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Unveiling the role of autophagy in albino plantlets of *Agave Angustifolia* Haw.

Tesis que presenta

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RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis de Alexis Iván Cadena Ramos titulado **Unveiling the role of autophagy in albino plantlets of *Agave angustifolia* Haw.** fue realizado en la Unidad de Biotecnología, en la línea de investigación de agrobiotecnología, en el laboratorio de epigenética del Centro de Investigación Científica de Yucatán, A.C. bajo la dirección de la Dra. Clelia De la Peña Seaman, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

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Este trabajo se llevó a cabo en la Unidad de Biotecnología del Centro de Investigación Científica de Yucatán, A.C. con el financiamiento del Consejo Nacional de Humanidades, Ciencias y Tecnologías (Proyecto CB2016-285898, Ciencia Básica 2016), bajo la dirección de la Dra. Clelia De la Peña Seaman.

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LISTA DE PRODUCTOS GENERADOS

I. Artículos científicos

- Cadena-Ramos, A., De-la-Peña C. (2024). Picky eaters: Selective Autophagy in Plant Cells. *The Plant Journal*, 117(2), 364-384.

II. Artículos de divulgación

- Cadena-Ramos A., De-la-Peña C., Limones-Briones V. (2023). Las Células de las Plantas También Reciclan. *Desde el Herbario CICY.* 15, 170-173.

III. Actividades de divulgación

- Club ambiental. Sesión 2: ¿Cómo ver los microplásticos en el agua? ¿Son un peligro para el zooplancton? Realizado en el Centro de Investigación Científica de Yucatán, A.C. Mérida, Yucatán, México. Marzo de 2022.

- Taller informativo: Luces, fotosíntesis, acción: el despertar de los cloroplastos. Municipio de Chapab, Yucatán, México. Octubre de 2023.

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ABBREVIATIONS

3-MA	3-Methyladenine
AIM	ATG8-Interaction motif
AMSH3	Associated molecule with the SH3 domain of STAM3
ATG	Autophagy-related gene
ATI-PS body	ATI1-GFP labels plastid-associated body
CAM	Crassulacean acid metabolism
CCD4	Carotenoid cleavage dioxygenase 4
CEP	Cepharanthine
CHMP1	Charged multivesicular body protein1
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
GBSSI	Granule-bound starch synthase I
PAS	Phagophore assembly site
PCD	Programmed cell death
PE	Phosphatidylethanolamine
PI	Phosphatidynositol
PtdIns3K	Phosphatidylinositol 3-kinase
PUB4	Plant U-BOX4
RCB	RuBisCO containing bodies
ROS	Reactive oxygen species
SAG12	Senescence associated gene 12
SAV	Senescence associated vacuole
SnRK1	Sucrose nonfermenting-1-related protein kinase 1
SSTG	Small starch granule-like structure
TAG	Triacylglycerol
TF	Transcription factor
TGD	Trigalactosydiacylglycerol
TOR	Target of rapamycin
TORC1	TOR complex 1
UPS	Ubiquitin proteasome system
VIPP1	Vesicle inducing protein in plastid 1

RESUMEN

La autofagia, un proceso celular fundamental en las plantas, desempeña un papel importante en el mantenimiento de la homeostasis celular mediante la degradación selectiva de orgánulos dañados, proteínas mal plegadas y otros componentes celulares. Esta tesis investiga los complejos mecanismos de la autofagia en *Agave angustifolia* Haw., enfocándose particularmente en su papel en la degradación de cloroplastos dentro del fenotipo albino. Los análisis del transcriptoma revelaron una regulación al alza significativa de los genes relacionados con la autofagia en los tejidos albinos, lo que indica una mayor actividad autofágica destinada a mitigar el estrés oxidativo y mantener la funcionalidad celular, especialmente en los cloroplastos inmaduros y dañados.

Se encontró que genes clave de la autofagia como *ATG2, ATG8, ATG12, ATI1 y NBR1* estaban prominentemente expresados en los tejidos albinos, sugiriendo su participación crítica en la degradación selectiva de los cloroplastos inmaduros. Este proceso es crucial para optimizar la utilización de nutrientes y la función celular bajo condiciones de estrés. Además, la adición de altas concentraciones de la auxina 2,4-diclorofenoxiacético (2,4-D) en el fenotipo albino indujo cambios fenotípicos específicos, incluyendo alteraciones en la formación de brotes y callos, y respuestas de desarrollo.

Asimismo, investigaciones utilizando el inhibidor de la autofagia 3-Metiladenina (3-MA) elucidan su papel en la inhibición de los miembros de la familia PI3K, interrumpiendo así el flujo autofágico y alterando los patrones de expresión génica asociados con la autofagia de los cloroplastos y el mantenimiento celular.

En general, esta tesis proporciona conocimientos exhaustivos sobre los mecanismos moleculares de la autofagia de los cloroplastos en *A. angustifolia* Haw., enfatizando su importante papel en la homeostasis celular, la adaptación al estrés y el reciclaje de nutrientes. Los hallazgos subrayan la importancia de la autofagia en la resiliencia de las plantas y proporcionan una base para futuras investigaciones sobre la manipulación de las vías de la autofagia para aplicaciones agrícolas y ambientales.

ABSTRACT

Autophagy, a fundamental cellular process in plants, plays an important role in maintaining cellular homeostasis by selectively degrading damaged organelles, misfolded proteins, and other cellular components. This thesis investigates the complex mechanisms of autophagy in *Agave angustifolia* Haw., particularly focusing on its role in the degradation of chloroplasts within the albino phenotype. Transcriptome analyses revealed a significant upregulation of autophagy-related genes in albino tissues, indicating heightened autophagic activity aimed at mitigating oxidative stress and maintaining cellular functionality, particularly in immature and damaged chloroplasts.

Key autophagy genes such as *ATG2, ATG8, ATG12, ATI1, and NBR1* were found to be prominently expressed in albino tissues, suggesting their critical involvement in the selective degradation of immature chloroplasts. This process is crucial for optimizing nutrient utilization and cellular function under stress conditions. Additionally, the addition of the high concentrations of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in the albino phenotype was found to induce specific phenotypic changes, including alterations in shoot and callus formation, and developmental responses.

Furthermore, investigations using the autophagy inhibitor 3-Methyladenine (3-MA) elucidated its role in inhibiting PI3K family members, thereby disrupting autophagy flux and altering gene expression patterns associated with chloroplast autophagy and cellular maintenance.

Overall, this thesis provides comprehensive insights into the molecular mechanisms of chloroplast autophagy in *A. angustifolia* Haw., emphasizing its important role in cellular homeostasis, stress adaptation, and nutrient recycling. The findings underscore the importance of autophagy in plant resilience and provide a foundation for further research into manipulating autophagy pathways for agricultural and environmental applications.

INTRODUCTION

Autophagy is a conserved biological process found in all eukaryotic organisms, which plays a crucial role in intracellular recycling by degrading proteins and organelles through the vacuolar pathway in plants and yeast, and the lysosomal pathway in animals (Su *et al*., 2020). In plants, autophagy operates at a basal level to maintain cellular homeostasis during growth and development. However, the process is upregulated under environmental stress conditions to promote plant survival (Yoshimoto and Ohsumi, 2018). Interestingly, three distinct forms of autophagy have been identified in plants: microautophagy, macroautophagy, and mega-autophagy (Su *et al*., 2020).

Microautophagy is a process in which organelles are taken up and invaginated into the tonoplast, forming an autophagic body that is subsequently released into the vacuolar lumen for degradation (Yang and Bassham, 2015). In contrast, macroautophagy involves the formation of a double membrane, or phagophore, around intracellular material, resulting in the formation of an autophagosome. This vesicle encapsulates various cellular components and transports them to vacuoles or lysosomes for degradation. The outer membrane of the autophagosome then fuses with the tonoplast, releasing the autophagic body into the vacuole for degradation (Yang and Bassham, 2015). Finally, mega-autophagy involves the release of hydrolases from the vacuole into the cytoplasm, leading to the degradation of various cellular components, such as the cytoplasm, organelles, plasma membrane, and parts of the cell wall, resulting in cell death (Su *et al*., 2020) (**Figure 1.1**).

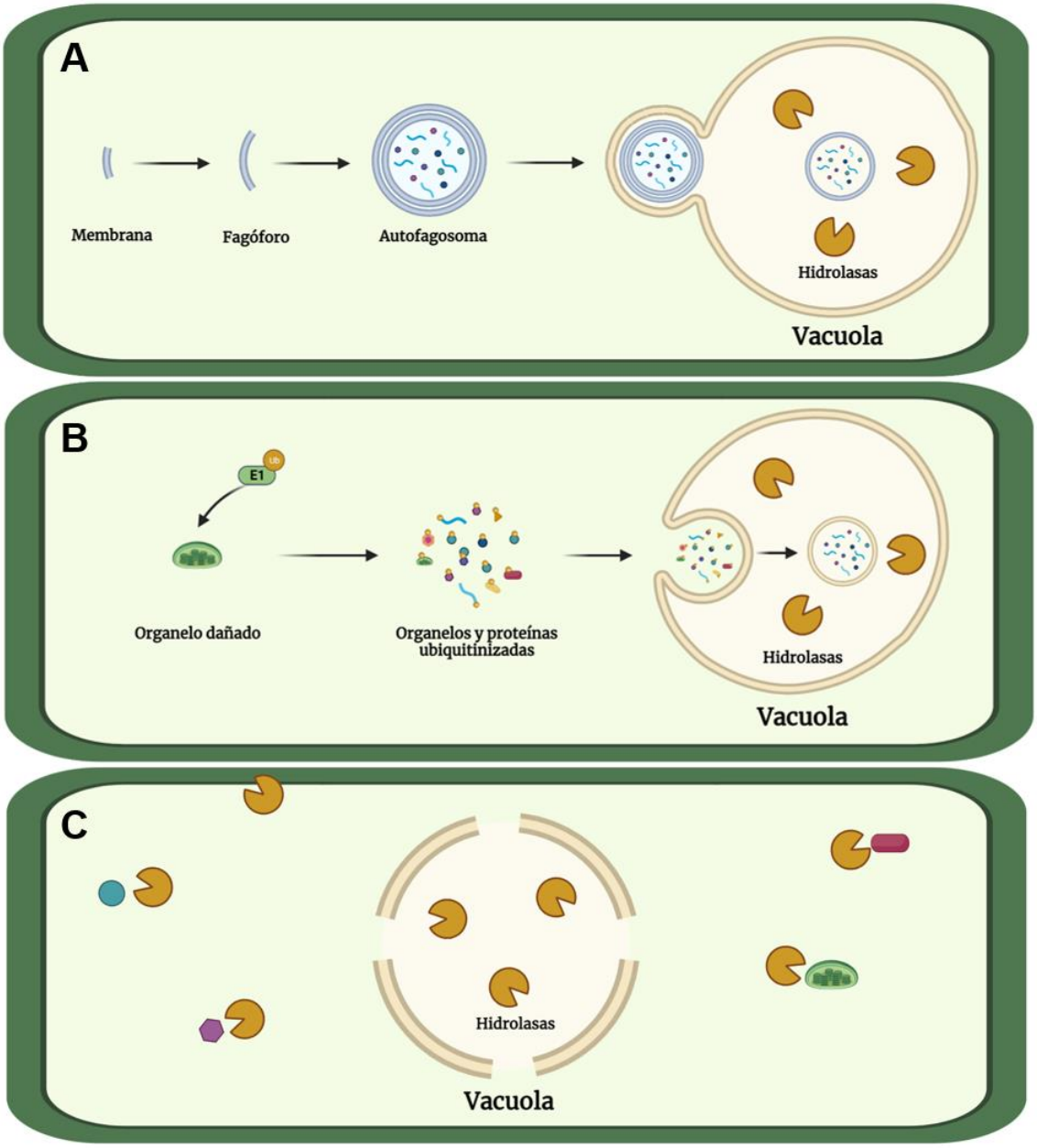


Figure 1.1. Mechanisms of autophagy described in plants. Macroautophagy (A) is a process that creates an autophagosome, a vesicle that carries cellular material to the vacuole for degradation. In contrast, microautophagy (B) involves the invagination of the tonoplast to engulf cytoplasmic components and transport them into the vacuole. Both macroautophagy and microautophagy lead to the formation of autophagic bodies and the degradation of cellular material by hydrolases in the vacuole. On the other hand, mega-autophagy (C) does not involve the formation of autophagosomes or direct transportation of cargo to the vacuole. Instead, it leads to

the rupture of the tonoplast and the release of vacuolar hydrolases into the cytoplasm, ultimately resulting in cell death. Figure obtained from Cadena-Ramos A., Limones-Briones V., and De-la-Peña C. (2023). Las células de las plantas también reciclan. Desde el Herbario CICY.

Autophagy is a complex process that encompasses distinct stages, including induction, cargo recognition, phagophore formation, phagophore expansion and closure, and autophagosome fusion and breakdown, all of which are tightly regulated by a group of genes collectively known as AUTOPHAGY-RELATED (ATG) genes. These genes can be classified into four core functional protein groups. The *ATG1/ATG13* kinase complex, for instance, is responsible for initiating autophagosome formation in response to nutrient scarcity and playing critical roles in membrane delivery, vesicle nucleation, and phagophore expansion and closure. The autophagy-specific class III phosphatidylinositol (PI) 3-kinase complex is another essential component required for autophagosome initiation and maturation. Additionally, the *ATG9* complex is involved in phagophore expansion, while the fourth protein group, the *ATG8/ATG12* ubiquitin-like conjugation system, plays a crucial role in phagophore expansion and maturation, ultimately leading to the formation of a fully mature autophagosome (Su *et al*., 2020).

Selective autophagy is a critical cellular process utilized by animal and plant cells to selectively eliminate specific cellular components. This process is mediated by interactions between *ATG8* genes and specific receptors that harbor the *ATG8*-interacting motif (AIM) (Zhuang and Jiang, 2019a). Autophagic receptors play a pivotal role in the regulation of selective autophagy of cellular components, such as organelles, proteins, and invading pathogens (Marshall and Vierstra, 2018). Autophagy plays a significant role in various physiological functions during different developmental stages in plants. These functions include regulating nutrient supply during seed and root (primary and secondary) development, participating in lipid metabolism by assisting in triacylglycerol (TAG) synthesis and lipid droplet (LD) degradation, acting as a negative feedback regulator of salicylic acid (SA) to limit senescence and programmed cell death (PCD), and facilitating the recycling of cellular material and remobilization of nutrients during oxidative stress response induced by NADPH oxidase-dependent or independent pathways (Su *et al*., 2020). Several organelles, including mitochondria, peroxisomes, lysosomes, and chloroplasts, are targeted by selective autophagy pathways in plants and are recognized by *ATG8*-binding proteins (Su *et al*., 2020).

Chloroplasts are a central organelle in plants, responsible for providing food and energy in the form of sugar or starch through photosynthesis (Jarvis and López-Juez, 2013). However, chloroplasts can become damaged under various stress conditions and produce toxic reactive oxygen species (ROS) or stress signals that affect plant growth (Nakamura and Izumi, 2018). To avoid accumulating these harmful substances, damaged or redundant chloroplasts are degraded through a process called chlorophagy, a form of selective autophagy. This process promotes cell survival and contributes to the natural turnover of aging chloroplasts, which helps to overcome early leaf senescence and cell death (Nakamura and Izumi, 2018). Chlorophagy can occur through several pathways, including the whole chloroplast pathway, RuBisCO-containing bodies (RCBs), plastid-associated bodies labeled by ATI1-GFP (ATI-PS bodies), and small starch granule-like structures (SSTGs) (Zhuang and Jiang, 2019a).

Albinism is a rare condition in plants that involves the partial or complete loss of chlorophyll pigments, which are necessary for light energy capture. This event leads to poorly developed thylakoid membranes and can significantly impair photosynthesis, ultimately resulting in the premature death of the plant before reaching maturity (Kumari *et al.*, 2009). The causes of albinism are diverse and may arise from various factors such as genotype, environmental conditions, meiotic abnormalities, hormonal imbalances, nuclear-plastid genome incompatibility, deletions in plastid DNA, and mutations in genes related to chlorophyll biosynthesis (Kumari *et al*., 2009). Additionally, epigenetic modifications can also contribute to the occurrence of albinism in plants (Duarte-Aké *et al*., 2023; Hernández-Castellano *et al*., 2020; Us-Camas *et al*., 2017). Autophagy mechanisms play a crucial role in selectively degrading incomplete chloroplasts and chlorophyll-binding proteins, which can contribute to the survival of the plant under these adverse conditions (Yamashita *et al*., 2021).

The mechanisms by which the ATG machinery interacts with different cellular pathways to regulate chloroplast turnover remain poorly understood. To bridge this gap in knowledge, the main goal objective of this study was to identify the key genes involved in autophagy in albino plantlets of *Agave angustifolia* Haw.

CHAPTER I

1.- BACKGROUND

1.1.- TYPES OF AUTOPHAGY

Intracellular protein quality control is an essential process for successful cell growth and development throughout the lifespan of a plant cell. This process involves a delicate balance between protein synthesis and degradation to ensure the removal of damaged components, the recovery of nutrients, and the elimination of destructive ROS from organelles such as chloroplasts, mitochondria, and peroxisomes (Bu *et al*., 2020).

Short-lived proteins and small molecules are typically degraded through the ubiquitin-proteasome system in the cytosol or by proteases within organelles. In contrast, degradation of long-lived cytosolic proteins, macromolecules, and larger structures, such as organelles, occurs through autophagy, a process commonly called "self-eating" (Floyd *et al*., 2015). Upon activation of the autophagic mechanism, cellular components, or "cargos," are transferred to the vacuole and degraded by hydrolases. The degradation products are then transported back into the cytoplasm for reuse by the cell (Su *et al*., 2020).

Various types of autophagy pathways have been identified in plant cells, including microautophagy, macroautophagy, and a third pathway called mega-autophagy, which may be specific to plants. Macroautophagy is the most extensively studied and prevalent form of autophagy in plants, while the other pathways remain less well-understood (Sieńko *et al*., 2020).

Macroautophagy is a fundamental process that operates at low levels to maintain normal cellular function but can be activated in response to environmental stresses and developmental signals. The initiation of macroautophagy involves forming a membrane structure called phagophore, which engulfs cytoplasmic material to be degraded. The phagophore expands by capturing the membrane from various cellular compartments, eventually closing to create an autophagosome, a double-membrane vesicle that contains

the autophagic cargo. The autophagosome is then delivered to the vacuole, which fuses with the tonoplast membrane, potentially aided by the cytoskeleton. The inner membrane and its contents are released into the vacuole as an autophagic body, broken down and recycled by vacuolar hydrolases (Floyd *et al*., 2015).

Microautophagy, on the other hand, is a type of autophagy that involves the direct invagination of the tonoplast membrane to form intravacuolar vesicles. This process allows for the direct endocytosis of cytoplasmic constituents and tonoplast components into the vacuole. There are three types of microautophagy, depending on the morphology of the vacuolar/lysosomal membrane deformation: lysosomal/vacuolar membrane engulfment, invagination, and invagination of late endosomes. However, the molecular mechanisms underlying microautophagy are not well understood (Sieńko *et al*., 2020; Floyd *et al.*, 2015).

Mega-autophagy, unlike the other types of autophagy, involves the degradation of cellular components by vacuolar enzymes without the formation of phagophores or transport of the cargo into the vacuole. Instead, rupture of the tonoplast releases vacuolar hydrolases into the cytoplasm, resulting in cell death. Due to its distinct mode of operation, mega-autophagy is sometimes referred to as autolysis rather than autophagy (Bu *et al*., 2020; Floyd *et al*., 2015).

The phenomenon of autophagy was first discovered in the late 1950s when researchers observed double-membrane vesicles containing cytoplasmic material being degraded by lysosomal enzymes following fusion with lysosomes in amino acid-deprived rat livers (De Duve and Wattiaux, 1966). In plants, autophagic structures were also identified in the late 1960s, with experiments showing the formation of autophagosome-like structures engulfing cytoplasmic components during vacuole biogenesis in embryo cells and root meristem (Marty, 1978). The process of microautophagy, involving the invagination and formation of intravacuolar vesicles, was documented in corn root meristem cells, and cytoplasmic components and chloroplasts were observed to be degraded in vacuoles during cotyledon and leaf senescence (Van Der Wilden *et al*., 1980). Chloroplast degradation was found to occur via microautophagy (Wittenbach *et al*., 1982). While these observations provided valuable insight into autophagy morphology and structural components, the molecular pathways involved in autophagy remained poorly understood.

