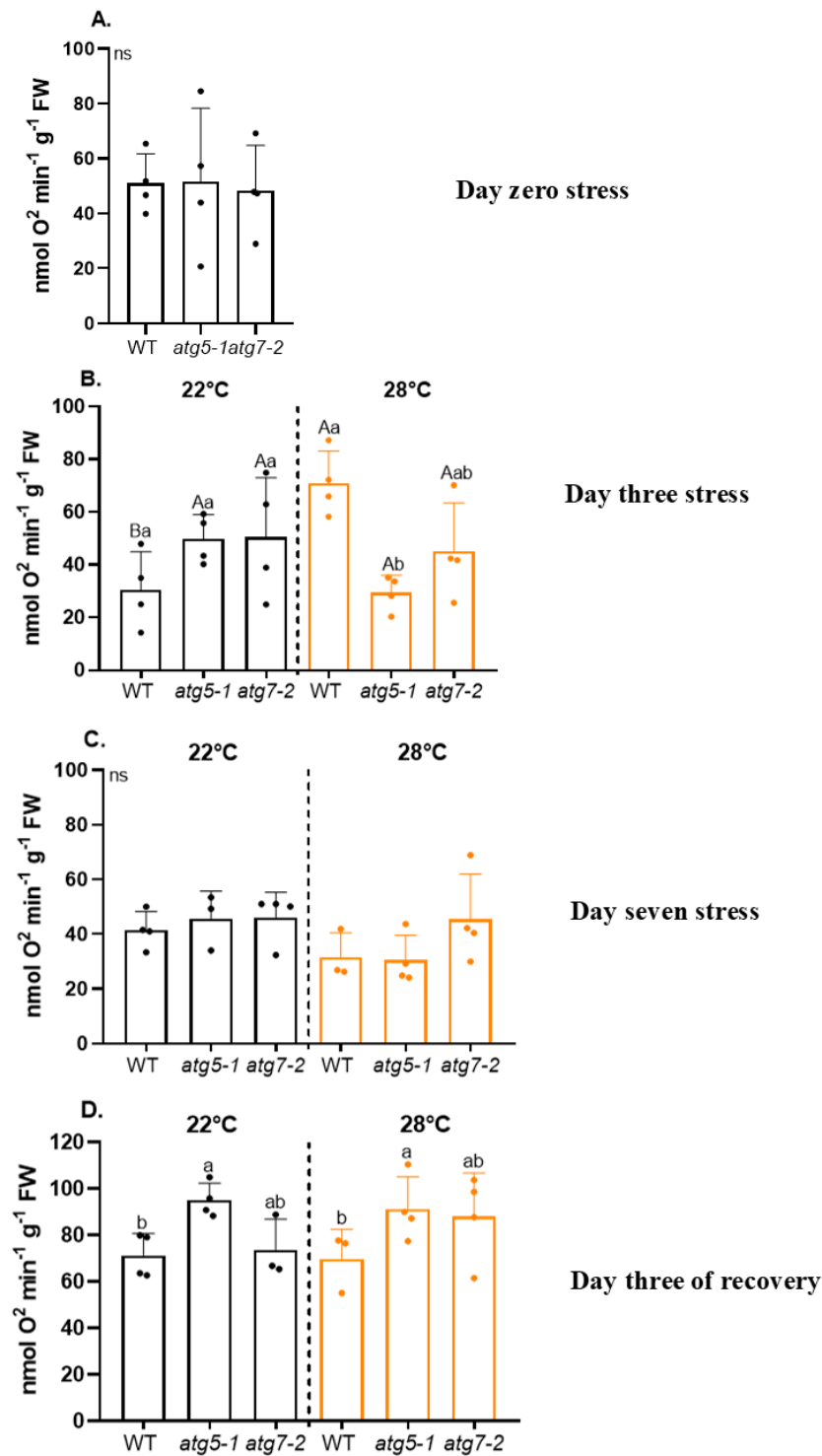


Figure S2. Oxygen (O_2) consumption in *A. thaliana* plants in response to zero, three and seven days of prolonged warming. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until bolting and subsequently subjected to prolonged warming (28°C day / 26°C night). A three-day post-stress recovery period was also analyzed. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data is presented as means $\pm$ standard error (SE) of four biological replicates per genotype. Abbreviation: ns: non-significant interaction; FW: fresh weight.

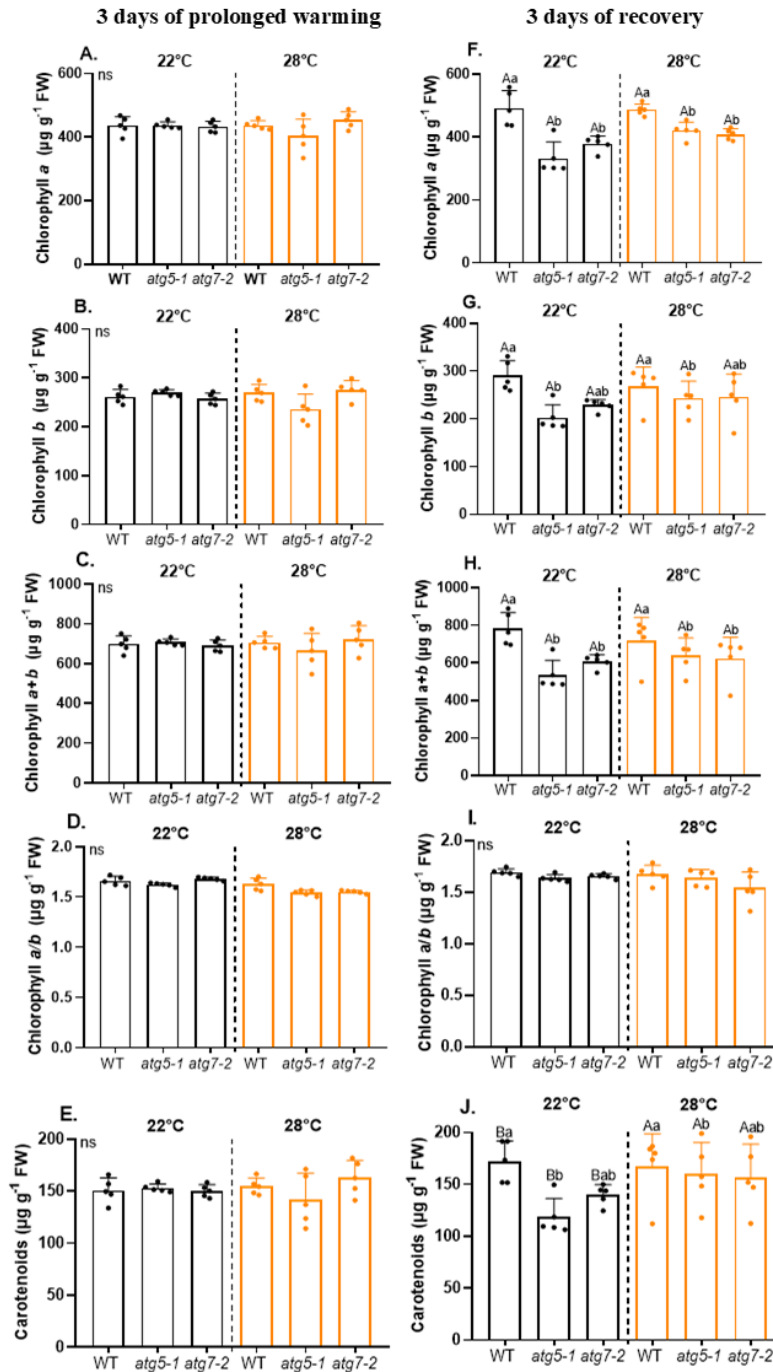


Figure S3: Prolonged warming does not affect pigment levels among the different genotypes. (A–E) Photosynthetic pigment levels in *Arabidopsis thaliana* plants in response to three days of prolonged warming. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until bolting and subsequently subjected to prolonged warming (28°C day / 26°C night). (F–J) A three-day post-stress recovery period was also analyzed. Chlorophyll *a*, chlorophyll *b*, total chlorophyll (*a*+*b*), chlorophyll *a*/*b* ratio, and carotenoid content were evaluated. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates, each consisting of five plants per genotype. Abbreviation: ns: non-significant interaction; FW: fresh weight.

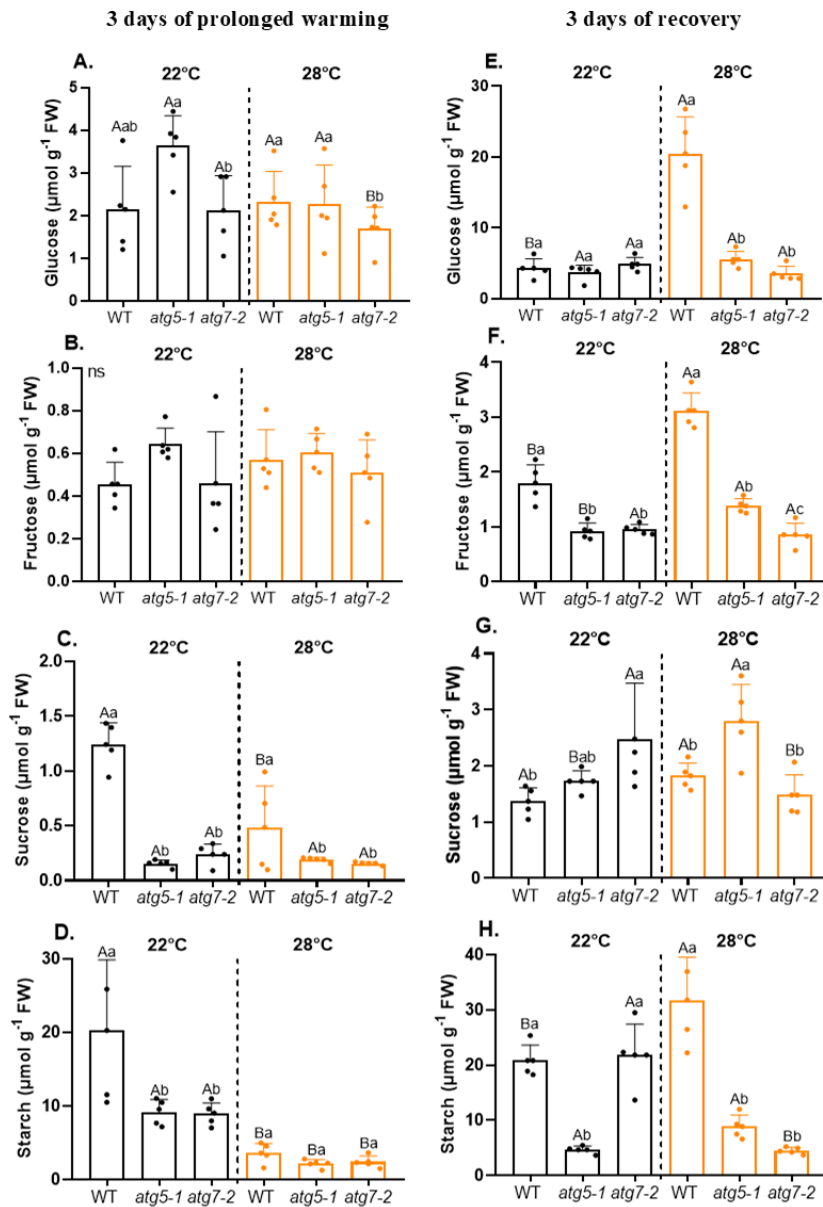


Figure S4: Sugar contents changed among the genotypes between three days of prolonged warming and three days of recovery. (A–D) Soluble sugar and starch levels in *A. thaliana* plants in response to three days of prolonged warming. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until bolting and subsequently subjected to prolonged warming (28°C day / 26°C night). (E–H) A three-day post-stress recovery period was also analyzed. The following soluble sugars were evaluated: glucose, fructose, sucrose, and starch. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: ns: non-significant interaction; FW: fresh weight.