1.2.- AUTOPHAGY MECHANISM

The elucidation of the molecular mechanisms underlying autophagy has been greatly facilitated by the discovery of autophagy in yeast and the identification of the essential AUTOPHAGY-RELATED (ATG) genes responsible for autophagosome formation (Fang *et al.*, 2021; Yoshimoto and Ohsumi, 2018). Genetic studies of yeast mutants that cannot accumulate autophagic bodies in the vacuolar lumen and are hypersensitive to nutrient deprivation led to the discovery of the genetic machinery of autophagy (Yoshimoto and Ohsumi, 2018). Since the first ATG genes were identified in yeast in 1993 (Tsukada and Ohsumi, 1993), genetic screening for autophagy-defective mutants in yeast and other fungi has identified 41 ATG genes that are critical for autophagy in these models (Wen and Klionsky, 2016). Similarly, approximately 40 ATG homologs have been identified in *Arabidopsis thaliana*, most of which are homologs of yeast ATGs. The core machinery of ATG genes for autophagosome formation comprises 18 ATG genes, including *ATG1-ATG10, ATG12-ATG14, ATG16-ATG18, ATG29*, and *ATG31*. In *Arabidopsis*, about 30 ATG homologs have been identified, but homologs of *ATG14, ATG17, ATG29*, and *ATG31* remain unidentified (Chung, 2019). The ATG proteins are divided into four core functional groups: (1) the *ATG1/ATG13* kinase complex (*ATG1, ATG13, ATG17, ATG29,* and *ATG31*), (2) the autophagy-specific class III phosphatidylinositol (PI) 3-kinase complex (*ATG6* and *ATG14*), (3) the ATG9 complex (*ATG2, ATG9*, and *ATG18*), and (4) the *ATG8/ATG12* ubiquitin-like lipidation/conjugation systems that are divided into lipidation (*ATG3, ATG4, ATG7,* and *ATG8*) and conjugation (*ATG5, ATG7, ATG10, ATG12,* and *ATG16*) (Su *et al*., 2020; Chung, 2019; Yoshimoto and Ohsumi, 2018; Floyd *et al*., 2015). (**Table 1.1**).

Table 1.1.- Autophagy is regulated by the ATG core functional groups, divided into four distinct groups. The first group is the ATG1/ATG13 kinase complex, which initiates the formation of the autophagosome and is regulated by the target of rapamycin (TOR) complex. The second group is the autophagy-specific class III phosphatidylinositol (PI) 3-kinase complex. The third group is the ATG9 complex, which is involved in phagophore expansion. The fourth group is the ATG8/ATG12 ubiquitin-like conjugation system, responsible for phagophore expansion and maturation.

FUNCTIONAL COMPLEX	GENES	FUNCTIONS	REFERENCES
ATG1 kinase complex	*ATG1*	Interacts with SNRK1 and TOR.	Huang *et al.*, 2019; McLoughlin *et al.*, 2020; Wang and Hou, 2022
	ATG11	Regulates ATG1/ATG13 signaling.	Li *et al.*, 2014; Zientara-Rytter and Subramani, 2020
	ATG13	Activates the *ATG1* kinase pathway. It is inhibited by TOR.	Yoshimoto and Ohsumi, 2018; Su *et al.*, 2020; Wang and Hou, 2022
	ATG101	Stabilizes ATG1/ATG13 complex.	Suzuki *et al.*, 2015
Class III PI3K complex	*ATG6*	Associated with autophagosome modeling.	Zhuang and Jiang, 2019; Yoshimoto and Ohsumi, 2018
ATG9 complex	*ATG2*	Membrane transport into the autophagosomes.	McLoughlin *et al.*, 2020; Yoshimoto and Ohsumi, 2018
	ATG9	Delivers membranes to the phagophores.	Yoshimoto and Ohsumi, 2018; Zhuang *et al.*, 2017; Su *et al.*, 2020
	ATG18	Deliver lipids into the forming autophagosomes.	Yoshimoto and Ohsumi, 2018; Su *et al.*, 2020
ELONGATION STEP			
ATG8-lipidation system	*ATG3*	Mediates the conjugation of ATG8 with phosphatidylethanolamine (PE), an essential step in autophagy.	Su *et al.*, 2020; Fang *et al.*, 2021
	ATG4	Regulates the ATG8-PE binding, activating and deactivating ATG8.	Yoshimoto and Ohsumi, 2018; Su *et al.*, 2020
	ATG7	Asssociated with chlorophagy pathways.	Nakamura *et al.*, 2018; Zhuang and Jiang, 2019; Yoshimoto and Ohsumi,

			2018
	ATG8	Ubiquitin-like, conjugates with PE to form nascent autophagosomal membranes.	Nakamura *et al.*, 2018; Chen *et al.*, 2019; Bu *et al.*, 2020
ATG12-conjugation system	*ATG5*	Conjugates with ATG12 and participates in ATG8-PE binding.	Nakamura *et al.*, 2018; Zhuang and Jiang, 2019)
	ATG10	Participates in the biogenesis of autophagosomal bodies.	Nakamura *et al.*, 2018; Yoshimoto and Ohsumi, 2018; Izumi *et al.*, 2019
	ATG12	Conjugates with ATG and participates in ATG8-PE binding.	Nakamura *et al.*, 2018; Izumi *et al.*, 2019; Su *et al.*, 2020
	ATG16	Interacts with ATG5.	Nakamura *et al.*, 2018; Izumi *et al.*, 2019; Su *et al.*, 2020

Although ATG genes are essential for the autophagy process, numerous complexes interact with the ATG machinery and play a crucial role in autophagy induction, cargo recognition, phagophore formation, phagophore expansion and closure, and autophagosome fusion and breakdown (Su *et al*., 2020) (**Table 1.2**). While several types of autophagy have been morphologically described in plants (Su *et al*., 2020; Yoshimoto and Ohsumi, 2018; Floyd *et al*., 2015), information is primarily available for macroautophagy, which can be divided into different stages, including autophagy induction, autophagosome formation, cargo selection, vesicle fusion, and vesicle turnover (Su *et al*., 2020; Yoshimoto and Ohsumi, 2018).

Table 1.2.- Multiple proteins associated with autophagic machinery reported in plants.

PROTEIN	**ATG INTERACTION**	**FUNCTION**	**REFERENCES**
TOR	*ATG13, ATG1, ATG9 and ATG18a*	Negatively regulates autophagy.	Yoshimoto, 2018; Su *et al.*, 2020
ATI1/ATI2	*ATG8*	It interacts through an *ATG8* interacting motif to transport the cargo into the vacuole. Participates in selective	Nakamura, 2018; Zhuang and Jiang, 2019; Izumi *et al.*, 2019

		autophagy.	
SnRK1	*ATG1*	Phosphorylates *ATG1* and the TOR complex subunit (RAPTOR) to inhibit TOR activity and initiate autophagy.	Su *et al.*, 2020
VPS34	*ATG8*	Adds a signal on the autophagic membranes to be recognized by the vacuole.	McLoughlin *et al.*, 2020
SH3P2	*ATG8*	Binds to PI3P and *ATG8*, regulating the autophagosome formation.	Yoshimoto and Ohsumi, 2018; Su *et al.*, 2020; Cadena-Ramos and De-la-Peña, 2024
ESCRT	*ATG8*	Participates in autophagosome fusion and degradation.	Su *et al.*, 2020; Cadena-Ramos and De-la-Peña, 2024

1.2.1.- Autophagy induction

In plant biology, the sucrose nonfermenting-1-related protein kinase 1 (SnRK1) is a critical regulator of the response to energy and nutrition depletion (Rodriguez *et al*., 2019). This energy-sensing system comprises a catalytic α-type subunit (KIN10 and KIN11 in *Arabidopsis*) and two noncatalytic subunits, β-type and γ-type (Polge and Thomas, 2007). SnRK1 is pivotal in maintaining homeostasis and activating the autophagy pathway during nutrient deprivation. Additionally, it regulates autophagy through the target of rapamycin (TOR) kinase complex, specifically TOR complex 1 (TORC1), which consists of TOR1/2, KOG*,* and TCO89. Under nutrient-replete conditions, this complex hyperphosphorylates *ATG13,* hindering the binding of *ATG1* with the *ATG13* subcomplex (*ATG13, ATG17, ATG29,* and *ATG31*) and thus resulting in autophagy inhibition. However, nutrient starvation inactivates TORC1, leading to *ATG13* dephosphorylation and *ATG1* hyperphosphorylation (Li and Vierstra, 2012). Consequently, *ATG1-ATG13* association and *ATG11* and *ATG101* form an active complex that activates the autophagy induction mechanism (Kamada *et al*., 2010). In *Arabidopsis*, the RAPTOR family is homologous to yeast KOG1 and serves as a target recognition cofactor for TOR. The RAPTOR family

comprises RAPTOR1/Raptor1B and RAPTOR2/Raptor1A (Floyd *et al*., 2015). Under nutrient-deprivation and stress conditions, TOR inhibits and SnRK1 activates *ATG1* kinase, which is essential for autophagic activation during short-term fixed-carbon starvation and nitrogen deprivation. Still, it is not necessary for autophagic activation induced by long-term fixed-carbon starvation. Other complexes involved in the induction of autophagy include the phosphatidylinositol 3-kinase (PtdIns3K) complex, which is associated with the early stages of phagophore formation and may play a role in the nucleation and recruitment of other ATG components. However, its precise function is unclear (Yoshimoto and Ohsumi, 2018). The *ATG1/ATG13* complex comprises the catalytic subunit *ATG1* that regulates autophagy in response to nutritional status. This complex activates autophagy through different steps, including the engagement of *ATG9* in autophagosome formation and phagophore modeling with *ATG8* and *VPS-34/ATG6/ATG14/VPS15* lipid kinase complex (Li and Vierstra, 2012). Multiple studies with mutants of the *ATG1* kinase complex have confirmed that there are two autophagic signaling pathways in *Arabidopsis* under fixed-carbon stress: *ATG1* short-term starvation and phosphorylation of *ATG6* by KIN10 in the downregulation or deficiency of the *ATG1* kinase complex (Huang *et al*., 2019a). The role of Rho GTPase signaling in plant autophagy is not yet clear. Recently, however, it was discovered that *Sec5* could bind to activated *ROP8* under stress conditions, creating the *Sec5-ROP8* complex, which promotes autophagosome formation by recruiting the *ATG1* and PI3K complex to the phagophore (Lin *et al*., 2021).

1.2.2.- Autophagosome formation

The autophagosome formation process involves expanding a membrane called phagophore, which facilitates cargo transportation to the vacuole/lysosome. This process begins at the phagophore assembly site (PAS), where most of the ATG proteins are co-localized (Floyd *et al*., 2015; Suzuki *et al*., 2013). In plants, autophagosomes recruit two main ATG complexes at the PAS: *ATG12-ATG5* and *ATG8-PE (*phosphatidylethanolamine*)*. The conjugation system responsible for this recruitment is like the ubiquitin-like conjugation system observed in cells. The *ATG12-ATG5* conjugation system requires the presence of *ATG7*, which acts as a ubiquitin-activating-like enzyme (E1) and activates *ATG12* by hydrolyzing ATP, forming a thioester bond between the C-

terminal glycine of *ATG12* and a cysteine residue on *ATG7* (Tanida *et al*., 2001, 1999). The C-terminal glycine of *ATG12* is transferred to a cysteine of *ATG10*, which functions as a ubiquitin-conjugating-like enzyme (E2), forming a new thioester bond and releasing *ATG7* (Shintani, 1999). *ATG12*'s C-terminal glycine forms an isopeptide bond with the amino group of a lysine in *ATG5*, releasing *ATG10* and producing the *ATG12-ATG5* conjugate. *ATG16*, an additional autophagy protein, interacts with *ATG5* within the *ATG12-ATG5* conjugate, forming a tetrameric complex (Kuma *et al*., 2002).

The ATG8-PE conjugation system requires the presence of three proteins: *ATG4* (cysteine protease), which removes the C-terminus of *ATG8*, leaving a glycine exposed; *ATG7* (E1-like enzyme), which activates *ATG8* by linking the exposed glycine of *ATG8* to an active cysteine in *ATG7*; and *ATG3* (E2 enzyme), which helps in the conjugation of *ATG8* with PE, which is recruited to the autophagosome membrane (Ichimura *et al*., 2004). The lipidation of *ATG8* is reversible by the action of the *ATG4* protease, which can deconjugate the *ATG8-PE* complex, recycle *ATG8*, and generate a conjugation cycle. This process is essential for autophagosome expansion and the normal functioning of autophagy (Nair *et al*., 2012).

The *ATG9* cycling system coordinates the delivery of lipids to the expanding phagophore along with *ATG2* (present in later stages of vesicle biogenesis) and ATG18 (Zhuang *et al*., 2017). The protein *SH3P2* plays a crucial role in autophagosome formation by binding with the PtdIns3K complex and interacting with *ATG8*. It is localized in the PAS and promotes the expansion or maturation of the developing autophagosome membrane and enclosure (Yoshimoto and Ohsumi, 2018; Zhuang *et al*., 2013).

1.2.3.- Cargo selection

The autophagy receptors p62 and mNBR1 play a crucial role in selective autophagy in mammals by recognizing and targeting the degradation of non-functional and ubiquitin-modified proteins in the form of aggregates, known as aggrephagy (Yoo *et al*., 2019; Kirkin *et al*., 2009; Pankiv *et al*., 2007). In Arabidopsis, a functional hybrid of p62 and mNBR1 called NBR1 has been identified (Svenning *et al*., 2011), while in tobacco, its ortholog is known as Joka2 (Zientara-Rytter *et al*., 2011). These orthologs bind to various isoforms of

ATG8 and help in targeting ubiquitinated proteins for selective autophagy, particularly under heat stress conditions (Zhou *et al*., 2013).

A distinct process of selective autophagy, known as proteaphagy, is crucial in removing damaged protein complexes (Marshall *et al*., 2015). This pathway can be triggered by two mechanisms: one involves nitrogen starvation and is regulated by *ATG1*, while the other is independent of *ATG1* and is activated by proteasome inhibitors such as MG132. Upon activation, *ATG1* becomes ubiquitinated and is recognized by proteaphagy receptors, such as RPN10 in Arabidopsis, which initiate the formation of autophagosomes (Marshall and Vierstra, 2018).

Plant peroxisomes are essential organelles that participate in many metabolic pathways, including beta-oxidation of fatty acids, glyoxylate cycle, and photorespiration (Su *et al*., 2019). The metabolic reactions within peroxisomes vary according to plant developmental stages, which require different types of enzymes (Young and Bartel, 2016). During early developmental stages, the glyoxylate cycle is critical for seed germination, while in green seedlings, enzymes associated with photorespiration replace those involved in the glyoxylate cycle (Hu *et al*., 2012). This adaptive process necessitates the activation of pexophagy, a selective autophagy mechanism that eliminates unwanted or damaged peroxisomes (Su *et al*., 2020). Pexophagy occurs at a higher rate than other types of selective autophagy and is tissue-dependent. The molecular mechanism that triggers pexophagy is still unclear. However, in Arabidopsis, peroxisomal membrane proteins PEX6 and PEX10 interact with *ATG8* through ATG8-interaction motif (AIM), suggesting their involvement in pexophagy initiation (Xie *et al*., 2016).

Other types of selective autophagy have been reported in plants. Under normal conditions, this process regulates the degradation of specific organelles such as protein complexes, endoplasmic reticulum, mitochondria, peroxisomes, ribosomes, and chloroplasts (Cadena-Ramos and De-la-Peña, 2024) (**Figure 1.2**).

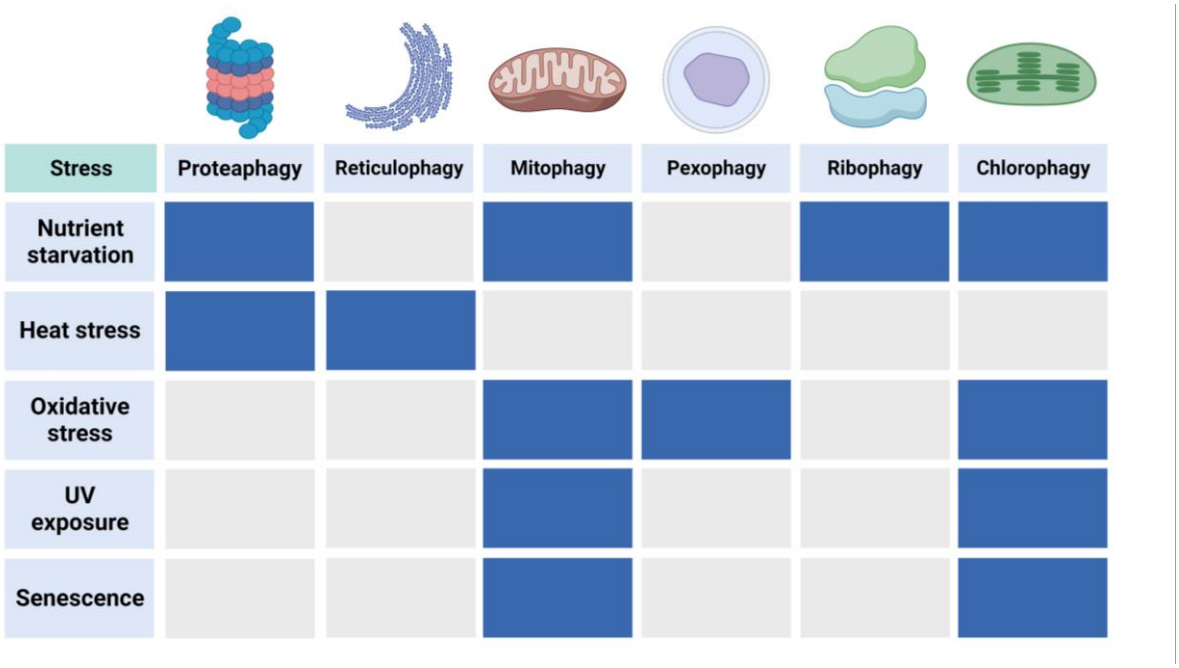


Figure 1.2.- Multiple targets of selective autophagy in plants. Numerous proteins that bind to ATG8 serve as receptors for selective autophagy, enabling the transportation of specific targets to autophagosomes for degradation during specific stress conditions.

1.2.4.- Vesicle fusion with tonoplast

Once the autophagosome is formed, FYCO1 binds to both *LC3/ATG8* and PI3P on the outer membrane of the autophagosome, facilitating its movement to the vacuole/lysosome (Pankiv *et al*., 2010). Previous research has indicated that *ATG8* can bind to microtubules, which suggests that microtubules may be involved in autophagosome movement to the vacuole (Ketelaar *et al*., 2004). Proper transportation of the autophagosome to the vacuole requires the presence of SNAREs, which are soluble NSF attachment protein receptors (Moreau *et al*., 2013). VT112, a v-SNARE protein (vesicle SNARE), plays a critical role in autophagosome fusion with the tonoplast (Surpin *et al*., 2003). ESCRT (endosomal sorting complex required for transport) is also necessary for autophagosome fusion and degradation. In Arabidopsis, AMSH3 (an associated molecule with the SH3 domain of STAM3) interacts with the ESCRT-III subunit *VPS2.1* (vacuolar protein sorting 2.1), which is essential for autophagosome transportation to the vacuole (Katsiarimpa *et al*., 2013). The autophagosome-vacuole fusion mechanism is regulated by a specific ESCRT component called FREE1 (FYVE domain protein required for endosomal sorting 1), which

interacts with the autophagy regulator SH3P2 and plays a crucial role in autophagosome-vacuole fusion in Arabidopsis (Zhuang *et al*., 2013).

1.2.4.1.- Vesicle turnover

After the autophagosome fuses with the vacuole, the resulting internal vesicle containing the cargo, known as the autophagic body, is released into the vacuole lumen, where it is degraded by a series of hydrolases into small molecules and amino acids for recycling (Marshall and Vierstra, 2018). To ensure proper degradation of the autophagic body, the vacuole needs to be acidic, and the presence of vacuolar hydrolases Pep4 and Prb1 is required (Nakamura *et al*., 1997). Vacuolar processing enzyme-γ (VPEγ) is a protease that initiates processing by releasing pro-proteins for cargo degradation (Rojo *et al*., 2003). The activity of hydrolases is dependent on the acidification of vacuoles, which is maintained by Vacuolar-type ATPases (V-ATPases), ATP-dependent proton pumps located in the tonoplast that continuously pump H+ into the vacuole to maintain an acidic environment (Kriegel *et al*., 2015; Sze *et al*., 1999). *ATG15* is a lipase that participates in the intravacuolar lysis of the autophagic body (Epple *et al*., 2001). *ATG22* regulates the transportation of amino acids back to the cytoplasm after the degradation process (Yang *et al*., 2006). *ATG8-PE* on the inner autophagosome membrane is degraded into the vacuole, while the outer membrane of *ATG8-PE* is cleaved by *ATG4* to release *ATG8* from PE for recycling (Su *et al*., 2020; Yoshimoto and Ohsumi, 2018; Yoshimoto *et al*., 2004).

1.2.4.5.- Brief description of the autophagy machinery

The complete process of autophagy is described in **figure 1.3**: Autophagy is negatively regulated by the TOR kinase complex, and during stress conditions SnRK1 or ROS inactivates TOR (**1**), leading to autophagy activation with the association of ATG1, ATG13, ATG11, and ATG101 (**2**). The autophagosome formation comprises membrane delivery, vesicle nucleation, and phagophore expansion and closure. ATG9, ATG2, and ATG18 are involved in the delivery of lipids to the expanding phagophore. Simultaneously, the *ATG12-ATG5* conjugation and the *ATG8-PE* lipidation systems are essential for autophagosome expansion (**3**). After the autophagosome closure, *ATG8-PE* and PI3P interact with *FYCO1* and *FYVE*, which promotes the autophagosome movement to the vacuole. Multiple protein complexes are involved during the autophagosome fusion with the tonoplast, including

SNAREs and *SH3P2*. After the delivery of the autophagic body, the hydrolase activity inside the vacuole degrades the cargo and produces small molecules and amino acids. These molecules are transported into the cytoplasm via the *ATG22* machinery. *ATG8* is deactivated and transported back into the cytoplasm with the *ATG4* activity (**4**).

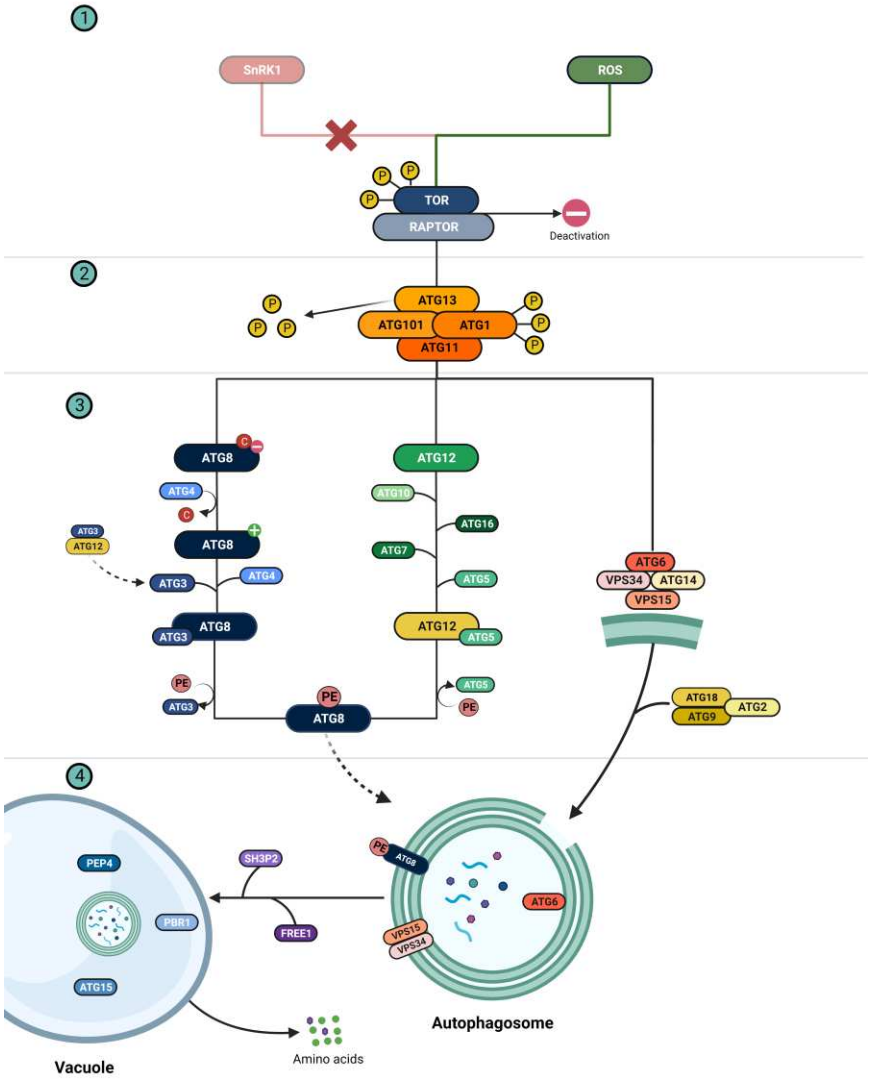


Figure 1.3.- The general autophagy process. Figure modified from Cadena-Ramos and De-la-Peña, 2024.

1.3.- SELECTIVE AUTOPHAGY AND STRESS

Autophagy, a fundamental process crucial for upholding cellular balance in plants, is preserved across species. It involves two primary pathways: selective and non-selective autophagy (Shaid *et al*., 2013). Selective autophagy maintains organelle and protein homeostasis under normal conditions. Nevertheless, when cells encounter stress, there is a noticeable increase in the activity of selective autophagy, specifically targeted at mitigating these stress factors (Shaid *et al*., 2013; Reggiori *et al*., 2012).

Within plants, selective autophagy hinges on the participation of ATG8 and its various isoforms. These proteins serve as docking sites on the autophagosome membrane, facilitating the selective recruitment of specific cargo (Cadena-Ramos and De-la-Peña, 2024). This process involves intricate interactions among selective autophagy receptors (SARs), ATG8, and the targeted cargo (Johansen and Lamark, 2020). Notably, unique autophagy receptors specific to plants have been identified, interacting with the autophagosome membrane via the ATG8-interaction motif (AIM) (Luo *et al*., 2021; Abdrakhmanov *et al*., 2020). Interestingly, most of these autophagy receptors are exclusive to plants, with only a few showing similarities to receptors in other organisms (Stephani *et al*., 2020; Michaeli *et al*., 2016). However, the ubiquitin-interacting motif (UIM) appears to be involved in specific receptors linked to proteasome degradation (Marshall *et al*., 2019). Among selective autophagy targets, proteophagy, reticulophagy, mitophagy, pexophagy, ribophagy, and chloroplagy are the most well-studied mechanisms described in plants (Cadena-Ramos and De-la-Peña, 2024).

1.3.1.- Proteaphagy

Selective degradation of proteasomes and misfolded/denatured proteins through autophagy is called proteaphagy. This process is important in preserving cellular balance and ensuring plant protein quality control. In response to constantly shifting environmental conditions, two pathways linked to protein degradation can be triggered: the ubiquitin-proteasome system (UPS) and selective autophagy mechanisms (Waite *et al*., 2022).

Ubiquitylation, a post-translational modification, stands as a pivotal mechanism for protein degradation within autophagy. This modification is primarily facilitated by UPS through E3 ubiquitin ligases that identify target proteins marked for degradation (Kirkin *et al*., 2009).

Ubiquitinated proteins are identified and broken down by the proteasome complex, which disassembles proteins and converts them into short peptides. These peptides are then further processed by peptidases, resulting in the general ion of amino acids that serve as essential sources of various metabolic pathways (Tomko and Hochstrasser, 2013; Hershko and Ciechanover, 1998). However, under conditions such as nitrogen starvation or exposure to protease inhibitors, ubiquitylated proteasome complexes accumulate and are recognized by the ubiquitin receptor RPN10, which directly interacts with ubiquitylated proteasomes and ATG8, leading to selective degradation of proteasome complexes (Marshall *et al*., 2015). Proteasome degradation through selective autophagy involves two pathways: ATG1-dependent and ATG1-independent, both of which require the core autophagy machinery to ensure effective protein degradation (Marshall *et al*., 2015).

Autophagy and the UPS system target distinct protein substrates. Proteasome complexes primarily degrade short-lived proteins and small molecules (Marshall *et al*., 2015; Glotzer *et al*., 1991), whereas autophagy is responsible for the degradation of long-lived cytosolic proteins, macromolecules, and large structures like organelles (Floyd *et al*., 2015). Despite these differences, both pathways utilize ubiquitination as a recognition signal, and they are interconnected systems capable of compensating each other's downregulation (Ji and Kwon, 2017; Nam *et al*., 2017).

The activity of autophagy is regulated by the negative regulator Target of Rapamycin Complex 1 (TORC1) under nutrient-rich conditions, whereby TORC1 phosphorylates ATG13 and ATG1 (Su *et al*., 2020). Interestingly, TORC1 also plays a role in activating the proteasome complex and was demonstrated in treatments with TORC1 inhibitors, which enhanced proteasome activity in humans and yeast (Zhang *et al*., 2013; Crespo and Hall, 2002). Activation of proteaphagy appears to take place during nutrient deprivation conditions, which leads to TORC1 inactivation by SnRK1 kinase (Waite *et al*., 2016; Marshall *et al*., 2015). It's plausible that alternative complexes could also trigger the proteasome and proteaphagy pathways. Prior studies have indicated that the accumulation of ROS can damage proteins and activate autophagy (Oikawa *et al*., 2022;

Shibata *et al*., 2013), which might then undergo ubiquitination and degradation via the UPS and proteaphagy through a potential TORC1-independent pathway (Cadena-Ramos and De-la-Peña, 2024).

1.3.2.- Reticulophagy

The endoplasmic reticulum (ER) is a crucial organelle in protein and lipid synthesis, quality control, and membrane production across eukaryotic organisms. Plants have developed specific regulatory mechanisms to uphold ER and cellular stability. Under normal conditions, the ER-associated degradation (ERAD) and the unfolded protein response (UPR) pathways act as quality control mechanisms (Molinari, 2021; Hetz, 2012; Vembar and Brodsky, 2008; Ellgaard and Helenius, 2003). However, during ER stress induced by the accumulation of unfolded or misfolded proteins, the UPR complex may fail to restore protein homeostasis (Howell, 2013; Bernales *et al*., 2006). In such scenarios, selective autophagy of the ER, termed reticulophagy (ER-phagy), is triggered. This process involves the sequestration and degradation of small ER fragments within the vacuole (Chen *et al*., 2020; Zeng *et al*., 2019; Yang *et al*., 2016). These mechanisms of ER homeostasis collaborate to protect the ER from stress-induced damage and ensure proper plant development and stress responses (Bao and Bassham, 2020; Yang *et al*., 2016). The endoplasmic reticulum have critical functions during autophagy by providing membranes for the autophagosome formation but also acts as a target for selective autophagy (Bao and Bassham, 2020). Reticulophagy can be triggered by heat stress or the application of exogenous ER stress inducers, leading to accumulation of misfolded proteins (Yang *et al*., 2016; Liu *et al*., 2012).

The initiation of ER-phagy relies on the presence of the ER stress sensor INOSITOL-REQUIRING ENZIME-1b (IRE1b) (Liu *et al*., 2012). Under normal conditions, the degradation of ER via autophagy is suppressed by REGULATED IRE1-DEPENDENT DECAY (RIDD). However, under ER stress, RIDD is inhibited, leading to autophagy activation (Bao *et al*., 2018). Golgi anti-apoptotic proteins, GAAP1 and GAAP3, contribute to cell survival by regulating IRE1 activity and influencing RIDD and autophagy responses (Zhu *et al*., 2019; Guo *et al*., 2018).

During specific stress conditions, different receptors engage with the autophagy machinery, potentially using distinct pathways for each adverse circumstance (Bao and Bassham, 2020). Moreover, there may exist interplay among receptors, where the absence of one receptor could be compensated by another (Cadena-Ramos and De-la-Peña, 2024). Further research is necessary to unveil the intricate details of reticulophagy and its significance in plant physiology and stress responses.

1.3.3.- Pexophagy

Peroxisomes are vital organelles in nearly all eukaryotic cells, crucial for cell functionality and maintenance (Pan *et al*., 2020). In plants, peroxisomes play pivotal roles in various metabolic pathways, including photorespiration, fatty acid oxidation, glycolate metabolism, hormone and cofactor biosynthesis, and polyamine catabolism (Kao *et al*., 2018; Rodríguez-Serrano *et al*., 2016). Additionally, peroxisomes generate and detoxify ROS and nitrogen-reactive species (RNS) as byproducts. Maintaining a proper balance between peroxisome production and recycling in imperative for plant survival, as an accumulation of peroxisomes can adversely affect cellular functions (Castillo *et al*., 2018; Mittler, 2017; Marinho *et al*., 2014). Pexophagy stands as a crucial process engaged in the degradation of excess or impaired peroxisomes, aiding in maintaining the delicate equilibrium between peroxisome turnover and production (Castillo *et al*., 2018; Mittler, 2017; Marinho *et al*., 2014). Ensuring metabolic homeostasis holds paramount importance for plant growth and development, specially under stressful conditions, where maintaining a proper balance between nutrient and energy sources is indispensable (Olmedilla and Sandalio, 2019). Pexophagy is the primary mode of selective autophagy to maintain metabolic homeostasis (Voitsekhovskaja *et al*., 2014; Yoshimoto *et al*., 2014).

While specific receptors for pexophagy in plants have yet to be reported, emerging candidates such as NBR1 show promise and may play a dual role in regulating both proteaphagy and pexophagy (Luong *et al*., 2022). Ubiquitination has been implicated in peroxisome degradation, as evidenced by the ubiquitination of 56 peroxisomal proteins associated with essential pathways related to photorespiration and ROS detoxification. (Akhter *et al*., 2023). Further investigations are essential to unravel the precise molecular mechanisms and regulatory pathways governing pexophagy in plants.

1.3.4.- Ribophagy

Ribosomes, the most abundant macromolecular cellular structures, are crucial in protein synthesis across all living organisms. However, it's imperative to maintain the appropriate protein quantity, particularly for adaptation during stress conditions (Warner, 1999). Ribosomal proteins are degraded before other cellular proteins under nutrient starvation conditions like nitrogen and amino acid starvation. This suggests their importance as the primary target of the ribophagy machinery, which involves ubiquitin proteases (Wyant *et al*., 2018; Kraft *et al*., 2008).

Although the exact mechanism of ribophagy in plants remains incomplete, previous studies have offered valuable insights into the process. It has been shown that ribosomal RNA (rRNA) degradation can occur through both macroautophagy and microautophagy pathways. Mutants lacking *ATG5* inhibit these pathways, while mutants lacking *ATG9* specifically impede the macroautophagy pathway of rRNA degradation (Floyd *et al*., 2015; Shin *et al*., 2014).

1.3.5.- Mitophagy

Mitochondria are indispensable organelles engaged in cellular respiration and energy production in eukaryotic cells, including plants (Nakamura and Izumi, 2021). They play a crucial role in cellular homeostasis by supplying ATP to various cellular processes, such as calcium signaling, cell growth, and cell death (Welchen *et al*., 2014; Osellame *et al*., 2012).

Maintaining mitochondrial health is crucial for plant growth and development, and the regulation of specific by-products, such as reactive oxygen species, is essential to prevent organelle malfunctions and stress conditions (Ashrafi and Schwarz, 2013; Murphy, 2009). Protein quality control (PQC) complexes play a significant role in regulating the degradation or repair of mitochondrial proteins to prevent the accumulation of free proteins associated with the respiratory chain (Ashrafi and Schwarz, 2013). Eukaryotic organisms employ three specific PQC mechanisms for degrading mitochondrial proteins: the mitochondrial unfolded protein response (UPRmt), the ubiquitin-proteasome system

(UPS), and selective autophagy, specifically mitophagy (Tran and Van Aken, 2020; Pickles *et al*., 2018; Bragoszewski *et al*., 2017; Quirós *et al*., 2016).

Mitophagy serves as a crucial mechanism for maintaining mitochondrial quality control by eliminating excessive, damaged, or nonfunctional mitochondria (Nguyen and Lazarou, 2021; Ashrafi and Schwarz, 2013). Two types of mitophagy have been described in yeast and mammals. The first type is ubiquitin-dependent mitophagy, where dysfunctional mitochondria are selectively ubiquitinated and recognized by the autophagosome for degradation within the vacuole. The second type is receptor-dependent mitophagy, where specific receptors on the outer mitochondrial membrane interact with the autophagosome membrane to facilitate mitochondrial degradation (Nakamura and Izumi, 2021; Nakamura and Otomo, *et al*., 2021).

Mitochondria and chloroplast fulfill dual roles in energy production and are closely associated with plant stress-response mechanisms. In situations where photosynthetic activity is reduced, such as when one leaf obstructs the light absorption of another, early senescence mechanisms are activated to recycle unused components from the leaf through autophagy (Ono *et al*., 2013; Wada *et al*., 2009). Early senescence and nitrogen starvation lead to a decrease in protein levels and the number of chloroplasts and mitochondria, which can serve as nitrogen sources (Izumi *et al*., 2015; Li *et al*., 2014; Wada *et al*., 2009).

During carbon or nitrogen starvation, small fragments or complete chloroplasts can be degraded, depending on the duration of darkness exposure. In the initial 2-3 days of darkness exposure, piecemeal chlorophagy is activated, targeting rubisco-containing bodies (RCB) and releasing free amino acids that can serve as alternative respiratory substrates for mitochondria. Prolonged exposure to darkness (over 6 days) triggers degradation of both whole chloroplasts and mitochondria (Hirota *et al*., 2018; Izumi *et al*., 2013; Wada *et al*., 2009; Keech *et al*., 2007). These findings unveil the sequential degradation of photosynthetic components during early darkness exposure, followed by the activation of complete chlorophagy and mitophagy pathways in the long term (Nakamura and Otomo, *et al*., 2021).

1.3.6.- Chlorophagy

Chloroplasts, essential organelles found in photosynthetic organisms such as plants and algae, play a pivotal role in capturing solar energy and facilitating the process of photosynthesis. Due to the intricate metabolic pathways involved, stringent regulation and quality control mechanisms are crucial for maintaining optimal chloroplast function (Cadena-Ramos and De-la-Peña, 2024). Additionally, in green tissues, chloroplasts serve as significant reservoirs for storing proteins, constituting approximately 50-75 % of the total protein and nitrogen contents in leaves (Ishida and Yoshimoto, 2008). Under growth demands and during stress conditions, chloroplasts can undergo degradation, serving as an alternative source of nutrients. While the breakdown of chloroplasts has been linked to prokaryotic proteasomes like the chloroplast proteasome complex (Clp) and the ubiquitin-proteasome system (UPS), selective autophagy, known as chlorophagy, has emerged as a crucial mechanism for chloroplast degradation in various stages of plant physiology (Wan and Ling, 2022).

Chlorophagy entails the selective degradation of specific components within chloroplasts, with one prominent target being ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), a critical enzyme involved in CO2 fixation during photosynthesis (Maheshwari *et al*., 2021; Parry *et al*., 2013). During senescence and under specific biotic and abiotic stress conditions such as high-light exposure, carbon starvation, darkness, and bacterial pathogens, RuBisCO is enclosed within autophagosomal membranes, forming Rubisco-containing bodies (RCBs) (Hanson and Hines, 2018; Dong and Chen, 2013). These RCBs are released through thin tubular extensions of the chloroplast called stromules or chloroplast projections, which are enveloped by the chloroplast membrane (Hanson and Conklin, 2020; Spitzer *et al*., 2015). The degradation of RCBs involves the transport of RuBisCO outside the chloroplasts through stromules, followed by an alternative autophagy pathway involving ATG8 and Charged Multivesicular Body Protein1 (CHMP1) for autophagosome maturation and transport into the vacuole (Spitzer *et al*., 2015). Interestingly, chlorophagy mediated by RCBs is implicated in regulating plastid morphology and division, and defects in CHMP1 have been shown to affect chloroplast morphology (Spitzer *et al*., 2015).

SSGL bodies, a different chlorophagy target, function as primary carbon reservoirs in photosynthetic organisms. Synthesized and stored within the chloroplast stroma during heightened photosynthetic activity, the undergo degradation at night. ATG proteins transports and break down of SSGL bodies through stromules, liberating sugars like glucose and maltose to serve as alternative energy sources during non-photosynthetic periods (Michaeli *et al., 2014*). The reliance on ATG proteins for SSGL body transport and degradation was evidenced through experiments conducted on *atg* mutants under dark conditions, where starch content notably increased, underscoring the pivotal role of autophagy in SSGL body breakdown (Malinova *et al*., 2018; Wang *et al*., 2013). Notably, the recognition patterns of SSGL bodies bear resemblance to those of RCBs, wherein starch molecules are similarly released through stromules (Wan and Ling, 2022). Furthermore, the presence of the marker granule-bound starch synthase I (GBSSI) serves to distinguish SSGL bodies, with its interaction with ATG8 observed in the autophagosomal membrane (Wang *et al*., 2013).