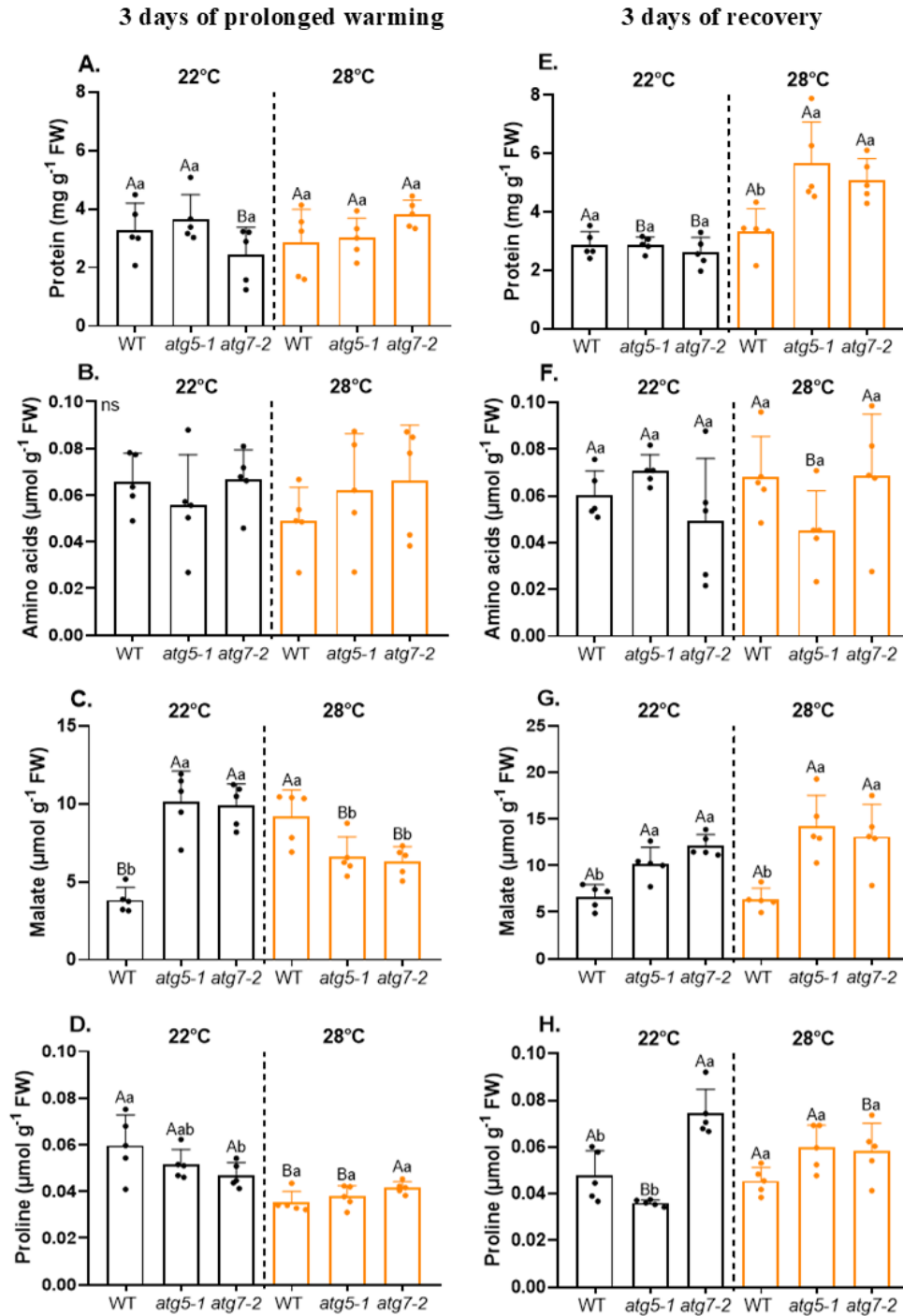


Figure S5. Under prolonged warming, nitrogenous compounds were not altered in *atg* mutant plants compared with WT plants. (A) Protein, (B) amino acid, (C) malate and (D) proline in *A. thaliana* plants in response to three days of prolonged warming. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under optimal conditions until bolting and subsequently subjected to prolonged warming (28°C day / 26°C night). A three-day post-stress recovery period was also analyzed. The following parameters were evaluated: (E) total proteins, (F) amino acids, (G) malate, and (H) proline. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different temperature treatments, while lowercase letters indicate comparisons among genotypes within the same temperature condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: ns: non-significant interaction; FW: fresh weight.

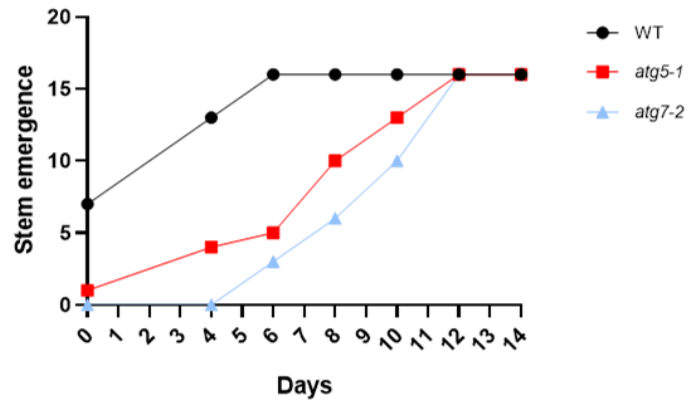


Figure S6: The wild type (WT) exhibited floral stem more quickly, while the *atg5-1* and *atg7-2* mutants showed late emergence. WT and *atg* mutants (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were compared regarding floral stem emergence prior to being subjected to seven days of prolonged warming. Data are presented as mean $\pm$ standard error (SE) from ten biological replicates per genotype.

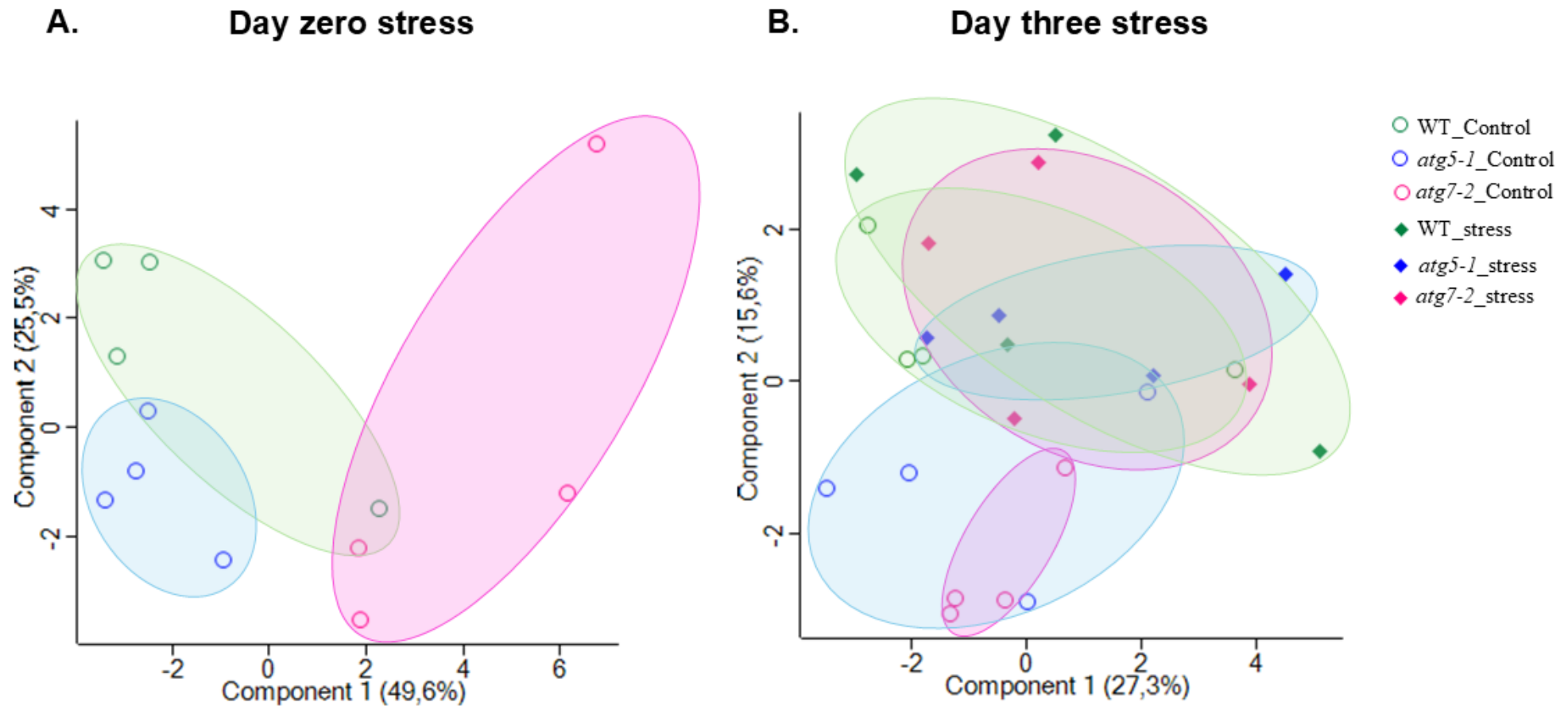


Figure S7: Principal component analysis (PCA) of metabolic profiles in *Arabidopsis thaliana* under prolonged warming. **(A)** PCA score plot at day zero (control conditions) showing separation among WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*), indicating genotype-dependent differences in basal metabolic profiles. **(B)** PCA score plot under three days of prolonged warming (28°C/26 °C), highlighting shifts in metabolic profiles driven by temperature and genotype. Each point represents an individual biological replicate. Open symbols correspond to control conditions, whereas filled symbols represent prolonged warming plants. Colors indicate genotypes: WT (green), *atg5-1* (blue), and *atg7-2* (pink). The percentage of variance explained by each principal component is indicated on the axes.

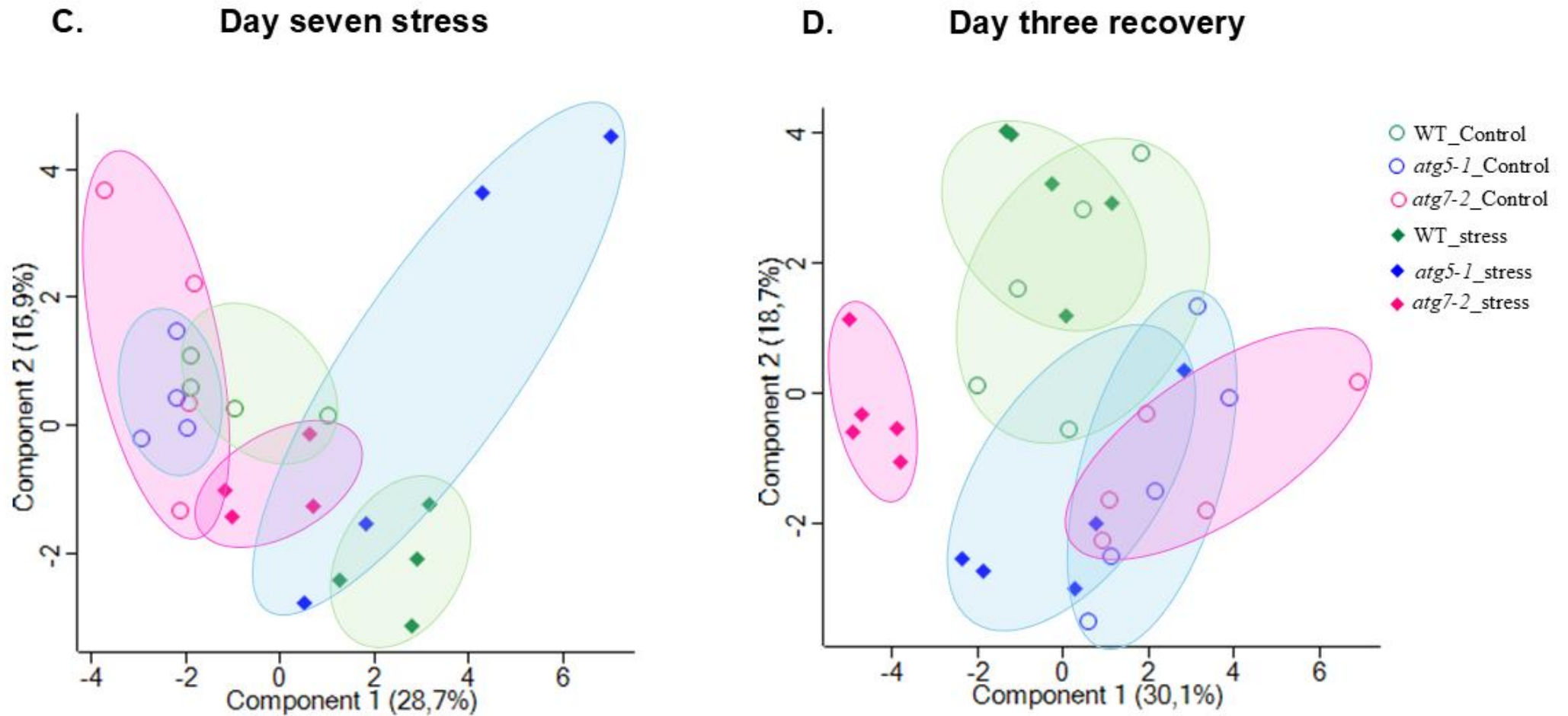


Figure S8: Principal component analysis (PCA) of metabolic profiles in *Arabidopsis thaliana* under prolonged warming and recovery. **(C)** PCA score plot under seven days of prolonged warming (28°C/26°C), highlighting shifts in metabolic profiles driven by temperature and genotype. **(D)** PCA score plot after three days of recovery prolonged warming, highlighting shifts in metabolic profiles driven by temperature and genotype. Each point represents an individual biological replicate. Open symbols correspond to control conditions, whereas filled symbols represent prolonged warming plants. Colors indicate genotypes: WT (green), *atg5-1* (blue), and *atg7-2* (pink). The percentage of variance explained by each principal component is indicated on the axes.

Table S1. Selected genes for expression analysis in *atg* mutants subjected to prolonged warming.

Gene	Locus	Description	“Primer Forward”	“Primer Reverse”
<i>EF1α</i>	At1g18070	Translation elongation factor EF1α (Constitutive)	TGGTTGTTATGCCGAACAAGGAAC	AAATTCTCACCCGGTCCTGCAC
<i>ACTIN</i>	At3g27000	Actin Related Protein 2(Constitutivo)	TCTTCCGCTCTTTCTTTCCAAGC	ACCATTGTCACACACACGATTGGTTG
<i>HSFA2</i>	At2g26150	Heat Shock Transcription Factor A2	GTAGCGGCTTCTTCATCTGTAGG	CAAGAAACCACCGTGTCCGT
<i>NBR1</i>	At4g24690	Next to BRCA1 gene 1	ACGAGTACAACCTGGAGCAGTC	TCACATCATCACAGAAGCCCATC
<i>HSP17.6</i>	At5g12030	Heat Shock Protein 17.6	CCCTTCAAGAGCTTACATGCGAG	ATCTCATCTCCTTTGATTCCAGGC
<i>WRKY75</i>	At5g13080	WRKY DNA-binding protein 75	AGTGGACCAAGAAGTGGTCGTG	GGTGGATTTCTCGATGGGATGC
<i>E3</i>	At5g57360	Ubiquitin ligase ZTL	GCGCCTTGTGGATTCGTTGT	AGCGGCAATTCCTCCGAGA
<i>GAP</i>	At1g12900	glyceraldehyde 3-phosphate dehydrogenase subunit 2	AACTCCTCTGCTCTTCCCTTTGC	TCCACCATTGCTTCTCATTGCAG
<i>FBA1</i>	At2g21330	Associated with carbon sequestration	CGCTACGCAGCTATTTCTCAAGAC	AGCCCAAACCTTCTCTGCAAC
<i>FUM1</i>	At2g47510	Fumarase of the tricarboxylic acid cycle	CACTTAACACAATCGCCACATCG	AGACCACATCTTGGGCCACTT

<i>PMDH1</i>	At2g22780	NAD-malate dehydrogenase 1 peroxisomal	TGAGCTTCCCTTCTTCGCATCG	TGCCTTCTCTAATCCCATCCTCTC
<i>bZIP9</i>	At5g24800	Basic leucine zipper 9	GACCTCATGAACCGGGATTACA	AAGGTCCAGCCGGAAACAATC
<i>bZIP24</i>	At3g51960	Basic leucine zipper 24	GAGGGCCAAACACTTCATGATCC	CCGACCTCTATGCCCCTAATTAAAC

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Chapter 2: Is Autophagy required for reproductive development in response to drought?

Abstract

Drought is one of the most detrimental environmental constraints affecting plant reproductive success and crop productivity. Although autophagy is recognized as a major mechanism promoting plant adaptation to water deficit, its role in coordinating physiological and metabolic responses during reproductive development remains poorly understood. This work investigated the contribution of autophagy to leaf metabolism, source–sink relationships and reproductive performance in *Arabidopsis thaliana* subjected to drought stress during the reproductive stage. Wild-type (WT) plants and the autophagy deficient mutants *atg5-1* and *atg7-2* were exposed to water deficit (50% of field capacity) after bolting and their physiological, biochemical, metabolic, and reproductive responses were evaluated. Although all genotypes experienced similar reductions in relative water content, the autophagy deficient mutants exhibited accelerated leaf senescence, enhanced degradation of photosynthetic pigments and profound alterations in carbon and nitrogen metabolism. These changes included impaired carbohydrate partitioning, disrupted amino acid homeostasis, and reduced nutrient remobilization, indicating severe metabolic imbalance under drought conditions. The disruption of leaf metabolic homeostasis was associated with impaired source–sink coordination, resulting in reduced pollen viability, fewer siliques, lower seed set and a marked decline in reproductive output. Despite the reduction in seed number, the seeds that successfully developed maintained normal germination capacity and individual seed weight, while displaying alterations in reserve accumulation and primary metabolism, suggesting preferential allocation of assimilates to a reduced number of developing seeds. Collectively, these findings demonstrate that autophagy could play a central role in coordinating carbon and nitrogen remobilization during drought, thereby maintaining metabolic homeostasis and ensuring efficient resource allocation to reproductive tissues. By integrating leaf senescence, source–sink regulation and reproductive development, autophagy is believed to sustain reproductive resilience under water deficit and contributes to plant adaptation to drought stress.

Keywords: Drought; Nutrient Remobilization; Reproductive Development; Senescence.

Introduction

Drought is one of the major limitations restricting agricultural production worldwide (Bao, 2020). Thus, there is an urgent need to improve plant performance under drought conditions (Tang & Bassham, 2021). Higher temperatures, changes in precipitation patterns, and more frequent and severe droughts are commonly cited as impacts of climate forcing driven by greenhouse gases (Tang & Bassham, 2021). Drought is generally defined as a deficit in water supply, that is, a shortage of water relative to plant demand, resulting in stress (Swann, 2018).

The effects of drought are associated with reductions in both biomass and productivity, resulting in stress to the plant, potential tissue damage, and, if the damage is extensive, mortality (Swann, 2018). Prolonged exposure to drought causes metabolic and physiological alterations, including changes in root and shoot biomass, relative water content, accelerated chlorophyll loss, leaf senescence, impaired mineral acquisition, and increased levels of reactive oxygen species and damaged proteins (Yang et al., 2021; Das et al., 2026). However, plants exhibit a wide range of responses to drought, including physiological and biochemical adjustments at both the cellular and whole-organism levels, making this phenomenon even more complex (Farooq et al., 2012). In response to stress, autophagy is responsible for the degradation and recycling of unwanted and toxic cytoplasmic components, contributing to stress tolerance in plants (Liu et al., 2009; Wang et al., 2015; Bao, 2020).

It has already been demonstrated that *atg5*, *atg7*, and RNAi-*AtATG18a* autophagy-deficient plants are more sensitive to drought conditions than WT plants, highlighting the role of autophagy in responses to these stresses (Liu et al., 2009; Tang & Bassham, 2021). Autophagy is activated through multiple mechanisms during water stress (Li et al., 2018; Chen et al., 2025). Several transcription factors are induced by drought to positively regulate the expression of autophagy-related genes, while autophagy is also post-translationally regulated through protein modification and stability (Avin-Wittenberg, 2018). In response to water deficit, autophagy selectively degrades aquaporins to adjust water permeability and removes excess heme and damaged proteins to reduce their toxicity (Tang & Bassham, 2021; Chen et al., 2025). Furthermore, autophagy may degrade regulators or components of hormonal signaling pathways and the COST1 protein to promote stress responses (Wang et al., 2015, Bao et al., 2020). During drought recovery, autophagy degrades drought-induced proteins to reestablish

cellular homeostasis (Tang & Bassham, 2021). While these degradative and protective mechanisms are relatively well characterized in vegetative tissues, the metabolic demands and cellular signaling networks switch dramatically during the transition to reproductive growth, where effective nutrient allocation becomes the primary determinant of survival (Qian et al., 2025).

Staple crops are particularly susceptible to water scarcity, especially during the critical stages of reproduction and grain filling. Water deficit disrupts flowering, gametophyte development, pollination, and seed formation, leading to infertility and substantial reductions in crop productivity (Qian et al., 2025). To maintain productivity and stability under these conditions, these crops rely on the coordinated implementation of morphological, physiological, and cellular adaptations (Khan et al., 2026). Autophagy stands out for its role in floral and seed development, tapetum degradation, pollen maturation and germination, and energy maintenance during microsporogenesis as well as in mediating source-to-sink nutrient remobilization from senescing leaves (Kuzuoglu-Ozturk et al., 2012; Avin-Wittenberg, 2018; Li et al.; Zhu et al., 2018). The absence of autophagy compromises productivity, whereas its overexpression increases seed yield, highlighting its important role in the reproductive aspects of plants (Avin-Wittenberg, 2018). However, little to nothing is known about how autophagy participates in reproductive responses under water deficit conditions. Therefore, this study aimed to investigate the role of autophagy in productivity under drought stress by characterizing the physiological and metabolic alterations occurring during the reproductive stage in autophagy-deficient plants (*atg* mutants) subjected to drought.