ATI1/2, also known as ATG8-interacting protein 1, functions as selective receptors pivotal in orchestrating the transit of chloroplast components to the vacuole, thereby facilitating the creation of ATI1-plastid-associated (ATI1-PS) bodies. These structures are discernible within both the ER and chloroplast membrane, where they interact with a cohort of 13 chloroplast proteins sourced from the stroma, thylakoids, or envelope (Michaeli *et al*., 2014). Importantly, the release of ATI1-PS bodies into the cytoplasm operates independently of ATG proteins but relies instead on direct binding with ATG8 for their subsequent conveyance into the vacuole (Michaeli *et al*., 2014).

Under typical circumstances, the full breakdown of chloroplasts is typically unnecessary (Evans *et al*., 2010). Nevertheless, nutrient deprivation, intense light exposure, UV treatment, and aging can trigger comprehensive chloroplast degradation. When chloroplasts become damaged or abnormal, they often manifest a swollen appearance and extensive ubiquitination, which signals autophagy activation (Woodson *et al*., 2015a).

It has been noted that the accumulation of reactive oxygen species (ROS) serves as the primary trigger for initiating the complete degradation pathway of chloroplasts via microautophagy, a process involving genes from the autophagy machinery such as ATG5, ATG7, and ATG8 (Zhuang and Jiang, 2019b). VIPP1, known as the vesicle-inducing

protein plastid1, plays a crucial role in regulating the repair of damaged chloroplast envelopes, mitigating chloroplast swelling, and facilitating membrane remodeling (Zhang *et al*., 2016, 2012). Strains lacking VIPP1 display heightened accumulation of swollen chloroplasts, whereas VIPP1 overexpression curtails chloroplast swelling and consequently suppresses the chlorophagy mechanism (Nakamura *et al*., 2018). Moreover, the ubiquitin-binding autophagy receptor NBR1, known for its involvement in peroxisome degradation, can identify and bind to photodamaged chloroplasts, leading to their degradation through a microautophagy-like pathway that operates independently of the conventional ATG machinery (Lee *et al*., 2023).

Multiple stress conditions have the potential to instigate either piecemeal or complete chlorophagy. Yet, the examination of selective autophagy receptors (SARs) remains at a nascent stage, necessitating further exploration and delineation. Unraveling the intricacies of the complete chlorophagy pathway poses a formidable challenge due to several factors. Firstly, not all chloroplast components undergo degradation simultaneously, and the specific pathway engaged is contingent upon the prevailing stress condition. Secondly, dissecting, and scrutinizing chloroplast dismantlement pathways in isolation proves intricate. Lastly, probing the chloroplast membrane system and its sub-compartments presents a formidable hurdle for researchers. Despite the hurdles associated with studying chloroplast turnover, considerable strides have been made in recent years, propelling us closer to comprehending this pivotal stress response mechanism (Cadena-Ramos and De-la-Peña, 2024)

1.4.- AUTOPHAGY AND GROWTH REGULATORS

Among eukaryotes, the autophagy-starting machinery is well preserved and is mainly triggered by nutritional and energetic changes or stress conditions, leading to SnRK1 and TOR interactions to activate autophagy, preserving the homeostasis between plant development and stress responses (Yang *et al*., 2023; Huang *et al*., 2019a; Jung *et al*., 2010).

Although the general autophagy mechanism is conserved, a great number of autophagy regulators are different between animals, yeasts, and plants (Rexin *et al*., 2015), suggesting that plants have developed plant-specific mechanisms to regulate autophagy. It

is well known that phytohormones are necessary for plant growth and development, also regulating stress responses (Liao *et al*., 2022), sharing these similar roles with autophagy, resulting in a strong relationship between autophagy and phytohormones. After phytohormone treatments, autophagy can be rapidly activated or suppressed to promote specific responses inside the plant (Rodriguez *et al*., 2020).

Abscisic acid (ABA) accumulates during stress conditions and is associated with stress responses inside the plant, regulating the turnover of critical proteins during stress conditions through the autophagy mechanism. The ABA presence can indirectly influence in post-translational modifications of ATG4 and ATG8 (Laureano-Marín *et al.*, 2020); salt and drought stress responses (Li *et al*., 2020; Zhang *et al*., 2015); and autophagy-associated seed germination (Honig *et al*., 2012), leading to autophagy activation and degradation of specific proteins involved in stress responses.

Brassinosteroids (BRs) are steroid phytohormones involved in cold stress responses through the interaction of ATG genes and BR transcription factors, including BRASSINAZOLE-RESISTANT1 (BZR1), leading to autophagy activation (Wang *et al*., 2019). However, BR can negatively regulate autophagy during specific conditions. Higher concentrations of BR decreased ATG gene expression levels by direct phosphorylation of ATG18 through BAK1 (Wang *et al.*, 2019), or indirect phosphorylation of ATG13a through Ser916 (Liao *et al*., 2023).

Auxins regulate several plant development processes, and its levels are affected by multiple stress conditions (Korver *et al*., 2018). The role of auxins during autophagy is regulated through TOR activation, leading to autophagy inhibition during nutrient deprivation, salt and osmotic stress (Pu *et al*., 2017). In the other hand, autophagy can modulate auxin metabolism, accumulation, and transport during root development (Liao *et al.*, 2022; Liu *et al.*, 2018).

Cytokinins (CK) and autophagy interactions have strong roles during leaf senescence, nutrient transport, root development, and stress responses (C. Liao *et al*., 2022). Autophagy seems to regulate CK content by degrading proteins involved in CK transport (Liao *et al*., 2022; Kurusu *et al*., 2017). Additionally, selective autophagy receptors can

target the type-A ARR proteins involved in negative regulation of CK signaling in *Arabidopsis* (Acheampong *et al*., 2020).

Ethylene (ET) can upregulate ATG4 and ATG8 expression during nutrient starvation (Okuda *et al*., 2011). The role of ethylene during drought stress is coordinated by the increasing of the mitochondrial alternative oxidase (AOX) and autophagy, where both seem to be important for ethylene-dependent drought responses (Liao *et al*., 2022; Zhu *et al*., 2018). The presence of ET triggers autophagy during pollination (Shibuya *et al*., 2013), ripening, and senescence of fruit and vegetables (Barry and Giovannoni, 2007). This upregulation seems to be a response to suppress ET activity during multiple processes stages in organ senescence (Liao *et al*., 2022).

1.5.- AUTOPHAGY INHIBITORS

Autophagy can be divided into three primary phases: autophagosome formation, autophagosome-vacuole fusion, and autophagic body degradation inside the vacuole (Yang *et al*., 2021). In recent years, various chemical inhibitors have been identified and utilized in various eukaryotic models to study autophagy (**Table 1.3**). Nevertheless, most of these inhibitors are not specific and can affect multiple cellular pathways (Pasquier, 2016).

Table 1.3. Main autophagy inhibitors.

Name	Function	Process associated
3-Methyladenine	PI 3-kinase inhibitor	Autophagosome formation
Wortmannin	PI 3-kinase inhibitor	Autophagosome formation
Verteporfin	PI 3-kinase inhibitor	Autophagosome formation
Cycloheximide	Protein synthesis inhibitor	Autophagosome formation
Konkanamycin A	Vacuolar H+-ATPase inhibitor	Autophagosome fusion
Bafilomycin A1	Vacuolar H+-ATPase inhibitor	Autophagosome fusion

1.5.1.- Autophagosome formation inhibitors

The early stages of autophagosome formation are associated with the phosphatidylinositol (PI) 3-kinase (3K) complex. This complex is crucial for recruiting autophagy machinery to specific membrane domains during autophagy progression. The PI3K inhibitor, 3-

methyladenine (3-MA), can block this recruitment pathway and prevent the formation of autophagosomes. Moreover, class III PI3K is required to activate autophagy and directly influences class I PI3K, which negatively regulates the process of autophagy (Chantranupong *et al*., 2015; Lee *et al*., 2010).

Previous research has verified that autophagy can be inhibited by 3-MA and wortmannin, which act by targeting class III PI3K (Yang *et al*., 2021). Both 3-MA and wortmannin affect both class I and class III PI3K complexes in both the short and long term. However, 3-MA's short-term effect inhibits starvation-induced autophagy, while its long-term effect under nutrient-sufficient conditions promotes the completion of the autophagy process. In contrast, wortmannin's short-term effect targets class I PI3K-induced autophagy, and its long-term effect affects class III PI3K. These findings suggest that wortmannin may be the preferred long-lasting PI3K inhibitor. In cellular experiments, a 20 mM concentration of 3-MA is dissolved into the medium and placed in a 37 °C incubator for 1 hour (Yang *et al*., 2021).

1.5.2.- Protein synthesis inhibitors

The primary role of cycloheximide is to impede protein synthesis in eukaryotic organisms (Yang *et al*., 2021). It works by directly interfering with the translocation steps of the protein synthesis pathway. Cycloheximide can act as a rapid and effective autophagy inhibitor in short-term experiments. However, once this compound is removed, autophagy inhibition is reversed (Lawrence and Brown, 1993).

1.5.3.- Autophagosome degradation inhibitors

Vesicular-type H+-ATPases (V-ATPase) are crucial for maintaining organelle function in lysosomes, vacuoles, inclusion bodies, and secretory vesicles. Konkanamycin A and bafilomycin A1 are specific inhibitors of V-ATPase, which alter the proton gradient of vesicles, resulting in an increase in the pH of acidic vesicles. This alteration prevents the fusion of autophagosomes with vacuoles/lysosomes, leading to the accumulation of autophagosomes in the cytoplasm (Izumi *et al*., 2017). In plants, bafilomycin A1 has been associated with Golgi swelling and an increase in apoptosis. Typically, a final concentration of 100 nM bafilomycin A1 is used, although lower concentrations may be

sufficient to inhibit autophagosome degradation. Treatment with bafilomycin A1 for 4 hours can completely block autophagy (Yang *et al*., 2021).

1.5.4.- Lysosomal/vacuolar inhibitors

The final phase following the fusion of autophagosomes with vacuoles or lysosomes is the degradation of the autophagic cargo. To initiate the degradation, the vacuoles or lysosomes need to be acidic, and the presence of several proteases is necessary (Nakamura *et al*., 1997). The resulting degradation products can be recycled by the cell. However, if the degradation process is obstructed inside the vacuole or lysosome, the autophagic cargo accumulates within these vesicles, resulting in the inhibition of autophagy (Yang *et al*., 2021).

The lysosomal/vacuolar lumen alkalizes can penetrate the vesicles, increasing the pH, inhibiting the vesicle fusion, and increasing the volume of the autophagosomes. Chloroquine, hydroxylated chloroquine, ammonium chloride, and cepharanthine (CEP), are the most common lysosomal lumen alkalizers used to inhibit degradation of the autophagic bodies in lysosomes. However, mostly of these inhibitors requires a high concentration to block autophagy (Yang *et al*., 2021).

Different lysosomal/vacuolar enzymes are involved in the degradation of the autophagic bodies, the cathepsins regulate the metabolic balance inside the vesicles by participating in autophagosome degradation. E64d can inhibits cathepsins B, H, and L, while pepstatin A is an inhibitor of cathepsins D and E, both inhibitors target directly lysosomal/vacuolar proteases (Moriyasu and Inoue, 2008). The presence of just one inhibitor cannot reduce the complete autophagosome degradation, for this reason is necessary to combine multiple inhibitors has been recommended (Yang *et al*., 2021).

1.5.5.- Other autophagy inhibitors

Previous studies demonstrated that exogenous mannitol could suppress the induction of chlorophagy. For instance, UV-damaged chloroplasts were cultured in Murashige and Skoog media with 0.33 M of mannitol. The results demonstrated that the addition of mannitol suppressed the induction of chlorophagy but was insufficient to repair envelope damage (Nakamura *et al*., 2018).

In contrast, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) negatively regulates autophagy in *A. thaliana* and tobacco. Specifically, GAPDH interacts directly with *ATG3*, thereby inhibiting its activity. However, under conditions of stress and ROS accumulation, GAPDH alleviates the inhibitory effect on *ATG3*, thereby activating autophagy (Han *et al*., 2015).

1.6.- THE *AGAVE* MODEL

The genus *Agave* includes more than 210 species distributed in the tropical and subtropical regions of the world, predominantly in dry and semiarid environments (Nava-Cruz *et al*., 2015; Tewari *et al*., 2014). Mexico is one of the greatest areas of *Agave* diversity and cultivation with more than 160 *Agave* species reported and distributed in almost all of Mexico states, except for Tabasco (Duarte-Aké *et al*., 2023; Figueredo-Urbina *et al*., 2021). Agaves can adapt to several environmental conditions (Pinos-Rodriguez *et al*., 2008), and have an important role in the right functioning of the systems where they grow, including soil erosion reduction, water infiltration, source of food for insects, reptiles, birds, and numerous benefits for human population (García Mendoza *et al*., 2019; Torres-García *et al*., 2019).

Agave species are characterized by their crassulacean acid metabolism (CAM), which implies the nocturnal CO_2 uptake and sugar production during the day to reduce the photorespiration and the loss of water, optimizing the plant adaptability to dry environments (Niechayev *et al*., 2019; Winter and Holtum, 2014). *Agave* plants show multiple features that allow them to survive in response to biotic and abiotic factors: morphological adaptations let the plant use the steam and leaf base as storage tissues; a unique flowering time that happens after approximately 12 years during the lifecycle of the plant; a particular root system that can transfix around 30 cm in the soil; and can reproduce through three different mechanisms (seeds, shoots from rhizomes, and seedlings from bulbils), where the main propagation mechanism is an asexual form throughout rhizomes (Us-Camas *et al*., 2017; Palomino *et al*., 2015, 2003).

In Mexico, *Agave* is used for multiple purposes, including food, beverages, forage, building, fibers, medicinal, ornamental, domestic, and pharmaceutical (**Table 1.4**). Tequila and mezcal, two of the most recognizable beverages in Mexico, are exported to the United

States, Germany, Spain, and Taiwan, the largest market for these beverages outside Mexico. The production of Agave-derived beverages like tequila and mezcal is estimated to increase by around 27.13 % and 7.99 % in the next decade, respectively (CRT, 2022; SIAP, 2021; and SADER, 2017).

Although the main usage of *Agave tequilana* and *angustifolia* is the production of fermented and distilled beverages, the discovery of bioactive compounds of *Agave angustifolia* species with potential antimicrobial, antifungal, antioxidant, anticancer, and anti-inflammatory activity have been considered by the pharmaceutical industry (López-Romero *et al*., 2018). Some *Agave* species are used during bioethanol production, which can be accomplished thanks to its CAM metabolism and high adaptability to dry environments, showing a significant biomass production in comparison with other bioethanol sources (Parascanu *et al*., 2021; Davis *et al*., 2011; Borland *et al*., 2009). In the food industry, *Agave* is used as a substitute for sugars and fats with numerous applications (Ortiz-Basurto *et al*., 2008).

Table 1.4.- Principal uses and products derived from *Agave.* Source: Centro de propagación de *Agave* del estado de Guanajuato, 2014.

Uses	Products	Part of the plant
Food	Sugar, stews candy, barbecue wrapping, mixiotes, white worms, red worms, pulque bread, and tortillas.	Stem, flowers, and leaves
Beverages	Aguamiel, honey, pulque, mezcal, tequila, sotol, bacanora, vinagre, syrup.	Stem and leaves
Agricole	Living fence, soil former, leader plant of ecosystems.	Entire plants
Forage	Bovine, caprine, porcine.	Leaves, flower scrapes, flowers, inflorescence
Medicinal	Anti-inflamatory and useful in cases of anemia.	Leaves honey and pulque
Domestic	Soap and detergents.	Leaves, stems, and roots stem.
Others	Drugs and steroidal products, ethanol production.	Leaves, roots, stems, and seeds leaves

Agave species have been considered important models to understand the adaptation mechanisms during biotic and abiotic stress (Us-Camas *et al*., 2017; Tamayo-Ordóñez *et al*., 2016). Despite the economic impact that *Agave* species have around the American continent, only a few initiatives for genetic improvement and transcriptomes analysis have been made. This lack of studies can be explained by the plant's morphology, long lifecycle, and polyploid genome, which can range from diploid (2n = 2x = 60) to octoploid (2n = 8x = 240) (Palomino *et al*., 2015, 2003; Robert *et al*., 2008). The study of the genus *Agave* is a promising topic with a high biotechnology potential over the coming years because of the need for new molecular understanding about how Agave plants modify its biochemical, genetic, or epigenetic makeup when exposed to numerous types of challenges (Nava-Cruz *et al*., 2015). Andrade-Marcial *et al*. (2024) published an important transcriptome analysis of the albino *Agave angustifolia* Haw, which will be useful over the next years, shedding light on the mechanisms at play in the emergence of these unusual plants.

1.6.1.- *Agave angustifolia* Haw.

Agave angustifolia Haw. is mainly used to produce mezcal beverages (Sánchez-Teyer *et al*., 2009). The polyploid level can vary from diploid to tetraploid (Sánchez-Teyer *et al*., 2009). *A. angustifolia's* lifecycle is between 20-25 years, reaching its reproductive stage around 7-8 years. It is a plant with morphological characteristics of succulence, forming a large rosette around 1.5 to 2 m tall and 1.5 to 2 m in diameter, with rigid leaves. The stem of the rosette is called "piña", which has an average fresh weight of 80kg (Ríos Ramírez *et al*., 2021; Cruz *et al*., 2013).

A. angustifolia can survive adverse environmental conditions, including dry, heat, high salinity, and low fertility, without limiting their growth and productivity (Andrade *et al*., 2007). The main propagation mechanism is through the rhizome, inflorescence bulbils, and plants obtained from plant tissue cultures (Arizaga and Ezcurra, 2002). The *Agave in vitro* culture requires the presence of essential nutrients, including N, P, K, Ca, Mg, S (macronutrients), and Fe, Cu, Mn, Zn, B, Mo (micronutrients) (Zuñiga *et al*., 2013).

The wild-type plants of *A. angustifolia* show a conserved genetic diversity compared to the cultivated plants. However, *A. angustifolia* plantlets cultured *in vitro* shown phenotypic changes, which is commonly known as somaclonal variation (SV) (Duarte-Aké *et al*., 2016;

Hernández-Castellano *et al*., 2020; Us-Camas *et al*., 2017; Sánchez-Teyer *et al*., 2009). SV has been associated with multiple factors during the *in vitro* culture of the explants, including environmental conditions (light, humidity, CO_2, and temperature); culture media components (carbon sources and growth regulators), and genetic or epigenetic changes (DNA methylation) (Duarte-Aké *et al*., 2016).

1.7.- JUSTIFICATION

Over the past decade, several research articles examining autophagy in plants have been published; these studies aimed to understand the expression of *ATGs* and how they interact with other proteins and pathways inside eukaryotic cells. The results of the vast number of investigations about autophagy have been significant in elucidating the function of *ATGs* during various stages of autophagy, including induction, recognition, phagophore formation, expansion, closure, and autophagosome fusion.

In prior studies conducted in our laboratory, *Agave angustifolia* Haw. exhibited elevated levels of reactive oxygen species (ROS), often linked to numerous stress conditions during *in vitro* culture. Particularly intriguing was the abundance of free amino acids in albino *A. angustifolia* plantlet, suggesting active autophagy processes within these albino cells. ROS likely catalyzes autophagy, facilitating the recycling of damaged organelles or proteins.

While autophagy has been studied in green plants, elucidating interactions between ATG machinery and protein complexes, its role in albino plants remains unexplored. Interestingly, the possible role of plant growth regulators in activating autophagy, presents a new direction for studying autophagy. Moreover, there is no reported scientific evidence about the specific role of autophagy in albino plants and its regulation of growth, development, and stress responses. The present thesis aims to fill this gap and serve as a foundational study for future autophagy research in albino models.

1.8.- RESEARCH QUESTIONS

I. What are the expression levels of autophagy-related genes (*ATGs)* in albino plantlets of *Agave angustifolia* Haw?

II. Are there differences in *ATGs* expression between green and albino plantlets of *A. angustifolia*?

III. What impact do autophagy inhibitor 3-MA have on albino plantlets?

IV. Does autophagy play a role in the survival of albino plantlets?

V. How do 2,4-D interact with autophagy?

1.9.- HYPOTHESIS

If the accumulation of immature/unused organelles or proteins activate autophagy, then the expression of autophagy genes will be upregulated in albino plants of *Agave angustifolia* Haw in response to degrading non-functional or immature organelles and proteins.

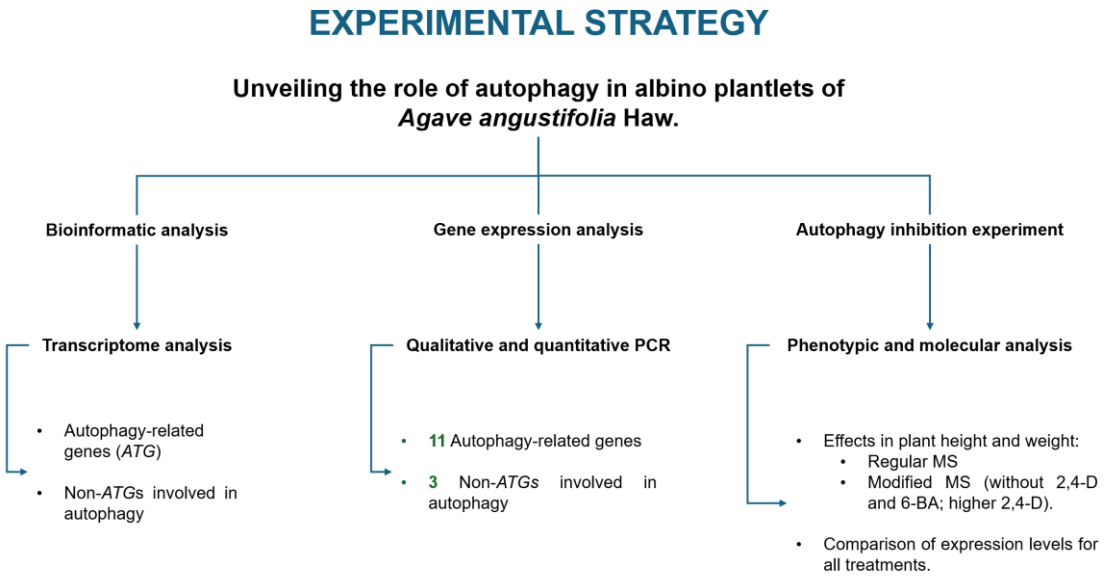
1.10.- GENERAL OBJECTIVES

Determine the role of autophagy in the growth, development, and survival of albino *Agave angustifolia* Haw.

SPECIFIC OBJECTIVES

- Compare gene expression levels associated with autophagy between albino and green plantlets.
- Evaluate the effects of the autophagy inhibitor 3-Methyladenine (3-MA) albino plantlets.
- Assess changes in autophagy-related genes (*ATG)* expression following exposure to 3-MA in albino plantlets.
- Evaluate the relationship between 2,4-D concentration and *ATG* gene expression.

1.11.- EXPERIMENTAL STRATEGY



CHAPTER II

2.1.- MATERIALS AND METHODS

2.1.1.- Plant cell culture

Agave angustifolia clone 26S cultures were maintained in a standard MS medium (Murashige and Skoog, 1962) at a pH 5.75. The medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.11 µM), 6-benzyladenine (BA; 22.2 µM) and gelled using agar (0.2 %) and gelrite (0.2 %) (Robert *et al*., 2006). Cultures started with 0.5 cm shoots, sub-cultivated every four weeks under the same conditions until reaching a 2.5 - 3 cm height. The culture was maintained under a photoperiod of 12 hours of light and 12 hours of darkness at 25 ± 2 °C.

2.1.2.- Autophagy inhibitors assay

Six treatments were evaluated on albino and green phenotypes of *A. angustifolia* over 28 days: **1)** Control: standard MS medium (pH 5.75) with 0.11 µM of 2,4-D and 22.2 µM of 6-BA; **2)** 3-MA treatment (5 mM) added to standard MS medium; **3)** Control without 2,4-D and 6-BA supplementation; **4)** 3-MA (5 mM) added to MS medium without 2,4-D and 6-BA; **5)** MS medium with 1.1 µM 2,4-D and 22.2 µM 6-BA; **6)** 3-MA (5 mM) added to MS medium supplemented with 1.1 µM of 2,4-D and 22.2 µM BA. All treatments were gelled using agar (0.2 %) and gelrite (0.2 %) (Robert *et al*., 2006), and maintained to photoperiod conditions of 12 hours of light and 12 hours of darkness at 25 ± 2 °C. Samples of the albino phenotype from all treatments were collected after 14 days (treatments 1 and 2) and after 28 days (treatments 3 to 6) for gene expression analysis through PCR. Treatments 3 to 6 were collected after 4 weeks to measure height and fresh weight to perform statistical analysis between phenotypes (albino and green). All samples were stored at -80 °C.

Weight and height measurements across all treatments within both phenotypes were assessed using 20 plantlets per treatment after 4 weeks. Statistical analysis employed R software (v 4.3.3) to conduct normality tests. Data conforming to a normal distribution (fresh weight) underwent student's t-test ($p \leq 0.05$), while non-parametric data (plant height) underwent Mann-Whitney U test.

2.1.3.- Bioinformatic analysis

The complete albino and green transcriptome of *A. angustifolia* (Andrade-Marcial *et al*., 2024) was analyzed to retrieve autophagy genes. Out of 270164 unigenes, 225 autophagy unigenes were identified. Repeated ATGs were filtered based on read count, gene length, and identity percentage per unigene. Differential expression (DE) analysis compared expression levels between green and albino leaf and meristem using Log2 fold change (>0.5). A heatmap was generated using the heatmapper platform (March 2023; www.heatmapper.ca) with Z score and clustering method of complete linkage and Euclidean distance measurement.

2.1.4.- Primer design

Primers for the autophagy genes associated with the activation, recognition of the cargoes, phagophore formation and closured, and autophagosome fusion and breakdown were designed from the *A. angustifolia* transcriptome. The bioinformatic tools used to design the primers were OligoCalc 3.27, OligoEvaluator and OligoAnalyzer. The sequences obtained are described in **Table 2.1**.

Table 2.1.- Primers designed for genes encoding proteins involved in autophagy regulation during multiple process steps, including autophagy activation, autophagosome formation, membrane transportation to the autophagosome, and autophagosome transportation into the vacuole.

Gene	Sequence (5'→3')	Size	TM (°C)
ATG1	*ATG1F:* AGCCTCTTGAAGGAGATCAC *ATG1R*: CGAGCTGCCTCATAAAATGC	193	58
ATG2	*ATG2F:* CTGAAGATGCACAACAAGGG *ATG2R:* GAAGCAGCAACAGGAGCAAT	185	58
ATG4	*ATG4F:* AAGTTGCTTGGGCACCGATTC *ATG4R:* GCACTTCATGAGGATCTAGG	204	58
ATG5	*ATG5F:* GCAGAACCAGAAAGACCATG *ATG5R:* CCCATTAATGACGTATGCTGC	141	58

ATG6	*ATG6F:* CCGGTAAACTTGATGAAGAGG *ATG6R:* GCCAAAATTGCATCCCTTTC	154	58
ATG8	*ATG8F:* CATCATTGCAGCAGTTGGTG *ATG8R:* CCCTGTGATTGTTGAGAAGG	187	58
ATG12	*ATG12F:* TAATTAACCACCAGCTTCCC *ATG12R:* GTCGCCAACTTCATCGAGAT	124	58
ATG13	*ATG13F:* TCGTCCTTTGTAGCTCCGTTC *ATG13R:* GATCATCGCCGGCATTAGAGA	165	59
ATG18	*ATG18F:* CCTGGACACTATAGATACCG *ATG18R:* CAGCTAGTGGTGAACGATGA	176	59
ATG101	*ATG101F:* GCAGATGCCTTGGAATGATG *ATG101R:* GTCAAGAACAAGCAGCAAACC	128	59
SH3P2	*SH3P2F:* GAACCTATTGAAAGCCCTGG *SH3P2R:* TGCCGTATCCTGTCATAACG	114	58
VPS15	*VPS15F:* TACCTGGTGACAACTTCCAC *VPS15R:* TACTAACGCTGTGGGATCTG	200	58
VPS34	*VPS34F:* CACCTATCCCCAAGATGTATG *VPS15R:* GCACCGGAGTATTGTGAACT	140	58

2.1.5.- Gene expression analysis by qualitative PCR and electrophoresis

To conduct gene expression analysis, total RNA was extracted from leaf and meristem tissues of albino and green plant phenotypes. Furthermore, RNA extraction was solely from albino phenotypes for each treatment in the autophagy inhibitor experiment. The TRI Reagent method was employed for RNA extraction following the manufacturer´s instructions (Sigma). RNA integrity was verified via agarose gel electrophoresis at 2.0 %, and its concentration was assessed with Nanodrop.

For cDNA synthesis, the single-stranded kit utilized an RNA concentration of 1000 ng to yield a final reaction volume of 20 µl. PCR reactions were made with 250 ng/µl of cDNA for each tissue, 2.5 µl of DreamTaq buffer, 0.5 µl of each primer (10 µM), 0.5 µl of dNTPs (10µM), 0.1 µl of DreamTaq polymerase, and 20.4 µl of nuclease-free water to obtain a total volume of 25 µl. The constitutive gene *18S* was used as a positive control. Qualitative analysis was made by agarose gel electrophoresis at 2.0 %, and densitometric measurements were made for each gene in all tissues.

For quantitative PCR analysis, four genes were selected based on their roles in the autophagy pathway: activation, membrane transport, autophagosome formation, and autophagosome transport. Additionally, the constitutive gene *18S* was used as a positive control. For RT-qPCR reactions, 0.3 µl (10 mM) of each primer, 12.5 µl of SYBR Green (Applied Biosystems), 0.05 µl of ROX solution, 1 µl of cDNA at 150 ng/µl, and 10.85 µl of nuclease-free water were used for each reaction. The amplification program consisted of Initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec with a Tm of 58 °C for 30 sec and 72 °C for 40 sec. Analysis of RT-qPCR reactions was performed using the StepOne Software v2.2.2-Real Time PCR system (Applied Biosystems).

CHAPTER III

3.1.- RESULTS

This thesis highlights *Agave angustifolia*'s complex autophagy mechanisms, revealing its vital role in cellular homeostasis and stress response. Autophagy, a conserved process in eukaryotes, degrades and recycles cellular components to maintain cellular integrity and function. This study investigates albino chloroplast. The study additionally explores how auxin 2,4-D affects albino tissue phenotypic changes, revealing its role in shoot and callus formation, and developmental responses. The autophagy inhibitor 3-Methyladenine (3-MA) was evaluated due to its inhibitory effects on PI3K family members, disrupting autophagy flux and altering gene expression patterns related to chloroplast autophagy and cellular maintenance**.**

3.1.1.- Autophagy inhibitors assay

Phenotypic changes were evident in treatments lacking growth regulators (**Figure 3.1**). Treatment 3 in albino plantlets exhibited normal growth. However, treatment 4 showed accelerated senescence, increased leaf fragility, and loss of turgor pressure in albino plantlets. The most notable characteristic in green plantlets was root formation, whereas in treatment 4 plantlets displayed minimal root development and slight leaf apex pigmentation loss (**Figure 3.1**). Significant weight differences were observed in green plantlets (Student T, P= 0.04145), while albino plantlets showed no significant weight or height changes (**Figure 3.2**)

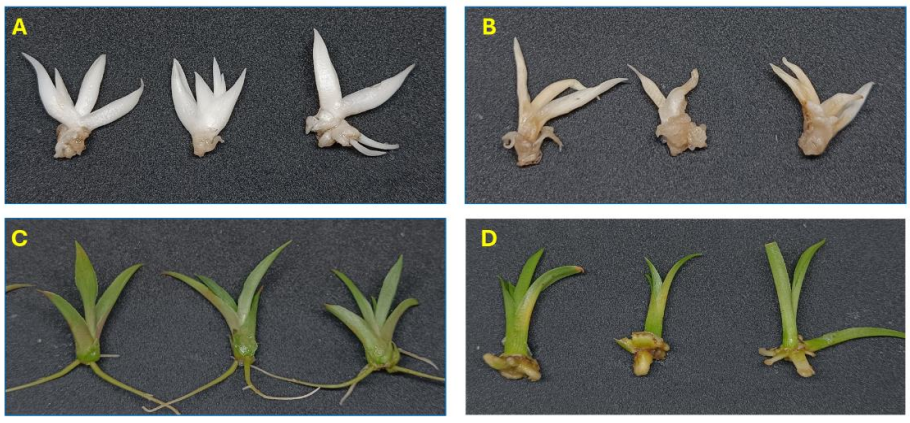


Figure 3.1.- Phenotypic variation observed in treatments without growth regulators. Control without 2,4-D and BA supplementation in albino (A) and green plants (C); Treatment with 3-MA and without 2,4-D and BA supplementation in albino (B) and green plants (D).

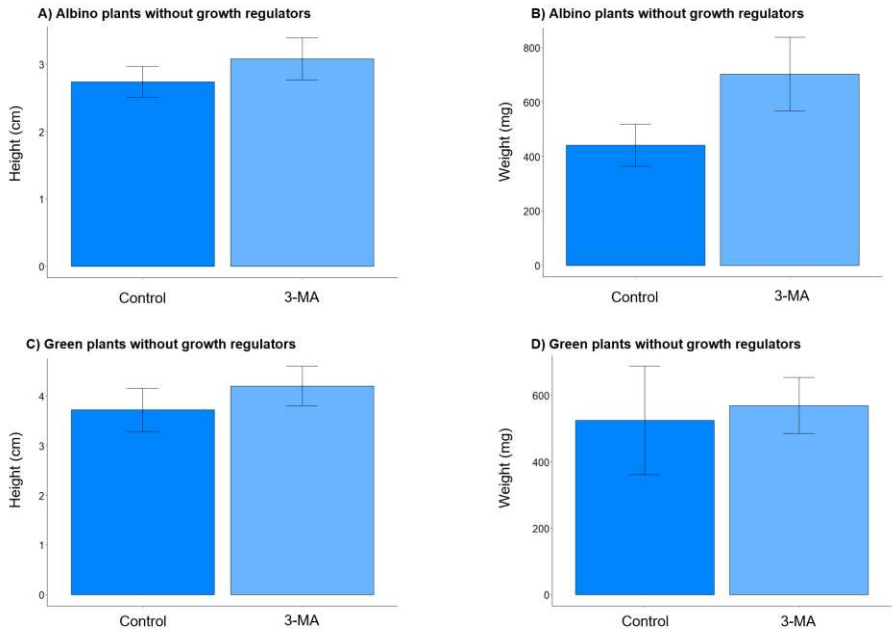


Figure 3.2.- Quantification of plant height and fresh weight for treatments 3 (control without 2,4-D and 6-BA supplementation) and 4 (3-MA added to MS medium without 2,4-D and 6-BA) in albino and green plantlets after 28 days. A) Average fresh weight between control and 3-MA treatments in albino plantlets; B) average plant height between control and 3-MA treatments in albino plantlets; C) average fresh weight between control and 3-MA treatmentes in green plantlets; D) average plant height between control and 3-MA treatments in green plantlets.

Treatments 5 and 6 exhibited intriguing differences as well. Treatment 5 in albino plantlets induced callus formation, whereas treatment 6 did not, showing slight senescence signs and turgor pressure loss instead. For green plants, treatment 5 led to a high proliferation of callus, while treatment 6 showed lower callus formation rates than treatment 5 (**Figure 3.3**). Statistical analysis indicated no significant differences in plantlet weight or height for both treatments in both green and albino phenotypes (**Figure 3.4**).

Figure 3.3.- Phenotypic changes observed in treatments with elevated 2,4-D concentrations. Control with 1.1 µM 2,4-D concentration in albino (A) and green plants (C); Treatment 3-MA supplemented with higher 1.1 µM 2,4-D in albino (B) and green plants (D).

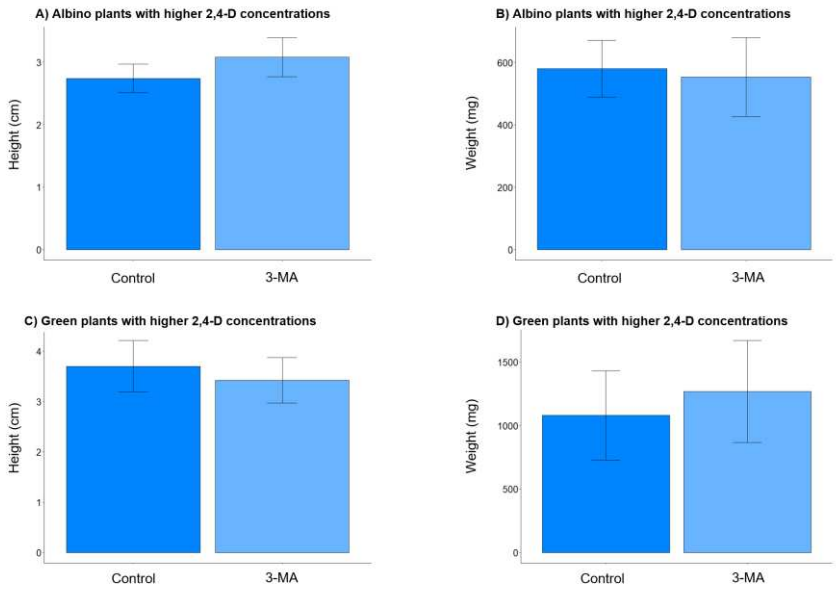


Figure 3.4.- Quantification of plant height and fresh weight for treatments 5 (MS medium with 1.1 µM 2,4-D and 22.2 µM 6-BA), and 6 (3-MA (5 mM) added to MS medium supplemented with 1.1 µM of 2,4-D and 22.2 µM BA) in albino and green plantlets after 28 days. A) Average fresh weight between control and 3-MA treatments in albino plantlets; B) average plant height between control and 3-MA treatments in albino plantlets; C) average fresh weight between control and 3-MA treatmentes in green plantlets; D) average plant height between control and 3-MA treatments in green plantlets.

3.1.2.- Bioinformatic analysis

Understanding the complex network of genes involved in autophagy is essential for unraveling the molecular mechanisms driving this process. Bioinformatic analysis is an important tool in predicting the functions of autophagy genes, providing valuable insights into the regulation of autophagy across different *Agave* phenotypes.

Out of a total of 225 unigenes found associated with autophagy, we identified 25 unigenes that met the filtering criteria based on read count, gene length and identify percentage. Specifically, 17 ATGs were found based on reported sequences of autophagy genes in *Arabidopsis thaliana* and *Asparagus officinalis*: *ATG1, ATG2, ATG3, ATG4, ATG5, ATG6, ATG7, ATG8, ATG9, ATG10, ATG11, ATG12, ATG13, ATG14, ATG16, ATG18,* and *ATG101*. Additionally, eight non-ATGs, such as *SH3P2, RAPTOR, SNRK, VPS15, VPS34, FREE1, NBR1* and *ATI1*, were found.

Differential expression analysis revealed notable findings: only four autophagy genes (16 %) showed higher expression in both green and albino meristem (*ATG1, ATG2, ATG101, FREE1*), whereas 14 autophagy genes (56 %) were overexpressed in the albino leaf (*ATG7, ATG8, ATG9, ATG12, ATG13, ATG14, ATG16, SH3P2, NBR1*) and meristem (*ATG3, ATG4, ATG5, ATG11,* and *ATI1*). The remaining 7 autophagy genes (28 %) showed higher expression in the green phenotype (*ATG6, ATG8, ATG10, VPS15, VPS34, SNRK1, RAPTOR*). (**Figure 3.5**). Interestingly, core autophagy genes (*ATG1, ATG4, ATG12, ATG13, ATG18, ATG101, SH3P2* and *ATI1*), were found to be over-expressed in the albino plant, suggesting an upregulated autophagy mechanism compared to the green plant. Heatmap analysis based on Z score for each gene further confirmed higher expression levels of autophagy genes in AL, GM, and AM tissues, with comparatively lower expression in GL tissues (**Figure 3.5**). This indicates an overexpression of autophagy in these tissues, possibly driven by the need to degrade more organelles and damaged proteins to maintain optimal nutrient conditions in albino leaves. Additionally, the over-expression of sucrose non-fermenting-1-related (SnRK1), a key player in autophagy activation during nutrient-deficient conditions, was observed in green tissues.