Methodology

Growth conditions

A. thaliana wild-type (Col-0) and mutant lines *atg5-1* (SAIL_129B079; Thompson et al., 2005) and *atg7-2* (GK-655B06; Hofius et al., 2009) were used to study the autophagic process. Seeds were disinfected in 1 mL of 70% (v/v) ethanol for 1 minute and then in 2.5% (v/v) sodium hypochlorite for 15 minutes. All sodium hypochlorite residue was removed by washing (6x) with sterile water. After disinfection, the seeds underwent the vernalization process at 4°C for 48 h and were distributed in sterile Petri dishes containing ½ MS culture medium (Murashige & Skoog, 1962). The plates were then kept in a growth chamber for 10 days under the same conditions described above.

After transplantation, WT *Arabidopsis thaliana* plants (Colombia-0) and mutants for the autophagic process (*atg5-1* and *atg7-2*) were grown in a growth chamber with a neutral photoperiod (12 h light: 12 h dark), temperature of 22 °C during the light period and 20 °C during the dark period for four weeks. Relative humidity was maintained at 60% and irradiance at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Treatment of drought

Drought was initiated consisting of maintaining the pots at 50% of water capacity for 21 days after the emergence of the floral peduncle in the reproductive phase (Huang et al., 2015). The pots in the control group were irrigated to 80% of their field capacity. The physiological and biochemical analyses described below were performed on plants at eight weeks of age.

Measurement of relative water content

The relative water content (RWC) of leaves was evaluated to monitor leaf hydration status over 14 days under irrigation at 50% field capacity. One leaf per replicate was excised and weighed to obtain fresh weight (FW). The leaves were then hydrated for 2 hours in Petri dishes containing distilled water and weighed to obtain turgid weight (TW). Finally, the leaves were dried in an oven at 72 °C for 72 hours and weighed to obtain dry weight (DW). RWC was calculated using the following equation:

$$RWC(\%) = \frac{FW - DW}{TW - DW}$$

Measurement of senescence parameters

Protein and chlorophyll content were determined photometrically as described in the literature (Bradford 1976; Porra et al. 1989), respectively. The ratio of F_v / F_m , which corresponds to the potential quantum yield of the photochemical reactions of PSII and represents a measure of the photochemical efficiency, was measured after dark adaptation of leaves for 30 min, as previously described (Oh et al. 1996).

Determination of metabolites in rosettes

The rosette discs were collected and homogenized and subjected to methanolic extraction. Briefly, 700 μL of methanol was added to the sample followed by shaking (750 rpm) at 80°C for 20 minutes. Afterwards, the samples were centrifuged, and the supernatant was separated from the precipitate. To the supernatant were added 375 μL of chloroform (99.9%) and 750 μL of milli-Q water. The samples were centrifuged and the

aqueous phase was separated for future analyses. In the soluble fraction, the contents of chlorophyll (Sulpice et al., 2009), glucose, fructose and sucrose (Ferne et al., 2001), total soluble amino acids (Cross et al., 2006), malate and fumarate (Nunes-Nesi et al., 2007) were determined. In the insoluble fraction the starch (Ferne et al., 2001) and protein (Bradford, 1976) contents.

Count of flowers, siliques, branches, seeds per silique and silique length

The number of flowers, siliques and branches per plant were analyzed. Siliques from WT and mutant plants were collected from five plants per genotype and clarified in 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS) solution (Yoo et al., 2012). The clarified siliques were used for silique and number of seeds per silique that were analyzed under a dissecting microscope (Stemi 2000-C, Zeiss) with an adapter (SOLIGOR tube for Canon A650 IS B52 Wide) to a 12.1-megapixel CANON camera (Power Shot A650 IS).

Pollen grain viability

Pollen viability was performed on WT and *atg* mutant plants by collecting flowers from five plants per genotype. The flowers were then dismembered by removing the petals and exposing the anthers to remove the pollen by mechanical force with the aid of a magnifying glass. The pollen was then stained with Alexander's histochemical reagent, which stains viable and nonviable pollen differently. The photos were taken and measured by microscope (Zeiss Vision Imager.A2 Axion 10 photo microscope). They were subsequently evaluated using the ImageJ program.

Seed count and weight per plant

The weight of a thousand seeds and the weight of all seeds produced by the same plant were quantified. The biometric data obtained from the photographic records were evaluated using the ImageJ program.

Seed germination test

Seeds of Col-0 plants and *atg* mutants from both the control and heat treatments were properly disinfested and germinated in MS medium for 60 hours. The seeds then underwent a stratification process for 48 hours at 4°C in the dark.

Subsequently, the plates were transferred to a growth chamber regulated at 22 ± 2°C. Evaluations were performed at intervals of 0, 12, 24, 36, 48 and 60 hours.

Germination percentage and rate, and germination speed index were evaluated. Five replicates with 50 seeds of each genotype were included in the analysis and the variables will be calculated according to Bruginski et al. (2009).

Determination of seed metabolites

Seeds of Col-0 plants and *atg* mutants from both the control and drought treatments were collected and embedded in historesin (Historesin Leica Microsystems, Heidelberg, Germany) according to the manufacturer's recommendations. Cross sections (5 µm thick) were obtained using an automatic rotary microtome (model RM2155, Leica Microsystems Inc., Deerfield, USA). Histochemical tests for the detection of seed storage carbohydrates, proteins, and lipids were performed using Lugol's solution (Johansen, 1940), Coomassie Brilliant Blue (Fisher, 1968), and Sudan Red B (Brundrett et al., 1991), respectively. For reserve quantification, the seeds were homogenized and subjected to methanolic extraction. In summary, 700 µL of methanol were added to the sample followed by agitation (750 rpm) at 80°C for 20 minutes. Afterwards, the samples were centrifuged, and the supernatant was separated from the precipitate. To the supernatant were added 375 µL of chloroform (99.9%) and 750 µL of milli-Q water. The samples were centrifuged and the aqueous phase was separated for future analyses. In the soluble fraction, the contents of sucrose (Fernie et al., 2001), total soluble amino acids (Cross et al., 2006), malate and fumarate (Nunes-Nesi et al., 2007) were determined. In the insoluble fraction the starch (Fernie et al., 2001) and protein (Bradford, 1976) contents. Seeds oil was extracted with hexane, and the recovered lipid fraction was subsequently weighed to determine seed oil content (Bligh and Dyer, 1959).

Experimental design and statistical analysis

The experimental design was completely randomized, consisting of two levels: control study (80% field capacity) and drought (50% field capacity), with five replicates. Each experimental unit consisted of ten plants.

The normality and homoscedasticity of the data were analyzed by the Shapiro-Wilk and Bartlett tests, respectively, both at 0.05 probability. Then, the data was subjected to analysis of variance (ANOVA) using the F test ($P \leq 0.05$). When significant, the characteristics were subjected to the Tukey test ($P < 0.05$). All statistical analysis of the data was performed using protocols developed in the R software (R Development Core Team, 2019).

Results

Reducing the water status to 50% of field capacity leads to changes in leaf area and root fresh weight

To evaluate the role of autophagy in plant responses to water deficit, WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were subjected to drought conditions (50% field capacity) for 21 days (Figure 1). Under control conditions, WT plants exhibited significantly larger leaf area compared to *atg* mutants, indicating reduced vegetative growth in the absence of autophagy. Under drought conditions, leaf area was reduced in all genotypes. However, WT plants maintained significantly larger leaf area than both mutants, which exhibited a more pronounced reduction (Figure 1B). A similar behavior was observed for root biomass (Figure 1C). Under control conditions, WT plants showed significantly higher fresh root weight compared to *atg* mutants. Under drought, WT plants showed a tendency towards increased root biomass, suggesting an adaptive response to water limitation, whereas *atg5-1* and *atg7-2* failed to exhibit a comparable increase, maintaining significantly lower root mass. Leaf relative water content (RWC) was markedly reduced under drought conditions in all genotypes (Figure 1D). However, no significant differences were observed among WT and *atg* mutants, indicating that all genotypes experienced similar levels of water deficit. Despite the morphological and growth differences, the maximum quantum efficiency of PSII (F_v/F_m) remained unchanged among genotypes under both control and drought conditions, suggesting that the photochemical efficiency of PSII was not significantly impaired by water deficit or autophagy deficiency (Figure 1E). Phenotypic observations further supported these findings, with *atg* mutants exhibiting more severe visual symptoms, including reduced size and increased leaf senescence under control and drought conditions compared to WT plants (Figure 1A). Overall, these results indicate that autophagy plays an important role in maintaining plant growth and biomass allocation. Although all genotypes experienced similar levels of dehydration, the reduced growth performance and impaired root development in *atg* mutants suggest that autophagy contributes to drought tolerance by supporting adaptive growth responses rather than directly affecting photosynthetic efficiency.