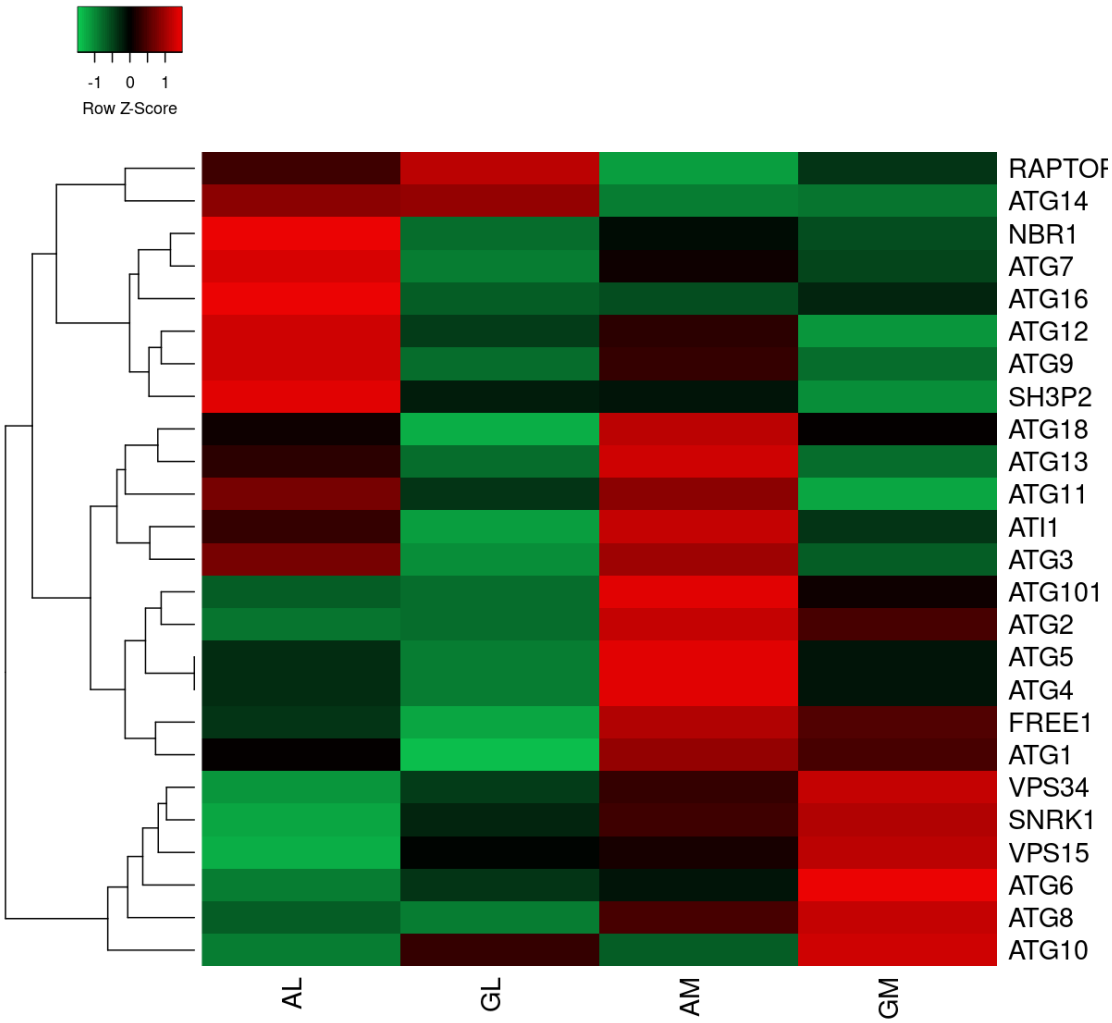


Figure 3.5.- Heatmap analysis of autophagy-associated genes in *Agave angustifolia* green and albino tissues. The graph was obtained by calculating the Z score. Albino leaves (AL), albino meristem (AM), green leaves (GL) and green meristem (GM).

3.1.3.- Gene expression analysis

The relative expression analysis of autophagy-associated genes was performed based on their roles in various stages of the autophagy process. Specifically, the focus was on genes involved in autophagy activation (*ATG1, ATG13, ATG101*), membrane transport (*ATG6, ATG18*), autophagosome modeling (*ATG6),* autophagosome formation (*ATG4,*

ATG8C, ATG8I), *ATG8* regulation (*ATG5, ATG12*), and autophagosome transportation (*SH3P2, VPS15, VPS34*). The results of this analysis are shown in **Figures 3.6** and **3.7.**

3.1.3.1.- Gene expression analysis in albino and green phenotypes

Activation of the autophagy machinery is regulated by *ATG1*, *ATG13* and *ATG101,* which are responsible for recruiting multiple *ATG* and non-*ATG* genes during autophagy. A higher expression level of *ATG101* was observed in albino tissues compared to green leaves. On the other hand, meristems showed differences in *ATG1* expression. Expression of *ATG13* remained consistent across both tissues analyzed. *ATG2, ATG6*, and *ATG18* are necessary for autophagosome formation and modeling, yet no significant differences were observed in their expression between albino and green leaves. However, higher expression of *ATG6 and ATG18* were observed in green meristems (**Figure 3.7**).

The ATG8-PE complex is the most important machinery for executing the autophagy process and consists of multiple gene components including *ATG4, ATG5, ATG8,* and *ATG12.* It was observed high expression levels of *ATG4,* and *ATG8* in the albino leaf tissues, with *ATG12* showing similar expression levels in both green and albino phenotypes (**Figure 3.7**). ATG8-PE pathway exhibited comparable expression in albino and green meristems, except for *ATG8,* which showed higher expression in albino meristems. Finally, autophagosome transportation is orchestrated by a group of non-ATG genes formed by *SH3P2*, *VPS15*, and *VPS34.* Notably, *SH3P2* showed higher expression levels in albino leaves, while expression levels remained consistent between albino and green meristems (**Figure 3.7**).

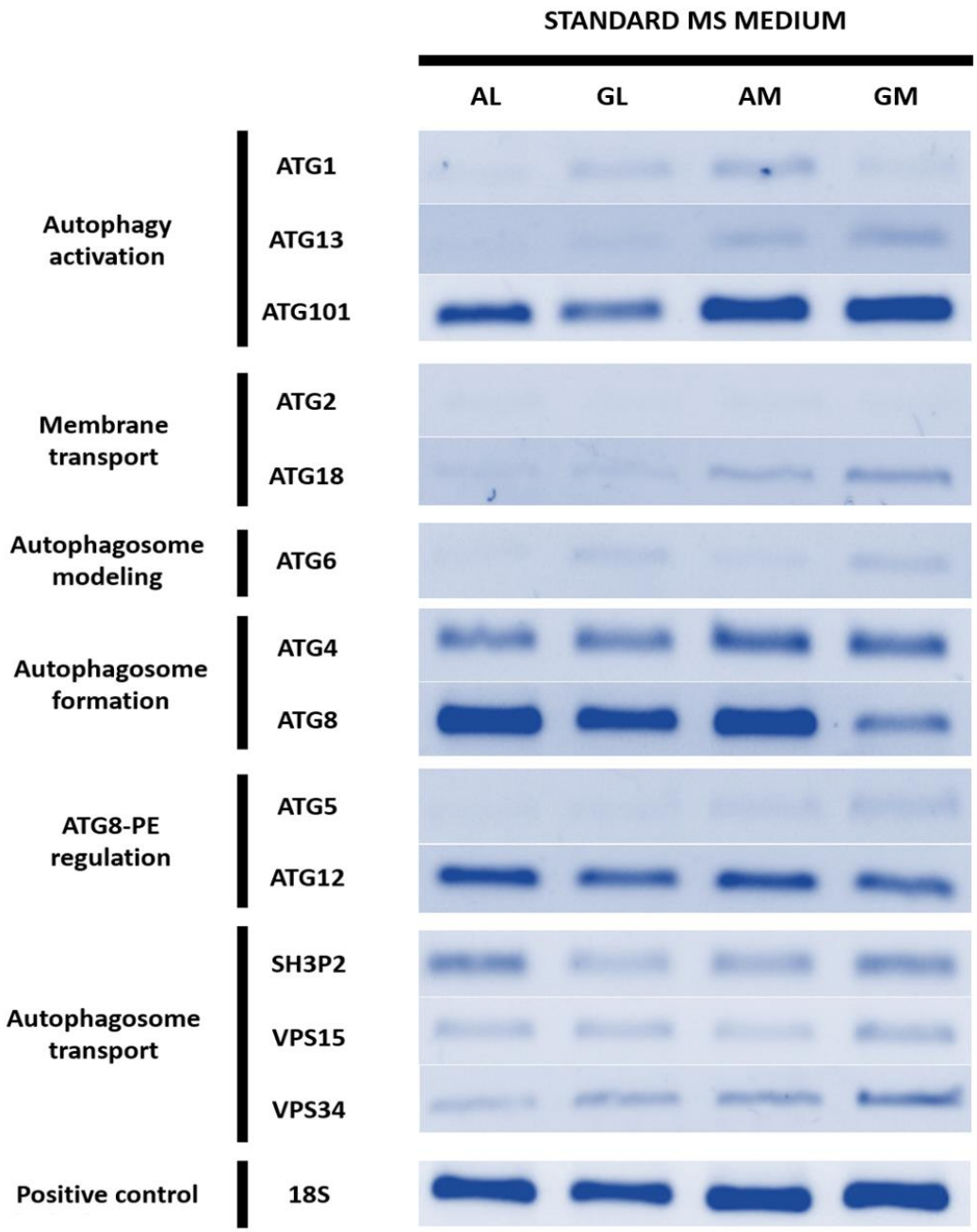


Figure 3.6.- Gene expression analysis of autophagy-associated genes in green and albino *Agave angustifolia* Haw. Albino leaf (AL), green leaf (GL), albino meristem (AM), and green meristem (GM), were analyzed.

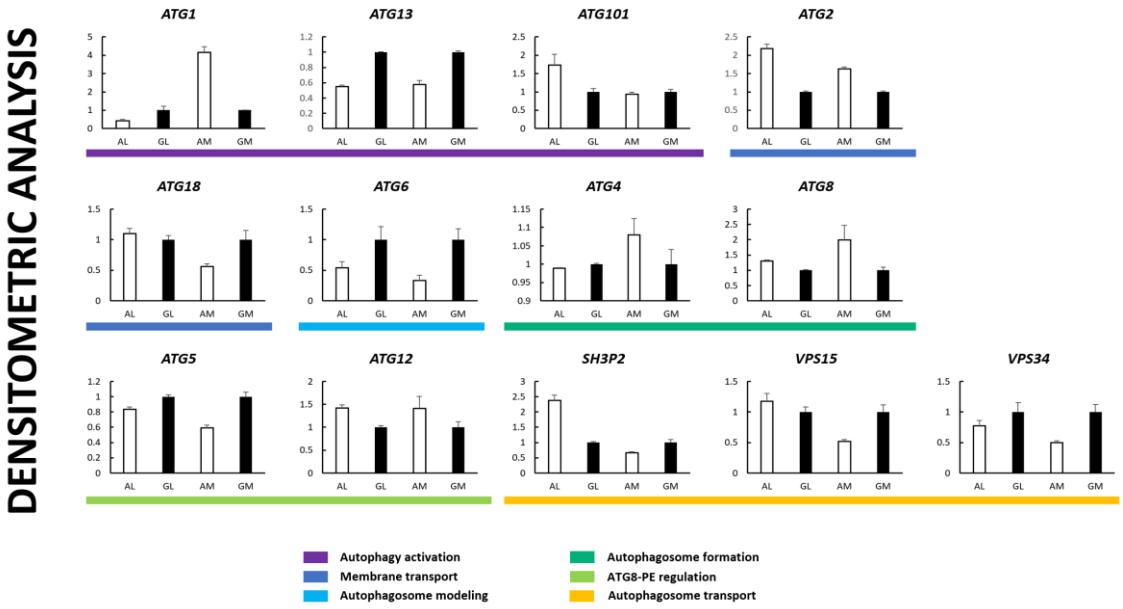


Figure 3.7.- Densitometric analysis of autophagy genes between albino and green tissues of *Agave angustifolia*. Expression levels were normalized using the GL tissue as the reference expression level. Albino leaf (AL), green leaf (GL), albino meristem (AM), and green meristem (GM). The graph was made using three qualitative PCR results to calculate standard error among replicates.

Results obtained from qualitative PCR analysis revealed that autophagy genes exhibit similar patterns between phenotypes, with genes associated with activation (*ATG1, ATG101*), membrane transport (*ATG2, ATG18*), ATG8-PE regulation (*ATG12*), and autophagosomes transport (*SH3P2*) showing over-expression in the albino phenotype.

Quantitative analysis of autophagy genes revealed higher expression of genes in green tissues, where only the *ATG8* gene showed an increase in its expression for the albino leaf. The *ATG13* and *VPS34* gene expression was higher in the green leaf, while *ATG18* remains equal for both phenotypes. Meristems exhibited the same expression in the *ATG8* and *ATG18* genes, while *ATG13* and *VPS34* expression was higher in green meristems (**Figure 3.8**).

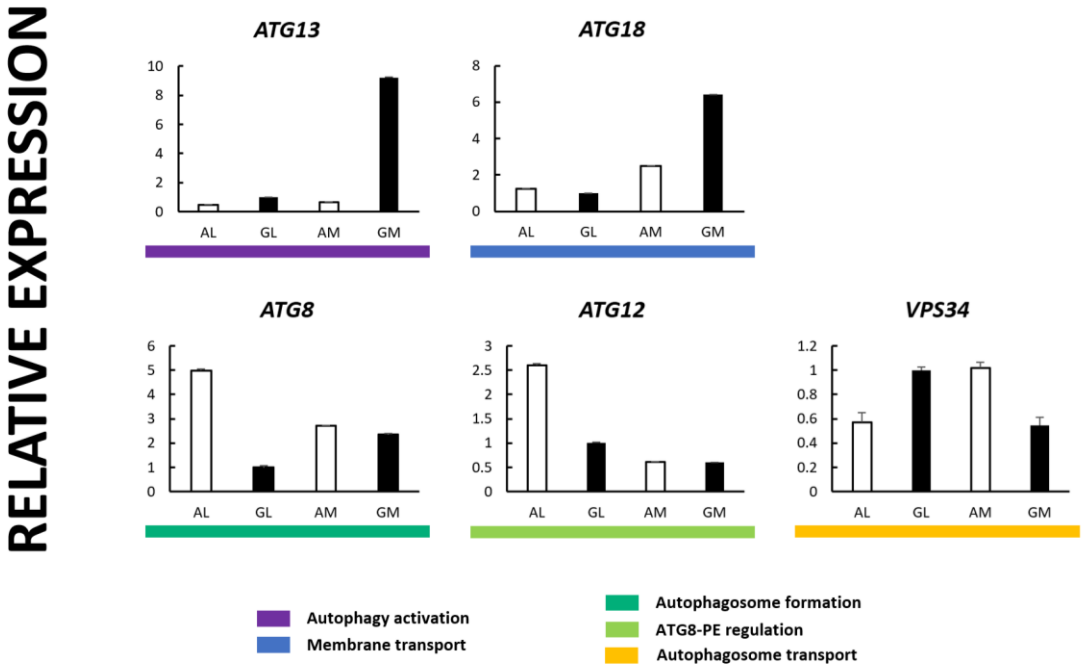


Figure 3.8.- Validation of five autophagy-related genes assessed in *Agave angustifolia* using qRT-PCR. Gene expression for each target was normalized by 2–ΔΔCT with GL tissue as the reference level. Albino leaf (AL), green leaf (GL), albino meristem (AM), and green meristem (GM). The graph was made using three quantitative qPCR replicates to calculate standard error.

3.1.3.2.- Gene expression analysis for autophagy inhibition assays

For treatments 1 and 2, expression of all autophagy genes decreased in MS medium supplemented with 3-MA in albino leaf. The pattern was very similar for meristems, with some exceptions of genes showing equal expression, including *ATG4, ATG5,* and *VPS34*. These results demonstrate the role of 3-MA during autophagy gene inhibition, with inhibition of the PI3K family (*VPS15, VPS34*) being its main target (**Figure 3.9** and **3.10**).

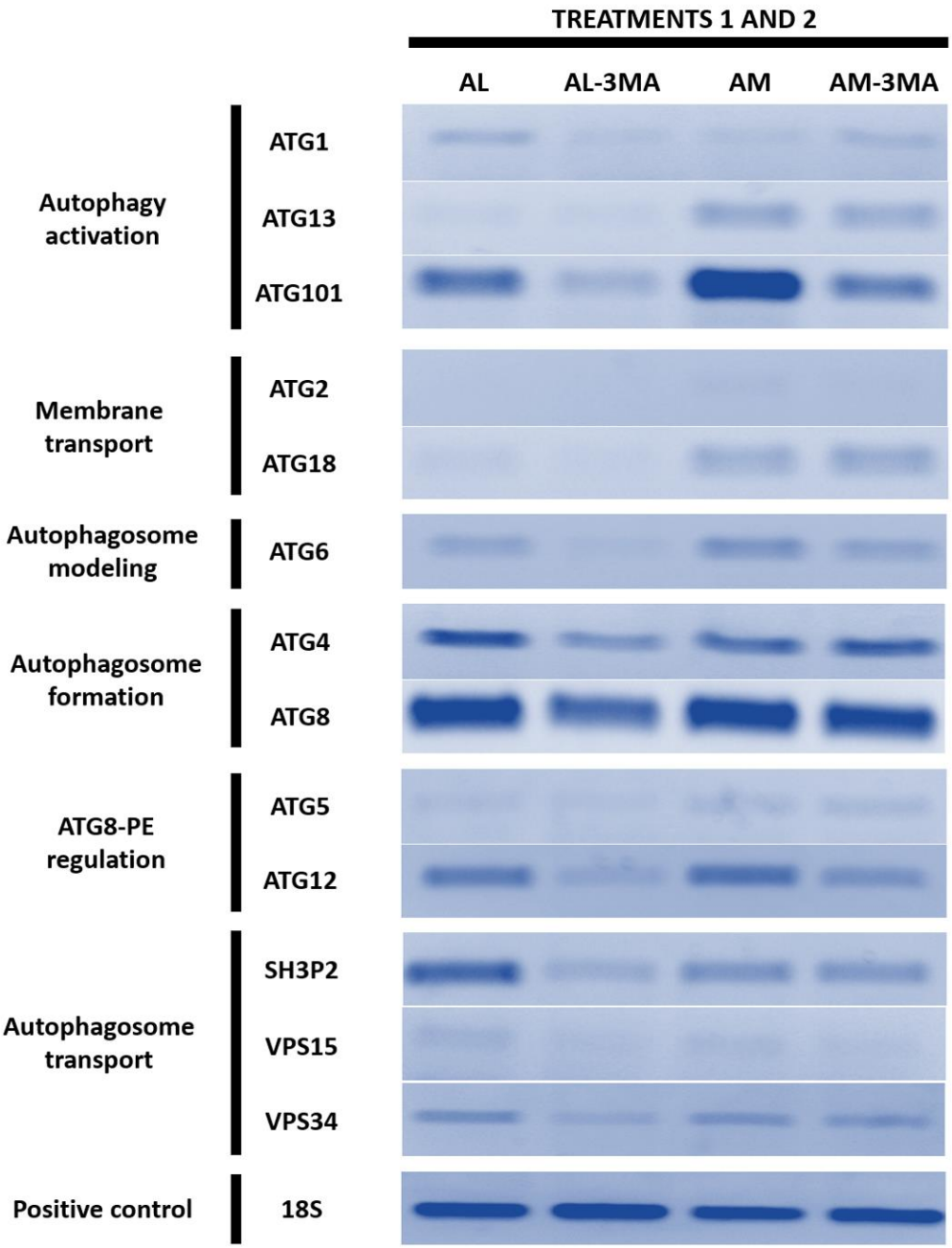


Figure 3.9.- Gene expression analysis of autophagy genes in treatments 1 and 2 of albino leaves. Treatment 1: standard MS medium (pH 5.75) with 0.11 µM of 2,4-D and 22.2 µM of 6-BA; Treatment 2: 3-MA treatment (5 mM) added to standard MS medium. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA).

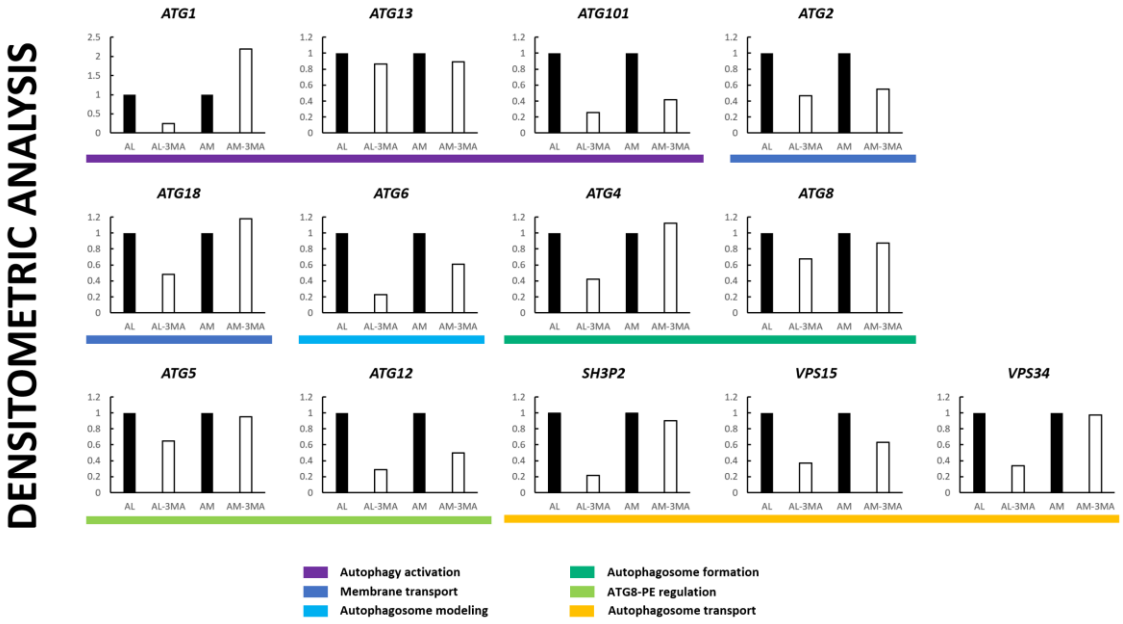


Figure 3.10.- Densitometric analysis of autophagy genes between albino leaves of *Agave angustifolia* in treatments 1 and 2. Expression levels were normalized using the AL tissue as the reference expression level. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA)**.** Densitometric analysis was made based on the results obtained in Figure 3.9.