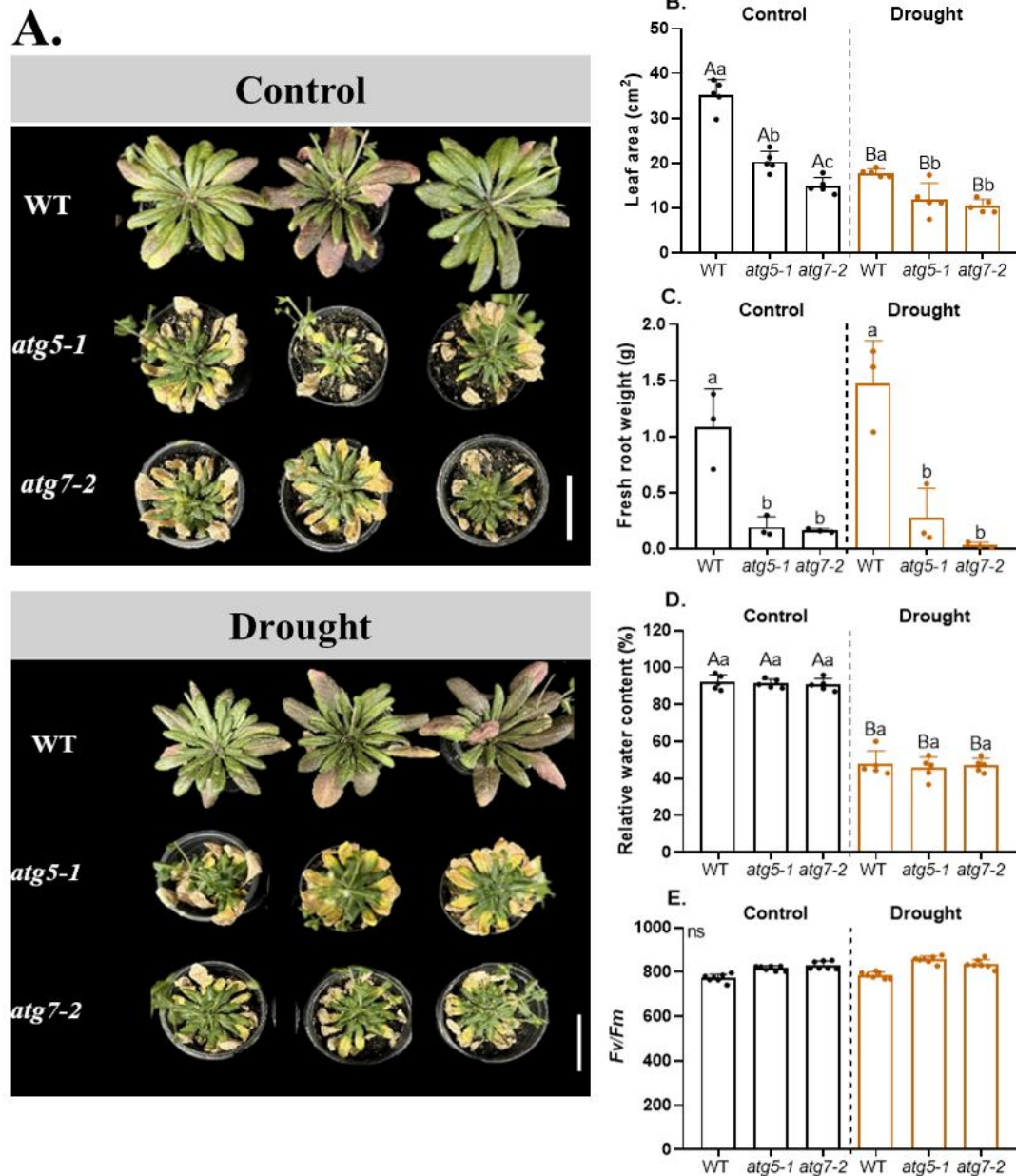


Figure 1: The absence of autophagy reduces leaf area and root biomass when plants are exposed to drought. **(A)** WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were compared 56 days after being subjected to 21 days of drought. The white scale bar represents 5 cm. The graphs show: **(B)** leaf area (cm²), **(C)** fresh root weight (g), **(D)** relative water content (%), and **(E)** maximum quantum efficiency of PSII (Fv/Fm) under control (80% field capacity) and drought (50% field capacity) conditions in autophagy-deficient mutants (*atg5-1* and *atg7-2*) compared to WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: ns: non-significant interaction.

Autophagy delays drought induced leaf senescence by preserving chlorophyll content

Total chlorophyll content was significantly affected by both genotype and drought stress (Figure 2A). Under control conditions, WT plants exhibited higher chlorophyll content than the *atg5-1* and *atg7-2* mutants. Drought significantly reduced chlorophyll content in all genotypes, with a more pronounced decrease in the autophagy-deficient mutants, which exhibited the lowest chlorophyll levels under stress. Since chlorophyll degradation is one of the major physiological hallmarks of leaf senescence, these results suggest that autophagy deficiency accelerates drought-induced leaf senescence. Protein content exhibited a distinct response (Figure 2B). Under control conditions, *atg5-1* accumulated significantly higher protein levels than WT and *atg7-2*, whereas no significant differences were observed between WT and *atg7-2*. Under drought conditions, both mutants displayed higher protein contents than WT, with no significant differences between *atg5-1* and *atg7-2*. Furthermore, drought did not significantly alter protein content within any genotype, indicating that despite the reduction in chlorophyll content, total protein levels were maintained during stress. Taken together, these results reinforce the role of autophagy in delaying drought induced leaf senescence.

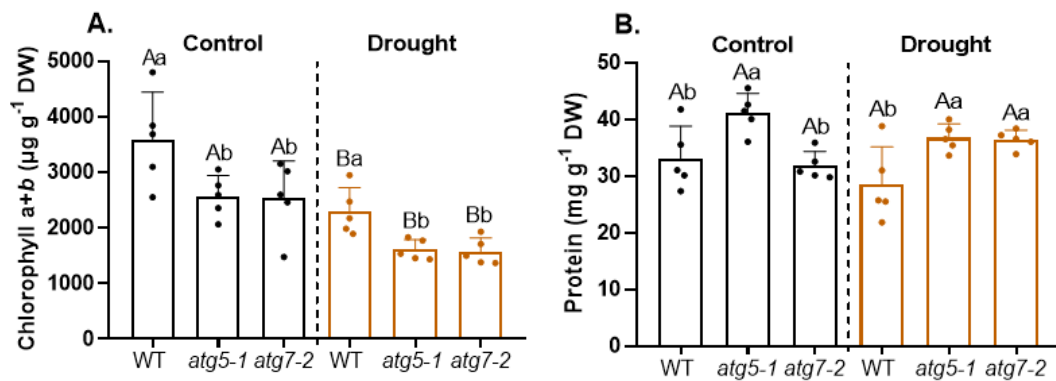


Figure 2: Autophagy aids in the preservation of photosynthetic pigments from control conditions through to drought. **(A)** Chlorophyll *a+b* and **(B)** Protein content in *A. thaliana* plants in response to drought. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under control (80% field capacity) and drought (50% field capacity) conditions in autophagy deficient mutants (*atg5-1* and *atg7-2*) compared to WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: DW: dry weight.

Autophagy coordinates carbon and nitrogen metabolism during drought stress

Drought stress markedly altered carbohydrate metabolism in WT and autophagy deficient plants (Figure 3). Glucose content was significantly increased in WT plants under drought compared with the control, whereas a smaller increase was observed in *atg5-1*, and no significant change occurred in *atg7-2* (Figure 3A). Under drought conditions, WT accumulated significantly higher glucose levels than both mutants. Fructose content also increased under drought (Figure 3B). While no significant differences among genotypes were detected under control conditions, drought induced greater fructose accumulation in *atg5-1*. Sucrose content was strongly enhanced by drought in all genotypes (Figure 3C). Under control conditions, sucrose levels did not differ among genotypes. Following drought treatment, all genotypes accumulated significantly higher sucrose contents, with *atg7-2* exhibiting the highest levels, although no significant differences were detected between WT and *atg5-1*. In contrast, starch content markedly declined under drought (Figure 3D). WT plants accumulated significantly higher starch levels than the autophagy deficient mutants under control conditions. However, drought almost completely depleted starch reserves in all genotypes, and no significant differences were observed among them under stress. Together, these results indicate that drought promotes starch degradation accompanied by the accumulation of soluble sugars, suggesting carbon remobilization during stress. The distinct patterns of sugar accumulation in the autophagy deficient mutants further indicate that autophagy contributes to carbohydrate homeostasis during drought-induced leaf senescence.

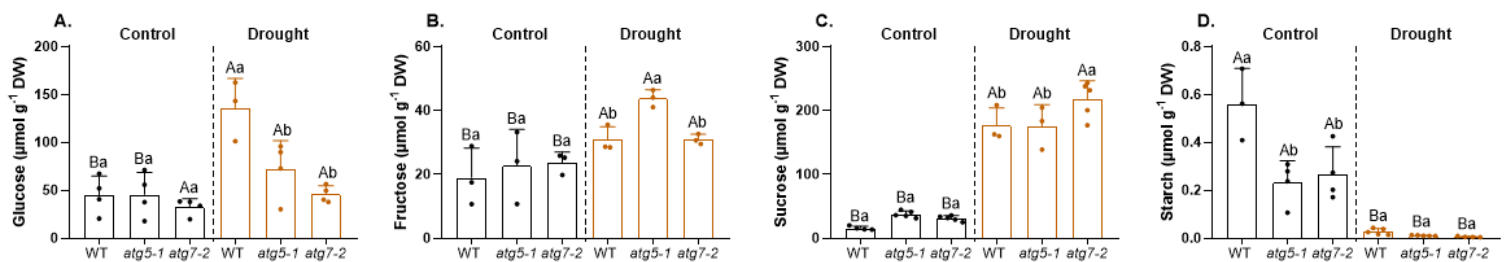


Figure 3: Autophagy modulates carbon metabolism under drought stress. **(A)** Glucose, **(B)** Fructose, **(C)** Sucrose and **(D)** Starch content in *A. thaliana* plants in response to drought. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under control (80% field capacity) and drought (50% field capacity) conditions in autophagy deficient mutants (*atg5-1* and *atg7-2*) compared to WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: DW: dry weight.

Drought stress differentially affected the accumulation of amino acids and organic acids in WT and autophagy deficient plants (Figure 4). Under control conditions, WT plants accumulated significantly higher amino acid levels than *atg5-1*, whereas *atg7-2* exhibited intermediate values. Following drought treatment, amino acid content increased in *atg* mutants, with *atg7-2* showing the highest accumulation (Figure 4A). Malate content was not significantly affected by either genotype or drought stress (Figure 4B). Fumarate content was significantly influenced by drought stress (Figure 4C). Under control conditions, there was no significant difference between the genotypes. Under drought, WT exhibited the highest fumarate content, while both autophagy deficient mutants displayed significantly lower levels. Proline accumulation was strongly induced by drought in all genotypes (Figure 4D). Under control conditions, proline content remained low and did not differ among genotypes. Following drought treatment, proline levels increased markedly in WT, *atg5-1* and *atg7-2* with no significant differences observed among genotypes. These results indicate that drought promoted metabolic reprogramming characterized by proline accumulation, whereas changes in amino acids and fumarate metabolism were more genotype dependent.

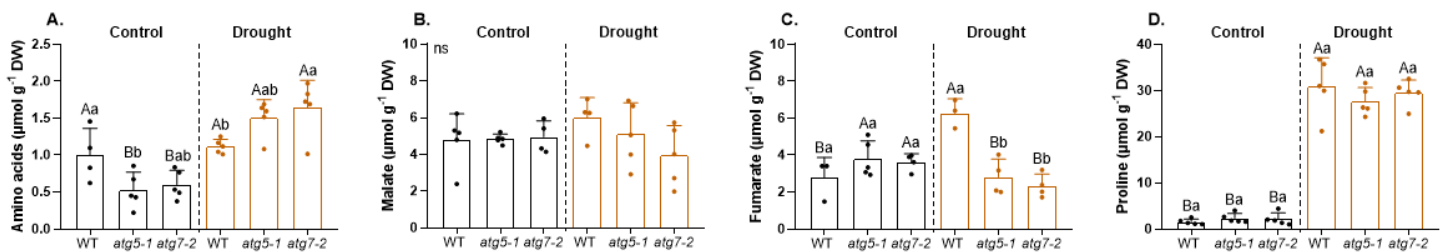


Figure 4: Drought promoted metabolic reprogramming characterized by amino acid and proline accumulation. (A) Amino acids, (B) Malate, (C) Fumarate and (D) Proline content in *A. thaliana* plants in response to drought. WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were grown under control (80% field capacity) and drought (50% field capacity) conditions in autophagy deficient mutants (*atg5-1* and *atg7-2*) compared to WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: DW: dry weight.

Autophagy supports reproductive development under water deficit conditions

To investigate the role of autophagy in reproductive development under water deficit, WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) were evaluated under control and drought conditions (Figure 5). Under control conditions, WT plants exhibited significantly greater height compared to *atg* mutants, with *atg7-2* showing the most pronounced reduction in growth. Drought stress led to a significant decrease in plant height in WT. However, WT plants still maintained higher values than both mutants under water deficit conditions (Figure 5A). The number of branches was not significantly affected by either genotype or drought stress, indicating that this parameter remained stable across treatments (Figure 5B). Reproductive parameters were strongly influenced by both genotype and water availability. Under control conditions, WT plants exhibited a higher number of open flowers compared to *atg5-1* and *atg7-2*. This trend was maintained under drought, where mutants showed a significant reduction in open flowers relative to WT (Figure 5C). In contrast, the number of floral buds showed a distinct pattern. Under control conditions, *atg7-2* displayed higher bud numbers compared to WT and *atg5-1*. Under drought stress, *atg7-2* exhibited a tendency for a further increase in floral buds, whereas *atg5-1* showed a tendency for a reduction, suggesting differential regulation of reproductive development among autophagy mutants (Figure 5D). Pollen viability was also affected by drought stress. Drought led to a reduction in viable pollen in all genotypes compared to the control, with *atg7-2* showing the lowest values, indicating a greater sensitivity to water deficit (Figure 5E).

Under control conditions, the number of siliques was higher in WT plants compared to autophagy-deficient mutants (*atg5-1* and *atg7-2*). Among the mutants, *atg7-2* exhibited the greatest reduction in silique number, indicating a more pronounced impairment of reproductive performance in the absence of autophagy. The imposition of drought stress resulted in a significant reduction in silique number in WT plants compared to control conditions. However, WT still maintained higher values than the autophagy-deficient mutants under drought (Figure 5F). In contrast to silique number, silique length did not differ significantly among genotypes or between water conditions, indicating that drought stress and autophagy deficiency predominantly affected silique production rather than their size (Figure 5G). The number of seeds per silique was affected by drought in WT and *atg5-1* genotypes, with *atg7-2* showing no difference compared to control condition. *atgs* mutants show reduction in response to stress, compared to WT plants

(Figure 5H). Analysis of flowering dynamics revealed that WT plants exhibited earlier floral stem emergence under control conditions. WT reached the maximum number of bolted plants more rapidly, whereas *atg5-1* and *atg7-2* showed delayed emergence. Among the mutants, *atg7-2* exhibited the most pronounced delay (Figure 5I).

Overall, these results demonstrate that autophagy plays a critical role in regulating plant growth, reproductive development, and flowering timing under drought conditions. The absence of autophagy compromises reproductive performance, delays developmental transitions, and reduces pollen viability, particularly under water deficit, highlighting its importance for maintaining reproductive fitness under stress.

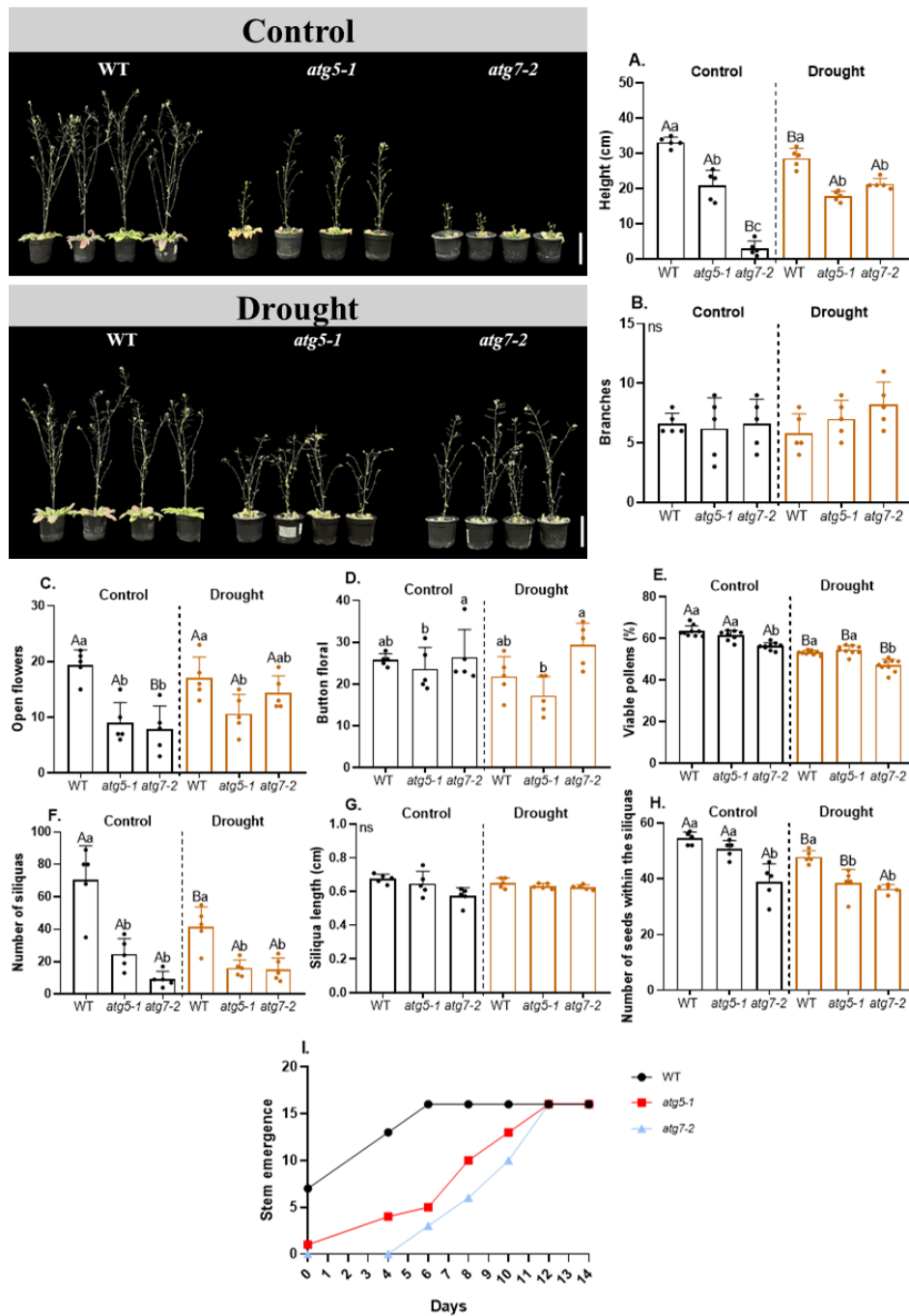


Figure 5: Autophagy supports reproductive development under water deficit conditions. WT and autophagy deficient mutants (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were compared to 56 days after being subjected to 21 days of drought. The white scale bar represents 5 cm. The graphs show plant (A) height (cm), (B) number of branches, (C) number of open flowers, (D) number of floral buds, (E) viable pollens, (F) number of siliques, (G) silique size (cm) and (H) number of seeds within the siliques under control (80% field capacity) and drought (50% field capacity) conditions in autophagy-deficient mutants (*atg5-1* and *atg7-2*) compared to WT. (I) bolting (floral stem emergence) under control (80% field capacity) before the application of stress. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: ns: non-significant interaction.

Autophagy deficiency primarily compromises seed production, an effect that is intensified under drought stress, while individual seed weight remains relatively unaffected (Figure 6). Under both conditions, the thousand-seed weight did not differ significantly between WT plants and autophagy-deficient mutants (*atg5-1* and *atg7-2*) (Figure 6B). In contrast, the total number of seeds was strongly influenced by genotype and water conditions. Under both control and drought conditions, WT plants exhibited a higher seed number compared to the autophagy mutants, with a progressive reduction observed in *atg5-1* and, more markedly, in *atg7-2* (Figure 6C). Under water deficit, a significant reduction in seed number was observed in WT and *atg5-1* compared to control conditions. Although there was a tendency for genotype clustering according to treatment within the germination rate analysis, germination speed index was not altered either among genotypes or by the different treatments (Figure 6D and 6E).

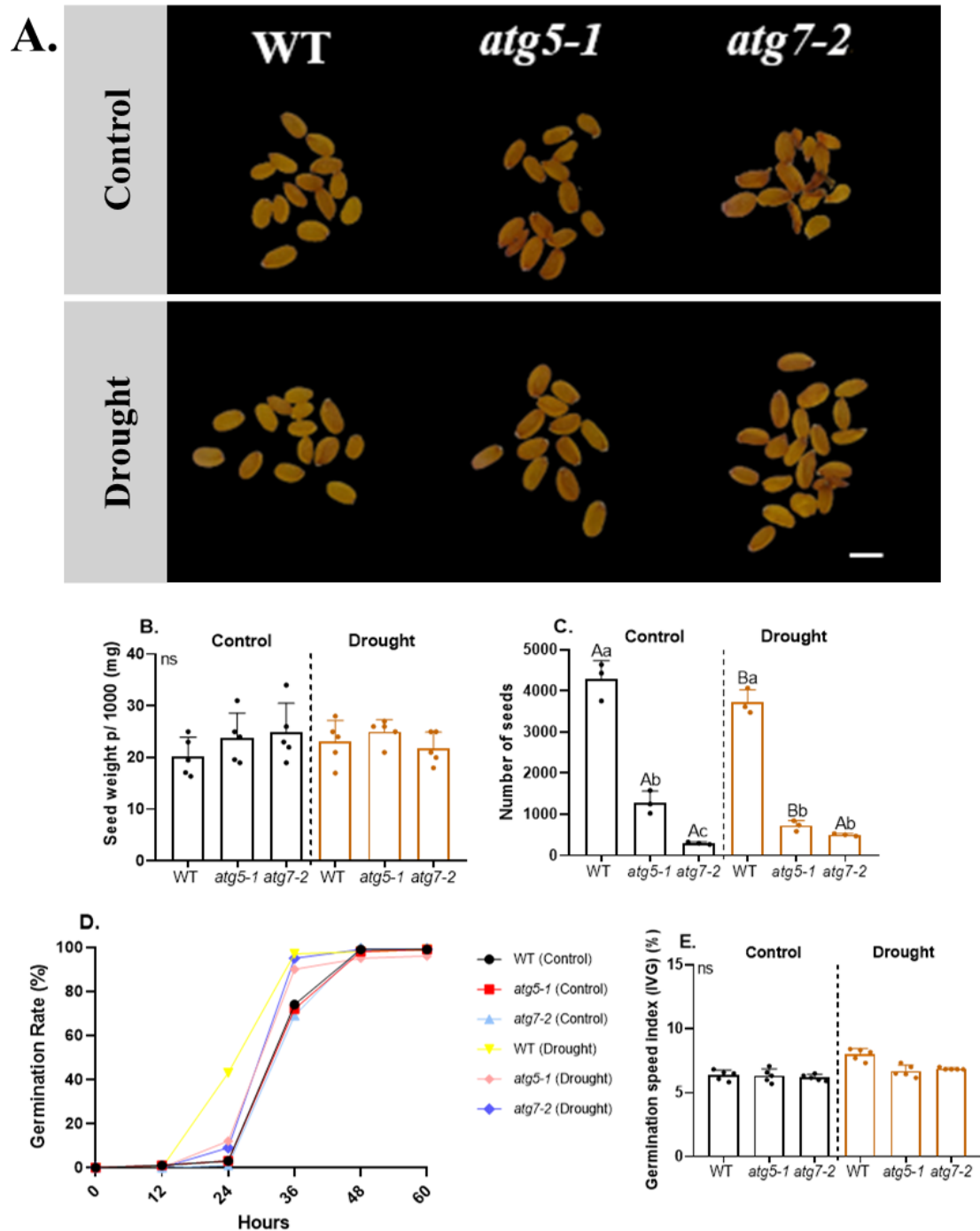


Figure 6: Only the number of seeds was altered among the different genotypes in response to drought. **(A)** WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) *A. thaliana* plants with impaired autophagic flux were compared to 56 days after being subjected to 21 days of drought. The white scale bar represents 1 mm. The graphs show **(B)** seed weight para 1000 (mg), **(C)** number of seeds, **(D)** germination rate (%) and **(E)** germination speed index under control (80% field capacity) and drought (50% field capacity) conditions of autophagy-deficient mutant plants (*atg5-1* and *atg7-2*) compared to the WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes under different water treatments, whereas lowercase letters indicate comparisons among genotypes within the same water condition. Data are presented as mean $\pm$ standard error (SE) from five biological replicates per genotype.