For treatments 3 and 4, no changes were observed in the activation genes of autophagy. Still, alterations were noted in genes associated with membrane transport (*ATG2*), with a reduction in expression observed for the treatment with 3-MA. Additionally, one of the genes responsible for regulating ATG8-PE (*ATG5)* showed reduced expression levels, as did genes involved in transporting the autophagosome to the vacuole (*VPS15, VPS34*). Expression levels in the meristem showed an increase in activation genes (*ATG1*, *ATG13*), membrane transport (*ATG18*), modeling (*ATG6*), formation (*ATG4*), and autophagosome transport (*VPS15, VPS34*) for the treatment with 3-MA, revealing an increase in the expression of autophagy genes despite the presence of the inhibitor in the medium (**Figure 3.11** and **3.12**).

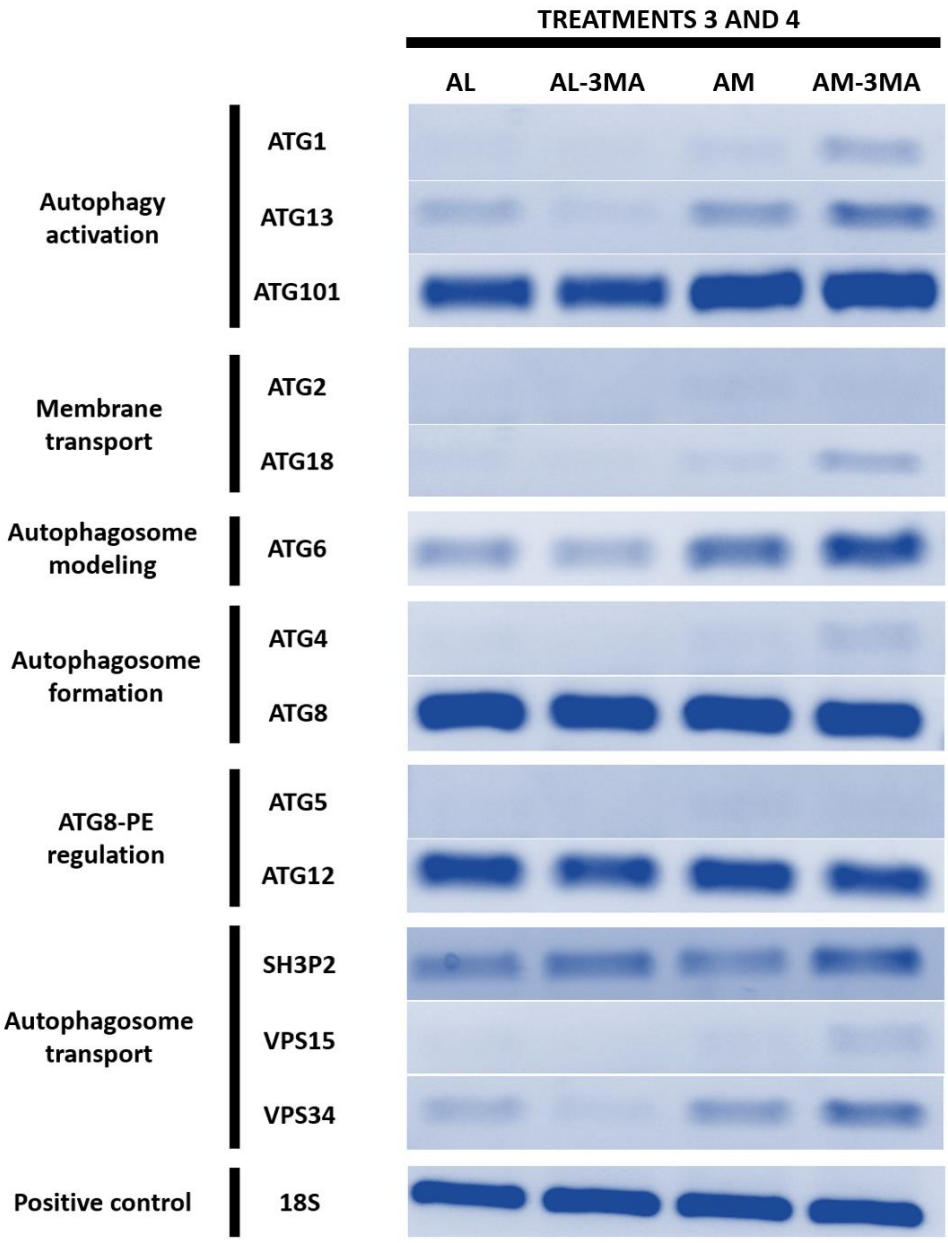


Figure 3.11.- Gene expression analysis of autophagy genes in treatments 3 and 4 of albino leaves. Treatment 3: standard MS medium without 2,4-D and 6-BA supplementation; Treatment 4: 3-MA treatment (5 mM) added to MS medium without 2,4-D and 6-BA. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA).

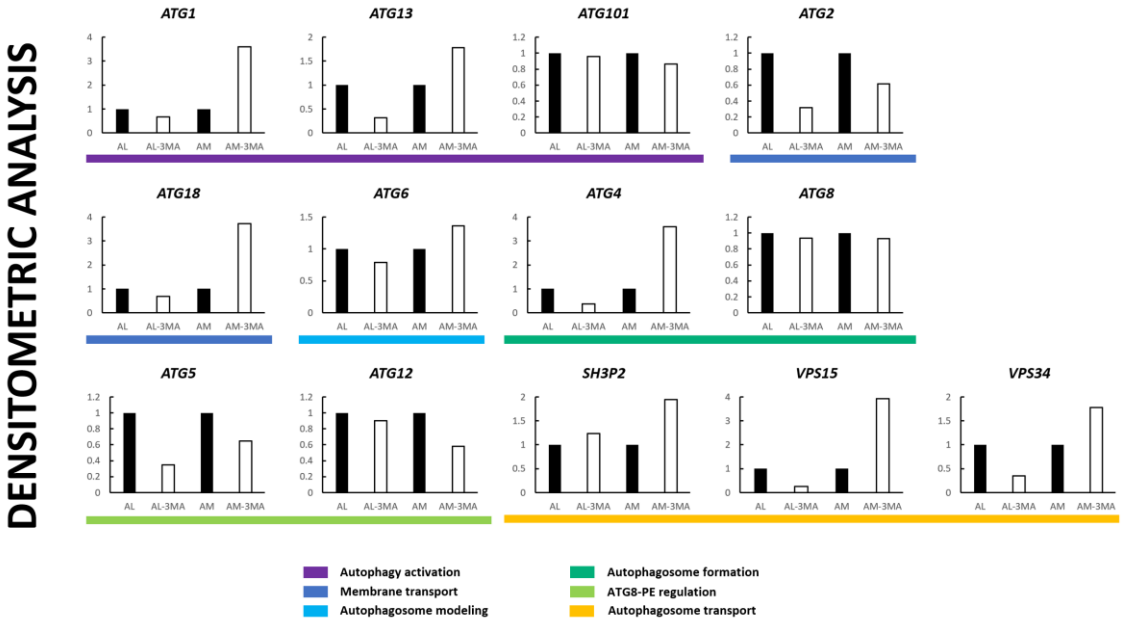


Figure 3.12.- Densitometric analysis of autophagy genes between albino leaves *of Agave angustifolia* in treatments 3 and 4. Expression levels were normalized using the AL tissue as the reference expression level. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA). Densitometric analysis was made based on the results obtained in Figure 3.11.

Treatments 5 and 6 yielded unexpected results. In treatment 5, the expression of almost all autophagy genes was reduced in the albino leaf compared to the treatment with 3-MA, except for the activation gene ATG101, which was upregulated in treatment 5. The results in the meristem were slightly similar to those observed in the leaves, showing an increase in expression for activation genes (ATG13, ATG101), as well as genes involved in membrane transport (ATG18), regulation of ATG8-PE (ATG5), and autophagosome transport (VPS15, VPS34) for the 3-MA treatment. Meristems exhibit the same behaviour with only *ATG2* and *ATG8* showing the same expression levels (**Figure 3.13** and **3.14**).

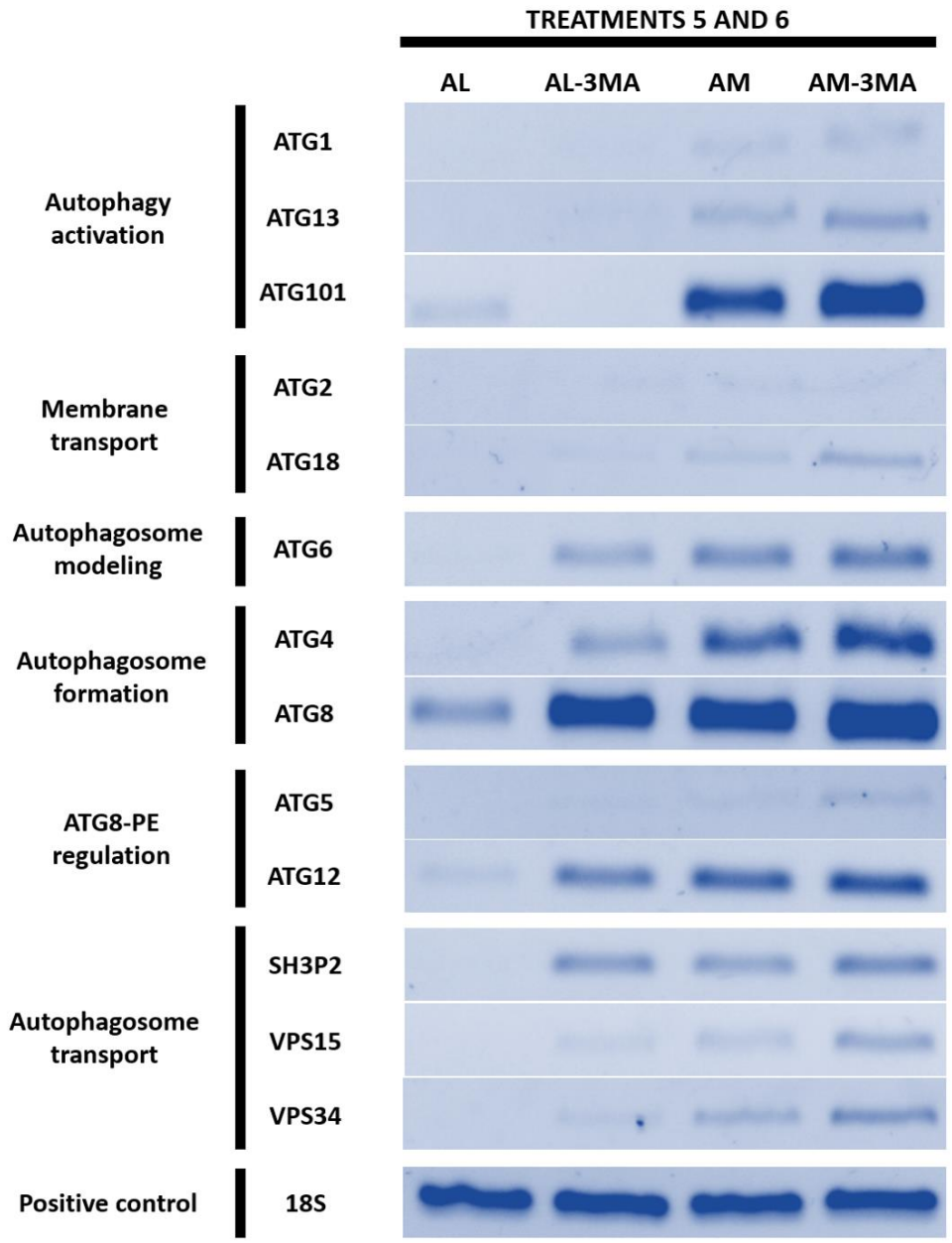


Figure 3.13.- Gene expression analysis of autophagy genes in treatments 5 and 6 of albino leaves. Treatment 5: standard MS medium with 1.1 µM 2,4-D and 22.2 µM 6-BA; Treatment 6: 3-MA treatment (5 mM) added to MS medium supplemented with 1.1 µM of 2,4-D and 22.2 µM BA. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA).

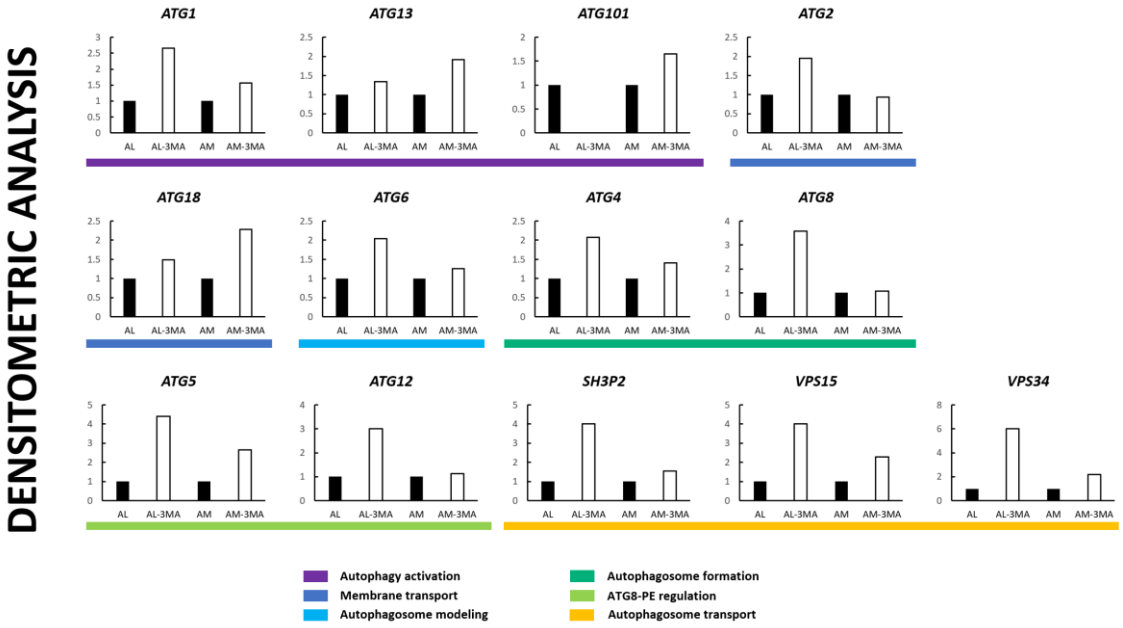


Figure 3.14.- Densitometric analysis of autophagy genes between albino leaves of *Agave angustifolia* in treatments 5 and 6. Expression levels were normalized using the AL tissue as the reference expression level. Albino leaf (AL), albino leaf collected from 3-MA treatment (AL-3MA), albino meristem (AM), albino meristem collected from 3-MA treatment (AM-3MA). Densitometric analysis was made based on the results obtained in Figure 3.13.

3.1.3.3.- Quantitative analysis for 3-MA treatments

The qPCR expression analysis revealed different expression patterns among treatments: for *ATG13*, higher expression was observed in treatment 1 compared to the remaining treatments. Overexpression of *ATG18* was observed in treatment 3. Treatment 4 exhibit higher expression of *ATG8*, and treatments 1 and 3 shown upregulation of *VPS34*. The most drastic results were observed in treatment 5 with lower expression of all genes analyzed by qPCR (**Figure 3.15**).

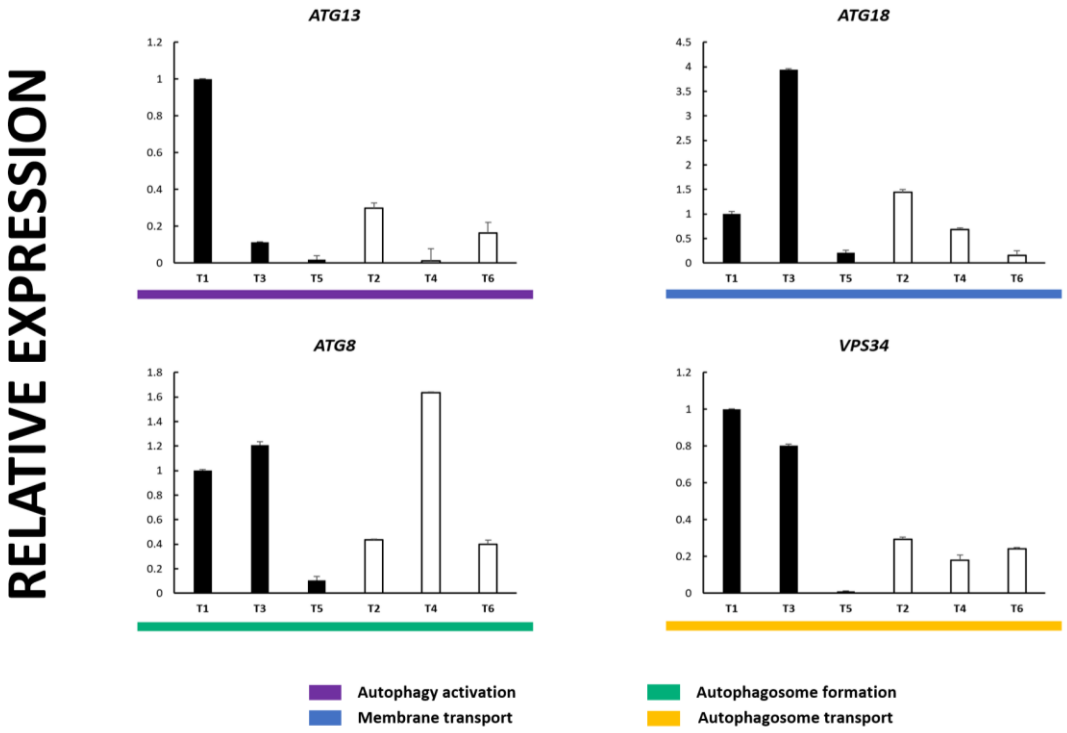


Figure 3.15.- Validation of four autophagy-related genes assessed in leaves of *Agave angustifolia* using qRT-PCR. Gene expression for each target was normalized by 2–ΔΔCT with AL tissue from treatment 1 as the reference level. Standard MS medium (T1), Standard MS medium + 3-MA (T2), Standar MS medium without 2,4-D and 6-BA (T3), Standard MS medium without 2,4-D and 6-BA + 3-MA (T4), Standard MS medium with 1.1 µM 2,4-D and 22.2 µM 6-BA (T5); 1.1 µM 2,4-D and 22.2 µM 6-BA + 3-MA (T6). The graph was made using three quantitative qPCR replicates to calculate standard error.

CHAPTER IV

4.1.- DISCUSSION

A bioinformatic analysis from the transcriptomic data and gene expression analysis was carried out on the albino and green phenotypes. The results of this analysis revealed that the expression of key genes involved in autophagy activation, autophagosome formation and transport, and genes involved in selective autophagy of chloroplasts was increased. The supplementation of albino plants with 3-MA was tested in order to gain an understanding of the effects of chemical inhibition of autophagy in the absence of growth regulators and in the presence of higher concentrations of 2,4-D. The findings led to the development of novel approaches regarding the specific role that autophagy plays during early senescence and the formation of callus. A discussion of the outcomes that were obtained from the experimental strategy is included in this section.

4.1.1.- Impact of autophagy inhibitor 3-MA on autophagy-related and PI3K family genes expression.

The phosphatidylinositol 3-kinase (PI3K) class I, II, and III, are a family of enzymes that play key roles in regulating the cell cycle, signaling, and development (Lee *et al*., 2010). PI3K activity is present in plants, animals, and yeast. However, plants and yeast only possess type III PI3K, also known as vacuolar protein sorting-associated protein 34 (VPS34) (Chantranupong *et al*., 2015). This enzyme forms the PI3K complex I (PI3KCI), which includes VPS34, VPS15, VPS30/ATG6/Beclin1, ATG14, and ATG38, and is crucial for autophagosome biogenesis (Qi *et al*., 2021; Nakatogawa, 2020). The PI3K complex II (PI3KCII), composed of VPS34, VPS15, VPS30/ATG6, and VPS38, is essential for ESCRT-mediated multivesicular body (MVB) formation, autophagosome-lysosome fusion, and the retromer complex function, which regulates retrograde trafficking from the endosome to the Golgi (Backer, 2016). VPS34 phosphorylates phosphoinositides at the third position of the inositol ring, producing phosphatidylinositol 3-phosphate (PI3P), which is crucial for endosomal trafficking (Wallroth and Haucke, 2018; Backer, 2008).