Histochemical and quantitative analyses revealed that drought altered the accumulation and distribution of seed storage reserves in WT and autophagy-deficient mutants (*atg5-1* and *atg7-2*) of *Arabidopsis thaliana* (Figure 7). Lipid staining with Sudan Red B demonstrated increased lipid accumulation in seeds of *atg5-1* and *atg7-2* plants under drought conditions compared with their respective controls, whereas WT seeds exhibited weaker staining under stress (Figure 7A). These observations were supported by the quantitative analyses, which showed that drought reduced seed lipids content in WT plants, while *atg7-2* maintained the highest lipid accumulation among the evaluated genotypes under stress conditions. In contrast, *atg5-1* displayed intermediate lipid levels under drought (Figure 7B).

Protein staining using Coomassie Brilliant Blue showed relatively homogeneous protein distribution among genotypes and treatments (Figure 7A). Quantitative analyses confirmed that total protein content was not significantly altered either by drought or among genotypes (Figure 7C), indicating that protein reserves were comparatively stable under stress conditions regardless of autophagic impairment.

Starch accumulation, detected by Lugol staining, exhibited clear genotype-dependent responses to drought (Figure 7A). Under control conditions, WT seeds accumulated higher starch levels than the autophagy-deficient mutants, particularly *atg5-1*. However, drought promoted a reduction in starch accumulation in WT seeds, while starch levels in *atg5-1* and *atg7-2* seeds remained relatively stable (Figure 7D). Consequently, differences among genotypes became less pronounced under drought conditions.

For total amino acids, the *atg5-1* mutant exhibited higher concentrations compared to WT under both control and drought conditions. Water deficit did not promote marked changes within each genotype (Figure 7E). However, the pattern of higher accumulation in *atg5-1* was maintained, suggesting alterations in nitrogen metabolism associated with autophagy deficiency. Malate levels did not differ significantly among treatments or genotypes (Figure 7F). Values remained relatively constant across all evaluated conditions, suggesting that malate-related metabolism was only slightly affected by drought stress and/or the absence of autophagy. Regarding fumarate, the *atg5-1* and *atg7-2* mutants showed higher concentrations compared to WT under both conditions. Under drought, WT exhibited the lowest fumarate levels, whereas the mutants maintained elevated values (Figure 7G). This pattern suggests a possible

reprogramming of organic acid metabolism in autophagy-deficient plants, independently of water availability.

Sucrose content did not show significant differences between the WT and *atg5-1* genotypes, whereas *atg7-2* displayed reduced values under control conditions. Under drought, an increasing trend was observed in the *atg5-1* mutant, while WT and *atg7-2* showed lower values, indicating greater sensitivity of this genotype to water deficit stress (Figure 7H).

Overall, these results demonstrate that drought differentially affects seed reserve composition and that autophagy deficiency alters carbon allocation during seed development, particularly influencing lipid and starch accumulation. The maintenance of higher lipid reserves in autophagy-deficient mutants under drought suggests that autophagy may contribute to the regulation of reserve remobilization and metabolic balance during reproductive development under water deficit conditions.

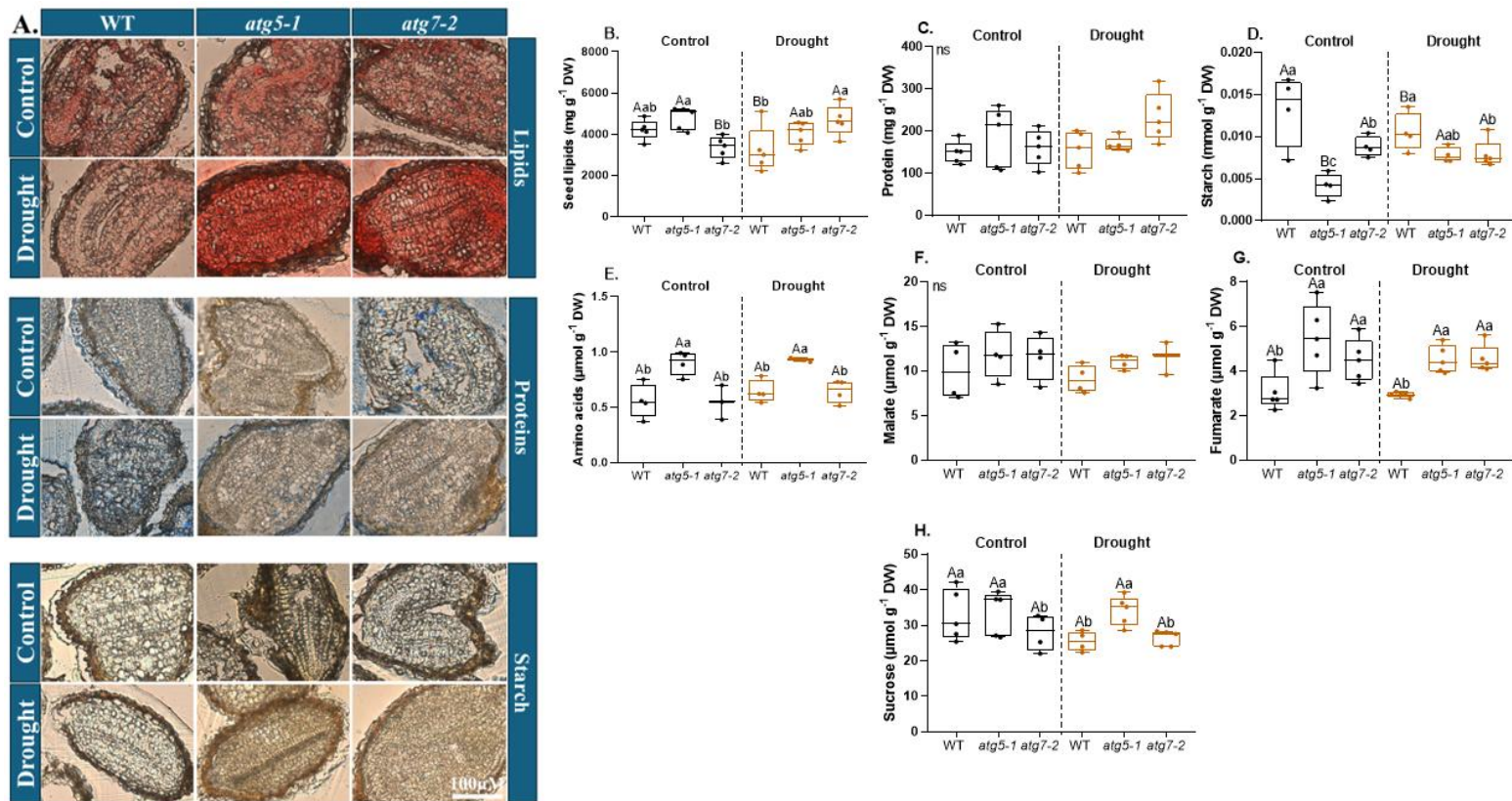


Figure 7. Histochemical and quantitative analyses of seed storage reserves demonstrate that drought affects reserve contents in autophagy deficient mutants (*atg5-1* and *atg7-2*). (A) Histochemical detection of lipids, protein and starch reserves in seeds from plants grown under control (80% field capacity) and drought (50% field capacity) conditions. Lipids were stained with Sudan Red B, proteins with Coomassie Brilliant Blue and starch with Lugol's solution. Scale

bar = 100µm. Quantification of **(B)** seed lipids, **(C)** protein, **(D)** starch, **(E)** amino acids, **(F)** malate, **(G)** fumarate and **(H)** sucrose content in seeds of WT, *atg5-1* and *atg7-2* plants under control (80% field capacity) and drought (50% field capacity) conditions in autophagy-deficient mutants (*atg5-1* and *atg7-2*) compared to WT. Different letters indicate statistically significant differences according to Tukey's test ($P < 0.05$). Uppercase letters denote comparisons among genotypes across different water conditions, whereas lowercase letters indicate comparisons among genotypes within the same condition. Data are presented as means $\pm$ standard error (SE) of five biological replicates per genotype. Abbreviation: ns: non-significant interaction. Abbreviation: ns: non-significant interaction; DW: dry weight.

Discussion

Despite recent advances in understanding plant responses to drought stress, the mechanisms regulating reproductive development under these conditions remain poorly understood. Indeed, most studies investigating the role of autophagy during drought have focused on responses occurring during the vegetative stage (Wang et al., 2015; Bao, 2020; Tang & Bassham, 2021), whereas the impact of water deficit on reproductive development and the mechanisms underlying fertility maintenance have received considerably less attention. In this context, the present study aimed to investigate the role of autophagy during the reproductive stage of *Arabidopsis thaliana* exposed to drought stress. Autophagy plays essential roles in plant adaptation to a wide range of abiotic stresses by promoting the recycling of damaged proteins and organelles, facilitating nutrient remobilization, and mitigating oxidative damage, thereby contributing to plant survival (Avin-Wittenberg, 2018). Under drought conditions, autophagy has been implicated in the degradation of aquaporins, the removal of heme and oxidatively damaged proteins, and the turnover of components involved in hormone signaling pathways (Wang et al., 2015; Tang & Bassham, 2021; Chen et al., 2025). Based on this evidence, we hypothesized that autophagy represents a central component of the adaptive responses to drought stress during sexual reproduction. Consistent with this hypothesis, our results demonstrate that autophagy acts as a key mechanism coordinating vegetative and reproductive development while regulating resource allocation under conditions of water limitation.

Autophagy Deficiency Induces Accelerated Leaf Senescence Without Impairing PSII Photochemical Efficiency

During drought stress, the relative water content decreased similarly across all genotypes, indicating that WT and autophagy deficient plants experienced comparable levels of water deficit (Figure 1D). Nevertheless, the *atg* mutants exhibited more

pronounced visual symptoms of leaf senescence and growth inhibition, suggesting a reduced capacity to physiologically adjust to drought stress. Moreover, *atg5-1* and *atg7-2* displayed reduced leaf area and lower root fresh weight even under well-watered conditions (Figures 1B and 1C), indicating that impaired autophagy could compromise basal plant development through its constitutive role in the recycling of proteins and damaged organelles, as well as in maintaining nutrient availability to sustain growth (Avin-Wittenberg, 2018; Signorelli et al., 2019; Li et al., 2026). Consistent with this notion, plants overexpressing *ATG5* and *ATG7* exhibit enhanced longevity, increased vegetative growth, and higher seed production compared with WT plants (Tang & Bassham, 2021). Interestingly, the maximum quantum efficiency of photosystem II (F_v/F_m) remained unchanged among treatments, indicating that PSII was not severely impaired by drought. These findings suggest that the phenotypic differences observed between genotypes are primarily associated with metabolic limitations and resource allocation rather than with photochemical dysfunction.

Autophagy Deficiency May Induce Leaf Metabolic Imbalance Under Drought

To better understand the metabolic basis underlying these foliar alterations, photosynthetic pigments and total protein content in the leaves were analyzed (Figure 2). Even under well-watered conditions, the *atg5-1* and *atg7-2* mutants exhibited significantly lower levels of total chlorophyll ($a + b$) than WT plants (Figure 2A). This constitutive phenotype indicates that autophagy functions as a housekeeping process essential for cellular homeostasis and for maintenance, even under favorable environmental conditions (Signorelli et al., 2019). Upon exposure to drought stress, all genotypes showed reduced pigment contents. However, the decline was more pronounced in the *atg* mutants, which exhibited the lowest absolute levels of chlorophylls. The accelerated degradation of photosynthetic pigments in autophagy deficient plants under drought is indicative of premature leaf senescence and uncontrolled cellular dismantling. Because autophagy mediates the selective removal of ROS damaged chloroplast proteins through chlorophagy (Izumi et al., 2017), disruption of this pathway in the *atg* mutants likely results in the accumulation of dysfunctional photosynthetic components, thereby accelerating chlorophyll loss. In contrast to the marked decline in photosynthetic pigments, total leaf protein content increased in the autophagy deficient mutants under drought, whereas WT plants showed lower protein levels (Figure 2B). This atypical protein accumulation likely reflects the inability of the *atg* mutants to efficiently degrade

and recycle structural and storage proteins, including Rubisco, during stress. Protein remobilization through autophagy is a key process for supplying carbon skeletons and nitrogen to support metabolism and reproductive development under adverse conditions (Masclaux-Daubresse et al., 2017). Therefore, the accumulation of total proteins in the mutants probably represents impaired protein turnover rather than enhanced protein synthesis, reinforcing the essential role of autophagy in nutrient remobilization during drought induced senescence.

The impairment of macromolecular recycling and nutrient remobilization in autophagy deficient plants had profound consequences for carbohydrate metabolism and carbon partitioning in leaves (Figure 3). Under drought stress, WT plants exhibited a marked increase in glucose and fructose contents, accompanied by substantial sucrose accumulation and an almost complete depletion of leaf starch reserves. This metabolic rearrangement represents a well-established adaptive response that promotes osmotic adjustment while protecting cellular structures under water deficit (Thalmann & Santelia, 2017). In contrast, the *atg5-1* and *atg7-2* mutants failed to accumulate glucose to the same extent as WT plants during drought (Figure 3A). Interestingly, leaf starch content, which was already significantly lower in the mutants under well-watered conditions, was depleted to similarly low levels under drought in both mutant and WT plants. The inability of the mutants to efficiently convert the remaining carbon reserves into free reducing sugars suggests that either the enzymatic machinery responsible for starch hydrolysis or the supply of carbon skeletons generated through autophagy-mediated recycling is compromised. Although both *atg* mutants accumulated sucrose in response to drought (Figure 3C), this increase occurred in parallel with markedly reduced glucose levels, suggesting an imbalance in carbon utilization rather than carbon availability. This metabolic profile may reflect impaired coordination between sugar synthesis, degradation, and utilization, processes that are tightly regulated by cellular energy signaling through the SnRK1 kinase in concert with the autophagy machinery (Soto-Burgos & Bassham, 2017). Together, these findings indicate that autophagy plays a critical role in maintaining carbon homeostasis during drought by coordinating reserve mobilization with sugar metabolism, thereby supporting metabolic adaptation under water-limited conditions.

The findings of leaf amino acids and organic acids (Figure 4) further support the occurrence of metabolic and energetic disturbances in autophagy deficient plants under

drought stress. Water deficit induced a pronounced accumulation of proline in both WT and *atg* mutants, confirming the activation of osmoprotective responses (Figure 4D). However, the total pool of free amino acids increased more markedly in *atg5-1* and *atg7-2* than in WT plants under drought conditions (Figure 4A). The elevated accumulation of free amino acids in the mutant leaves, together with their higher total protein content and lower monosaccharide levels, suggests that the absence of functional autophagy disrupts nitrogen remobilization and carbon metabolism during drought stress. Rather than reflecting efficient nutrient recycling, the accumulation of both proteins and amino acids likely indicates impaired protein turnover combined with a reduced capacity to export or metabolize these compounds (Fait et al., 2006). Collectively, these findings indicate that autophagy-deficient leaves undergo severe metabolic imbalance during drought, in which carbon and nitrogen resources are inefficiently remobilized and remain trapped in metabolically less accessible pools. Such impaired nutrient recycling is likely to compromise the metabolic support required by developing sink tissues, particularly during the reproductive stage, when the demand for carbon and nitrogen is substantially increased.

Autophagy Deficiency May Impair Floral Transition, Pollen Viability and Reproductive Success Under Drought Stress

These limitations became even more evident during the reproductive stage. Even under well-watered conditions, the *atg* mutants exhibited delayed floral stem emergence, reduced plant height, and fewer open flowers than WT plants (Figure 5), indicating that autophagy contributes to the proper transition from vegetative growth to reproductive development (Masclaux-Daubresse et al., 2017). Under drought stress, although plant height and the number of open flowers were only moderately affected, pollen viability was more severely reduced in the autophagy deficient mutants, particularly in *atg7-2* (Figure 5E). These findings suggest that impaired autophagy compromises key processes required to maintain male fertility under water deficit (Yan et al., 2023). Pollen development is among the most energy demanding processes in the plant life cycle and relies on a continuous supply of carbon, nitrogen, and metabolic energy (Yan et al., 2023; Qian et al., 2025). In the absence of functional autophagy, the reduced capacity for intracellular recycling is likely to limit the availability of these resources for reproductive tissues, rendering anthers and developing pollen grains more susceptible to drought induced damage (Masclaux-Daubresse et al., 2017). Consequently, the reduced pollen

viability observed in the *atg* mutants is expected to impair fertilization efficiency, thereby contributing to the decline in reproductive performance under drought stress (Mehmood et al., 2025).

Although silique length was largely unaffected by either genotype or drought treatment, the autophagy-deficient mutants produced significantly fewer siliques and fewer seeds per silique than WT plants (Figure 5). These results indicate that the primary reproductive impairment associated with autophagy deficiency occurs before fruit development, affecting processes such as floral development, fertilization, and early seed establishment (Qian et al., 2025). The preservation of silique length despite the marked reduction in seed number suggests that autophagy is more critical for reproductive success than for fruit growth, reinforcing its role in sustaining reproductive competence under drought conditions.

Trade-Offs in Seed Development: Autophagy Deficiency May Reduce Seed Quantity While Preserving Nutritional Quality for Efficient Germination Under Drought

The total number of seeds produced was drastically reduced in both *atg5-1* and *atg7-2* under well-watered and drought conditions (Figure 6C). In contrast, individual seed weight, as well as seed germination percentage and germination rate, remained unaffected (Figures 6B and 6E). These findings indicate that autophagy deficiency primarily compromises seed production rather than the physiological quality of the seeds that are successfully formed (Lornac et al., 2020). In other words, although impaired autophagy substantially reduces reproductive output, the seeds that develop retain their germination capacity, suggesting that seed vigor is largely preserved despite the reduction in reproductive success.

Seed reserve accumulation was also influenced by both autophagy deficiency and drought stress. Histological analyses revealed changes in the distribution of lipids, proteins, and starch among genotypes under drought, indicating alterations in seed filling (Figure 7A). Biochemical analyses further showed that oil and protein contents were maintained or even increased in the autophagy deficient mutants under water deficit, whereas starch content was significantly lower in *atg5-1* and *atg7-2* than in WT plants (Figures 7B–D). When considered together with the marked reduction in seed number, these results reveal an important physiological pattern. The decrease in the number of reproductive sinks may have allowed the available assimilates to be distributed among fewer developing seeds, thereby contributing to the maintenance or even enhancement of

reserve accumulation on a per-seed basis (Bennett et al., 2012). Thus, although autophagy is required to maximize overall seed production, its impairment does not necessarily compromise the biochemical quality of the seeds that successfully develop. Instead, a portion of the resources that would otherwise have been allocated to additional seeds may have been redirected to increase reserve deposition in the remaining seeds (Erllichman et al., 2023).

Under drought conditions, *atg5-1* seeds accumulated higher levels of sucrose, amino acids, and fumarate than WT seeds, whereas malate content remained relatively unchanged (Figure 7E, G, H). The accumulation of sucrose suggests alterations in carbon utilization and partitioning during seed filling (Baud et al., 2005). Likewise, the increased amino acid content indicates changes in nitrogen metabolism and possibly in the remobilization of nitrogenous compounds from maternal tissues (Baud et al., 2002). The higher fumarate content further points to modifications in respiratory metabolism and tricarboxylic acid (TCA) cycle activity during seed maturation (Fait et al., 2006). The concomitant accumulation of sucrose and free amino acids in *atg5-1* seeds under drought provides further evidence of altered source sink relationships and reserve metabolism. Under water deficit, the accelerated leaf senescence observed in the autophagy deficient mutants (Figure 1) is likely to enhance the remobilization of carbon and nitrogen from senescing leaves toward the limited number of developing reproductive sinks (Masclaux-Daubresse et al., 2017). However, the persistence of these metabolites in their soluble forms, together with the significant reduction in starch content (Figure 7D), suggests that although assimilate transport from maternal tissues was maintained, the conversion of imported carbon into storage compounds may have become less efficient in the absence of functional autophagy. The increased fumarate content may also reflect a metabolic adjustment in which excess carbon skeletons are preferentially redirected toward mitochondrial respiration or osmotic homeostasis during seed development (Fait et al., 2006). Collectively, these findings indicate that autophagy not only influences the supply of assimilates to developing seeds but also contributes to their efficient metabolic conversion into storage reserves under drought conditions.

Overall, our findings support a central role for autophagy in coordinating the physiological and metabolic adjustments required to sustain reproductive development under drought stress. In WT plants, autophagy appears to integrate chloroplast maintenance, nutrient recycling, carbon remobilization, and metabolic homeostasis,

thereby ensuring the continuous supply of assimilates to reproductive sinks. In contrast, impairment of autophagy triggered premature leaf senescence, disrupted protein turnover and carbohydrate metabolism, and altered carbon and nitrogen partitioning, ultimately compromising pollen viability, seed set, and reproductive output. Although the reduced number of developing seeds partially compensated for these limitations by preserving seed biochemical composition, this compensatory response was insufficient to restore reproductive performance. Together, these results indicate that the primary function of autophagy during drought extends beyond cellular protection, acting instead as a key regulator of source sink coordination that links leaf metabolic homeostasis with reproductive success. This work expands the current understanding of autophagy during plant reproduction and highlights its pivotal role in maintaining reproductive resilience under water limited conditions.

Conclusion

These results demonstrate that autophagy is essential for maintaining reproductive performance in *Arabidopsis thaliana* under drought stress. Although all genotypes experienced similar levels of water deficit, autophagy deficient mutants exhibited accelerated leaf senescence, impaired chloroplast maintenance, disrupted carbon and nitrogen metabolism and reduced nutrient remobilization, ultimately compromising the supply of assimilates required for reproductive development. These metabolic disturbances were associated with reduced pollen viability, lower seed set, and a marked decline in reproductive output. Despite these reductions, the biochemical composition and germination capacity of the seeds that were successfully developed were largely preserved, suggesting that autophagy primarily influences reproductive success by sustaining seed production rather than seed quality. Collectively, our findings establish autophagy as a potential regulator of leaf metabolic homeostasis during the reproductive stage. This study provides new insights into the role of autophagy during the reproductive stage and highlights its potential as a target for improving reproductive resilience and crop productivity under water limited conditions.

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