The autophagy inhibitor 3-MA is known for its ability to target and inhibit the phosphatidylinositol 3-kinases family class I and III (PI3K) through noncompetitive inhibition (Liu *et al*., 2017). 3-MA has been widely used in autophagy inhibition experiments to measure the responses against stress conditions without autophagy.

Plants exposed to 3-MA treatments exhibit morphological changes, as well as high susceptibility to stress conditions, and eventually cell death (Li *et al*., 2019; Takatsuka *et al*., 2004).

At the genetic level, it has been reported in mammals that 3-MA can inhibit the expression of genes such as 1A/1B-light chain 3 (*LC3*) and Beclin1 (*BECN1*), which are homologs of *ATG8* and *ATG6* in plants, respectively (Wang *et al*., 2022). However, besides the *ATG8* and *ATG6*, no other specific genes inhibited in plants by 3-MA have been reported. Our analyses conducted on treatments with inhibitors revealed the role of 3-MA in the inhibition of autophagy genes in the albino phenotype, showing the same expression pattern in treatments with regular MS medium and MS medium without growth regulators, where the control treatment exhibited higher gene expression of autophagy genes compared to the MS media supplemented with 3-MA (**Figure 3.10** and **3.12**). Reduction of autophagy gene expression could be explained by the interactions between PI3K and PAS (phagophore assembly site): PAS is a space in the cytoplasm where proteins involved in autophagosome formation are recruited, along with the addition of the ATG8-PE tag involved in autophagosome recognition and transport (Xu *et al*., 2011; Lee *et al*., 2008; Fujiki *et al*., 2007). Without the activity of PI3K, it is possible that autophagy-associated proteins may not be recruited, and the autophagosome may not be transported due to the inhibition of class III PI3K, which cannot produce lipid phosphatidylethanolamine (PE), inhibiting the formation of ATG8-PE conjugation (Zhao *et al*., 2022). The inhibition of PI3K could indirectly lead to reduce mRNA levels of autophagy genes due to cell inability to perform and conclude the autophagy pathway.

The effect of the inhibitor 3-MA on the PI3K family genes was confirmed through expression analyses, which showed a reduction in the genes *VPS15* and *VPS34* in most of the treatments (**Figures 3.9 and 3.11**). Additionally, previous research has indicated that 3-MA can inhibit the protein activity of VPS15, VPS34, and ATG8-PE, influencing protein concentrations and affecting plant growth and development (Li *et al*., 2019).

Results observed in treatments 3 and 4 (**Figure 3.12**) show that several autophagy genes are upregulated after 3-MA exposure. However, phenotypic evidence suggests that 3-MA exposure leads to accelerated senescence (**Figure 3.1**). It is possible that 3-MA inhibitory effect was active during the first weeks of exposure, inhibiting autophagy and leading to accelerated senescence. After prolonged exposure to autophagy inhibitors, 3-MA activity

might be decreased, resulting in activation of autophagy genes in response to early senescence processes taking place inside the cell (Avila-Ospina *et al*., 2014; Wu *et al*., 2010). Further studies are necessary to evaluate the transitory effects of 3-MA in *ATG* expression to evaluate when autophagy activity is reactivated.

4.1.2.- 3-MA and its role during callus formation.

The results obtained in treatment 5 (**Figure 3.3, 3.13**) shed light on the interactions between autophagy and phytohormones, specifically the synthetic auxin 2,4-D. Auxin presence can inhibit autophagy by triggering the TOR pathway, this results in TOR activation and, consequently, autophagy deactivation (Li *et al.*, 2020). However, another approach that still needs to be studied is the possibility of autophagy inhibition through auxin response factors (ARF), where ARF, specifically ARF1, ARF8, and ARF9 directly binds to *ATG8* promoter to regulate their expression. This inhibition mechanism is based on evidence that *cis* elements of ARFs can be found in the *ATG8* promoters in *Arabidopsis* (Wang *et al*., 2020). Previous evidence suggest that is conceivable that the external administration of elevated concentrations of 2,4-D may result in a dual suppression of autophagy, mediated by both the TOR pathway and ARFs binding to ATG promoters (Li *et al*., 2020; Wang *et al*., 2020). This proposed hypothesis could elucidate the observed downregulation of autophagy-related genes identified in the gene expression analysis conducted in our treatment with higher 2,4-D concentrations (treatment 5) (**Figure 3.13**).

The response of *A. angustifolia* to higher 2,4-D concentrations was previously explored (Cancino-García *et al*., 2020), where 0.5 µM of exogenous 2,4-D reduces *ARF15* and *ARF29* expression, two key *ARFs* associated with embryogenesis (Larrieu *et al*., 2022), leaf expansion and senescence (Li *et al*., 2023), and lateral root development (Marin *et al*., 2010). This downregulation of *ARFs* may be associated with callus formation (Cancino-García *et al*., 2020). Additionally, studies where plants were treated with 40 M of exogenous 2,4-D of *Arabidopsis* plantlets showed increased expression of *ARF3, ARF5, ARF7, ARF8,* and *ARF19* (Schuetz *et al*., 2019). For treatments 5 and 6 (**Figure 3.13**), synthetic auxin concentrations were 1.1 µM, two times higher than concentrations used in previous studies (Cancino-García *et al*., 2020). It is possible that, given the auxin concentrations, *ARF's* expression could be higher, and specifically, ARF8 might be interacting with ATG transcription factors, resulting in suppression of autophagy genes as observed in our results.

Although this ARF role during autophagy inhibition can provide an alternative route for autophagy and auxin interactions, further research is warranted to understand the interactions between ARF and ATGs. To date, only *ATG8* has been reported to be possibly inhibited by ARFs. Therefore, exploring which ATG genes are regulated by ARFs represents a topic of significant interest for further investigation.

One of the most interesting results was treatment 6 (**Figure 3.3**), where callus formation was inhibited even when the culture media conditions were supposed to promote this process. A lower expression of the *ATG* genes was expected due to the theoretical double inhibition condition present in the culture media supplemented with auxin and 3-MA. On one side, auxins suppress autophagy through TOR pathways. However, alternative routes (*ATG6*) that do not depend on the autophagy activation machinery can be triggered when TOR complex is activated, and autophagy is necessary: even with TOR activation, it was reported that *ATG6* can be phosphorylated, leading to an alternative autophagy activation pathway (Huang *et al*., 2019). With 3-MA presence, autophagosomes cannot be completed and transported into vacuoles, thus blocking the secondary mechanism of autophagy activation by *ATG6* previously mentioned (Petiot *et al*., 2000). Genetic analysis revealed a different pattern than expected, with higher expression of *ATG* genes in treatment 6 compared to treatment 5. This result, although unexpected, can be explained through the dual role of 3-MA. Under stress conditions (such as oxidative stress), 3-MA can inhibit autophagy (Wu *et al*., 2010). However, under nutrient-sufficient conditions (regular culture media with stable carbon sources), prolonged treatment of 3-MA is reported to promote autophagy flux (Wu *et al.*, 2010). In this way, the induction of autophagy genes following exposure to 3-MA could be attributed to the cessation of 3-MA's inhibitory effect. There have been no documented experiments involving exposure to 3-MA for longer than one week (Wu *et al*., 2010; Takatsuka *et al*., 2004). It is plausible that during the initial weeks of 3-MA exposure, the expression levels of *ATGs* were diminished. However, once 3-MA became inactive and in response to environmental cues, there was a significant upregulation in the expression of *ATGs*.

The findings from treatments 5 and 6 underscore the significant contribution of autophagy and its involvement in callus inhibition (**Figures 3.3 and 3.14**). Activation of autophagy impedes callus formation in plants subjected to callus-inducing treatments, whereas suppression of autophagy leads to an augmentation in callus formation. It was recently reported that autophagy-deficient plants exhibit higher callus formation in comparison to

wild type in *Arabidopsis* (Rodriguez *et al*., 2020). This reinforces our results and explains that lower expression levels of autophagy genes are directly linked with callus formation (**Figure 3.14**).

One intriguing question pertains to how cells of albino agave plants utilize the elevated concentration of 2,4-D present in the culture medium. One possible explanation could be attributed to endosomes, a collection of organelles that sort and delivery internalized material from the cell surface from the Golgi to the lysosome or vacuole (Contento and Bassham, 2012). Auxin carriers localized are reported to be delivered and recycled from the plasma membrane (PM) through endosomes (Michniewicz *et al*., 2007). PI3P is essential for endosomal trafficking and has been documented to be involved in endosomal recycling of PIN1 (Steinmann *et al*., 1999). PI3P's regulatory functions in endomembrane trafficking are primarily facilitated by PI3P effectors. These effectors bind to PI3P and, either directly or indirectly through protein interactions, fulfill various downstream roles, including cargo selection, membrane curvature generation, vesicle transportation, scaffold tethering, and signaling (Schink *et al*., 2013).

PI3P is documented to localize predominantly in late endosomes, whereas phosphatidylinositol 3,5-biphosphate (PI(3,5)P2), generated from PI3P, is typically detected in early endosomes (Armengot *et al*., 2016; Hirano *et al*., 2016). *Arabidopsis* plants lacking PI(3,5)P2 shown reduced auxin uptake (Hirano *et al*., 2016), this evidence could explain the phenotype observed in treatment 6 (**Figure 3.3**). Reports are indicating that 3-MA exhibits crosstalk with endocytic pathways (Raudenska *et al*., 2021). Following exposure to 3-MA, PI3K is inhibited, leading to a reduction in PI3P levels. (Wu *et al*., 2010), without PI3P is not possible to form PI(3,5)P2 which is key for early endosome formation (Hirano *et al*., 2016), disrupting the endosomal trafficking pathway of auxin influx and efflux carriers, resulting in cell inability to take up exogenous 2,4-D and reducing callus formation in our *Agave angustifolia* Haw model (**Figure 4.1**). The inhibition of PI3P synthesis could be confirmed by qPCR analysis where *VPS34*, a key element of PI3K complexes, shows lower expression levels in treatment 6 when we compare it with other treatments with 3-MA (2 and 4) (**Figure 3.15**).

Additional evidence about the role of PI3K is explained through VPS38, which functions as a subunit that interacts with VSP35 within the PI3K complex II. Mutants lacking *vps38* display a variety of abnormal phenotypes linked to deficiencies in vesicle trafficking and

membrane integrity, including enlarged endosomes and disrupted gravitropism resulting from irregular endosomal movement of the PIN family (Lee *et al*., 2018; Liu *et al*., 2018). For this evidence, it might be necessary to explore the expression levels of *VPS38* genes in our albino phenotype to determine whether this element of the PI3K is also disrupted after supplementation with 3-MA.

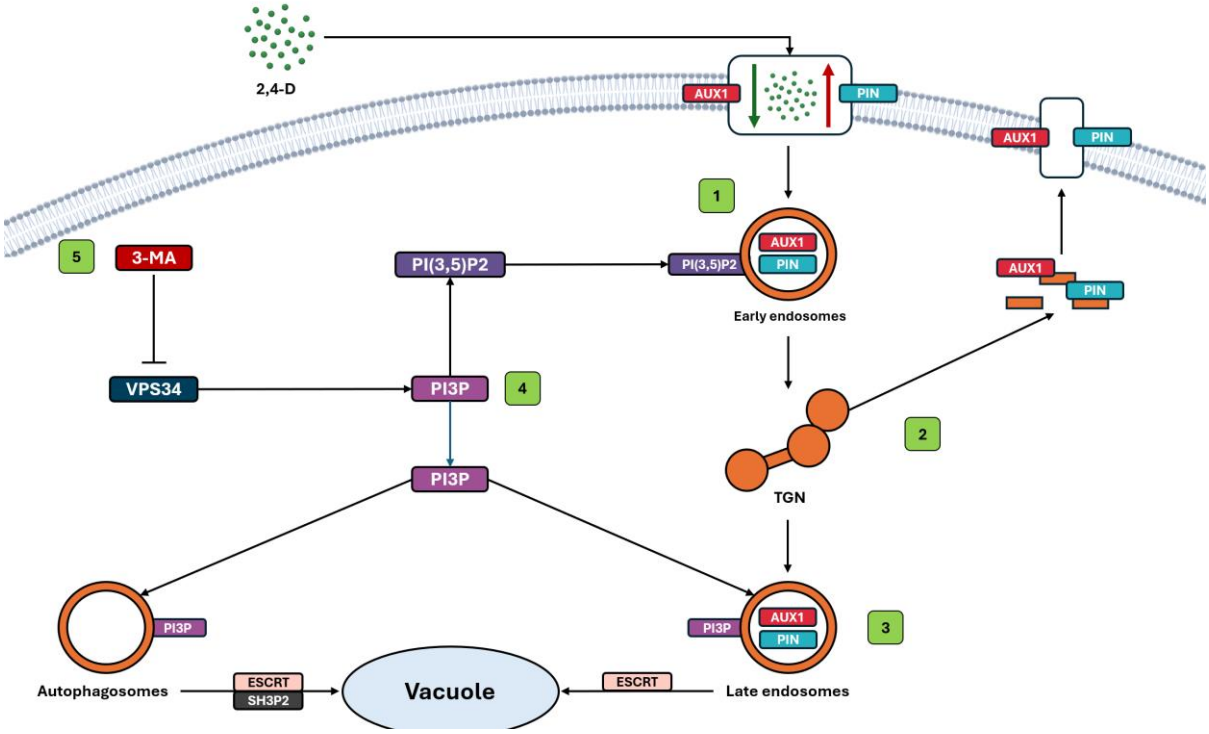


Figure 4.1.- Proposed scheme about the role of 3-MA in albino plants after exposure to the callus-inducer medium. **1)** 2,4-D is transported into the cell mainly by influx carriers, including AUX1/LAX, these carriers are delivered to and recycled from the plasma membrane via endosomes. Early endosomes are constituted of PI(3,5)P2, which is produced from PI3P by the enzymatic action of phosphatidylinositol 3-phosphate 5-kinase. **2)** Plant trans-Golgi network (TGN) decides the fate of early endosomes, which can either progress along the degradative pathway or be returned to the plasma membrane. **3)** Late endosomes are transported into vacuoles for degradation through ESCRT complexes. **4)** PIP3 is a lipid second messenger formed by PI3K, this second messenger is attached to autophagosome and late endosome membranes, interacting with cytoplasm transporters to redirect endosomes into vacuoles. **5)** Exposure to 3-MA leads to PI3K class III inhibition, blocking the production of PI3P and endosome/autophagosome formation, causing severely impaired inhibitions of auxin uptake.

4.1.3.- Transcriptome results revealed a higher accumulation of autophagy genes in the albino phenotype.

Autophagy is a fundamental process in eukaryotic cells responsible for transporting cytoplasmic material to vacuoles for degradation. This process has been recognized as a mechanism that promotes cell survival by eliminating damaged organelles and proteins under stress conditions, as well as its ability to participate in responses against pathogens (Wang *et al*., 2021; Shaid *et al*., 2013). During normal conditions, autophagy operates at basal levels to maintain cellular homeostasis and prevent damage caused by the accumulation of deteriorated cellular components (White *et al*., 2015).

The findings in this thesis reveal an upregulation of genes associated with autophagy in the albino phenotype (**Figure 3.5)**, reflecting increased expression across different stages of the process. Specifically, the overexpression of genes such as *ATG1, ATG13*, and *ATG101* suggests increased activation mechanisms in the albino plant, along with the regulation of *ATG18* involved in membrane transport to the autophagosome (Kotani *et al*., 2018). Autophagosome formation requires the involvement of *ATG4*, a key gene in the activation of *ATG8*, which, in turn, is essential for subsequent autophagy stages. The regulation of *ATG12*, crucial for the binding of ATG8-PE and transport of the autophagosome to the vacuole, is overexpressed in the albino phenotype. Additionally, the over-expression of *SH3P2*, which facilitates autophagosome transport to the vacuolar tonoplast through interaction with ATG8-PE, VPS15, and VPS34, indicates an overall upregulation of autophagy in the albino phenotype that includes all stages from activation to final transport.

Despite the evidence obtained in transcriptome results, one unknown remains in the air and that is knowing what type of autophagy is being carried out within the albino plant cells. However, upregulation of specific selective autophagy genes associated with chlorophagy, including ATI1 (ATG8-interacting protein 1) and NBR1 (Neighbor of Brca1), provide clues about possible chloroplasts degradation mechanisms through ATI1-PS bodies (Michaeli *et al*., 2014) or microautophagy (Yamamoto and Matsui, 2024), respectively.

4.1.4.- First insights into chlorophagy pathways

Chloroplast turnover is essential for plastid transition, nutrient recycling, and reactive oxygen species (ROS) production. Beyond breaking down damaged chloroplasts or those used as an energy source during stress, autophagy also targets immature chloroplasts, dismantling them to repurpose their components as nutrients (Norizuki *et al*., 2023). Given autophagy's role and its specificity for damaged or immature organelles, it is plausible that the immature chloroplasts reported in the albino phenotype are degraded through chlorophagy (Andrade-Marcial *et al*., 2024; Woodson *et al*., 2015b). It has been reported that specific autophagy genes participate during the microchlorophagy pathway, including *ATG8* (responsible for cargo recognition), *ATG5* and *ATG7* (which seems to participate in chloroplast enclosure with the vacuole) (Nakamura and Izumi, 2019). Additionally, the ATG-independent pathway orchestrated by the ubiquitin-binding NBR1 autophagy receptor can recognize and bind to damaged chloroplasts, resulting in chloroplast degradation through microautophagy pathways (Lee *et al*., 2023).

Gene expression analysis revealed that most of the ATG genes reported to be involved in microautophagy pathway are upregulated in the albino phenotype (**Figure 3.7** and **3.8**), with higher expression in *ATG2*, *ATG8*, and *ATG12*, suggesting a possible degradation pathway for immature chloroplasts. Furthermore, transcriptome analysis (**Figure 3.5**) revealed that *ATI1* and *NBR1* (key receptors associated with forming ATI1-PS bodies and ATG-independent chloroplast microautophagy pathways, respectively), are upregulated in the albino phenotype. Evidence gathered from our expression analyses and transcriptome results revealed possible chloroplast degradation routes that are ATG-dependent/independent. However, further analysis is necessary to evaluate the expression of NBR1 and ATI1 in PCR tests to confirm its role during autophagy in the albino phenotype.

CHAPTER V

CONCLUSIONS AND PERSPECTIVES

5.1.- CONCLUSIONS

- The albino phenotype exhibit differences in the expression of autophagy genes involved in autophagy activation (*ATG101*), membrane transport (*ATG2, ATG18*), ATG8-PE regulation (*ATG12*), and autophagosome transport (*SH3P2*) in comparison with the green phenotype.

- There is a possible chloroplast microautophagy mechanism being carried out in the albino phenotype, as the genes involved in chlorophagy are upregulated in *A. angustifolia* transcriptome (*NBR1* and *ATI1*), PCR (*ATG2, ATG5*), and qPCR (*ATG8* and *ATG12*) results.

- The expression levels of autophagy genes decreased in treatments of standard MS and standard MS without 2,4-D and 6-BA supplemented with 3-MA. However, 3-MA was reported to have a dual role during autophagy inhibition and activation, this statement was confirmed as treatment 6 with higher 2,4-D and supplemented with 3-MA exhibit higher expression of all autophagy genes analyzed in comparison with treatment 5 with higher 2,4-D.

- 3-MA supplementation could be responsible for callus inhibition through the blocking of PI3P production, which is involved in endosomal trafficking pathways associated with auxin carriers.

5.1.- PERSPECTIVES

- We recommend analyzing the expression of selective autophagy receptors to elucidate the specific type of autophagy occurring in the albino phenotype under normal conditions.
- Analyze the effect of a different autophagy inhibitor, such as wortmannin, under the same conditions explored in this thesis.
- Elucidate which autophagy genes interact with ARF during autophagy inhibition under 2,4-D supplementation.
- Employ detection of autophagy genes through western blot to confirm the inhibition of these proteins by 3-MA.
- Examinate autophagy activity through confocal microscopy analyses or transmission electron microscopy (TEM)
- Carry out a temporary course of the effect of 3-MA in a period of 28 days to understand when 3-MA activity is reduced.
- Identify changes in 2,4-D concentration after exposure to 3-MA.
- Analyze the epigenetics marks associated with autophagy and the effect after 3-MA exposure.

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