The Dependency of Stromules on Subcellular Structures and their Interactions in *Nicotiana tabacum*

Die Abhängigkeit der Stromuli von subzellulären Strukturen und deren Wechselwirkungen in *Nicotiana tabacum*

Final thesis to obtain the academic degree

Bachelor of Science

Botanical Institute

Department of Molecular Cell Biology

Faculty of Chemistry and Life Sciences

Karlsruhe Institute of Technology (KIT)

Submitted by

Fabio Mühlberg

August 30, 2021

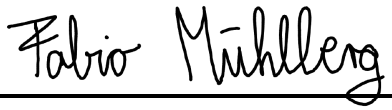
Examiner: Professor PhD Peter Nick

Co-Examiner: PhD Michael Riemann

Supervisor: M. Sc. Toranj Rahpeyma

Declaration of Authorship

I hereby certify that this thesis has been composed by me and is based on my own work, unless stated otherwise. No other person's work has been used without due acknowledgement in this thesis. All references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

Date: 30.08.2021 Signature: 

Fabio Mühlberg

Acknowledgments

I want to thank Peter Nick for the opportunity to compose my bachelor thesis at the botanical institute in the department of molecular cell biology. You were always very invested in everything going on in the lab, as well as quick to offer your help regarding any difficulties. You set the standard high as to how to think and write scientifically. My deepest respect goes to you and I will always value your professional opinion.

I want to thank Michael Riemann for undertaking the task of co-examiner for this thesis.

Next, to my supervisor, Toranj Rahpeyma, thank you so much for your continuous support. You are a genuinely great person to be around. I am sure that you will achieve great things in life. I wish you all the best in your continuing career.

I want to also thank Sabine Purper and Christian Metzger for helping me around the laboratory and answering my questions. To everybody else in the laboratory, the atmosphere and synergy you create are very inviting and open.

Lastly, my ever-supportive family and friends, I appreciate your consistent support throughout my studies and all the love you continue to give to me. I hope to make you proud.

Table of Contents

1 Introduction

To some extent the function of stromules and their relation to other subcellular structures is unknown. But stromules certainly play a key role in the stress response of plants. Stromules are defined as stroma-filled tubules found in angiosperm species. They extend from the surface of all kinds of plastids and can be observed in a wide range of different vascular and nonvascular plants. Stromules have been observed to form by inducing a various range of conditions, like for example salinity, light or drought stress. Nevertheless, a more gaugeable and efficient approach is to use other stromule-inducing substances like phytohormones (Sampath Kumar et al. 2014).

1.1 *Nicotiana tabacum*

The plant that is researched on in this thesis is *Nicotiana tabacum L.* or more commonly known as cultivated tobacco of the *Solanaceae* family. It is native to tropical and subtropical America and was brought to Europe in the 16th century. French botanist Jaques Dalechamps has first named it *Herba nicotiana*, which was also adopted by Lineé. Tobacco was originally used as a decorative plant, but later its multipurpose abilities have been discovered. Its leaf extract was used as biological pest control and later its alkaloid properties have been identified. Nowadays, the alkaloid named nicotine is a popular insecticide when extracted. When left in leaves and dried, it is also used to make smokable products like cigarettes (Oropeza 2005).

Its cultivar *Bright-Yellow-2* (*BY-2*) is a cell line that is highly homogenous and has a fast cell doubling time of 14 hours. It produces plant calli, which are unorganized parenchyma cells. These calli are initiated by plant cell regulators, such as auxin. For this reason, it is a model system for plant cell cycle and genetic engineering studies. With the use of stable transformation via *Agrobacterium tumefaciens*, the cell line is optimized for advancing in research. The *BY-2* cell line produces protoplasts that are a multifunctional tool that covers a variety of possibilities. In this work, for example, these protoplasts are perfect to study on stromule formation, because they emerge from any kind of plastids. By using protoplasts it is facilized to generalize the results to different plastid types and therefore to a variety of plants (Bhat and Thompson 2004).

1.2 Plastids

Plastids are the most characteristic attribute of plants compared to other eukaryotes. It is hypothesized that they have originated in the process of symbiogenesis between a procaryotic host cell and cyanobacteria. This process is called the endosymbiotic theory, it includes, inter alia, the origin of the mitochondria (Zimorski et al. 2014). The main characteristics of plastids are their double membrane and their separate circular DNA, which is not stored in the nucleus (Herrmann and Possingham 1980). Plastids perform metabolic and biosynthetic reactions, for example, fatty acid synthesis or carbon fixation. They are a partially autonomous organelle that has their own cell division, but because of some syntheses, which have been made reliant on the nucleus, they seem less independent as previously thought (Hoober 2012).

1.2.1 Movement

Chloroplasts dominate the research of plastid motility because they are easier to observe and to expose to stress conditions, like, for example, light stress. Nevertheless, there are also some non-green plastids studies. They have suggested that plastid movement is dependent on actin filaments (AF) in higher plants and on microtubules (MT) in lower plants. Angiosperm species have been observed to have their plastid motility to be exclusively actin-based (Wada et al. 2003). Mosses on the other hand depend on the combination of AF and MT (Sato et al. 2001). With the discovery of the CHUP1 gene, this was newly broad up and researched on. Plastids in *Arabidopsis thaliana* mutants that are missing CHUP1 accumulated at the bottom of the cell, which is the same plastid behavior as when treating the wild type with the anti-AF drug latrunculin B. A research showed that after immunolabeling with actin monoclonal antibodies, that plastids align with AF but not with MT (Kandasamy and Meagher 1999). On the other hand when MT were inhibited the plastid mortality increased, meaning MT prevent plastid movement (Serlin and Ferrell 1989).

1.3 Stromules

Stromules are stroma-filled tubular projections that form from the envelope membrane of plastids. They have been discovered more than 100 years ago by Gottlieb Haberlandt and Gustav Senn (Haberlandt 1888; Senn 1908). Their research was recently facilitated using fluorescent protein markers and live-cell imaging. They have been observed to interconnect two or more plastids possibly allowing transfer components through the stroma. Stromules differ in diameter and length. They have been observed to reach a diameter between 0.35 to 0.85 µm and a length up to 200 µm. Meaning there are estimated to transport molecules up to 560 kDa. Studies have shown that stromules are estimated to transport ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) but no macromolecules as such as proteins or DNA (Schattat et al. 2012).

Stromules constitute around 50 % of the plastids' surface (Sampath Kumar et al. 2014). This and other research have shown that two connecting plastids are not forming a new compartment, rather they just move their envelope membrane to each other. They used photoconvertible mEOS, of which its green fluorescence can be converted to red by using UV-light. It was observed that after converting the fluorescence of one plastid to red, the signal does not change in the plastid that is connected (Hanson and Sattarzadeh 2013).

The highly dynamic nature of stromules expresses itself in extending, retracting, bending and branching (Gray et al. 2012). All these movements were discovered to be the result of the actin cytoskeleton, which is powered by the activity of myosin (Sattarzadeh et al. 2009). Stromule regions have been shown to have high cytoplasmic streaming activities that change frequently in direction (Gunning 2005). Regarding the plastid size and density, stromules form accordingly. If there are many plastids clustered, stromules form in shorter versions, and if plastids are distributed, stromules form in longer versions (Waters et al. 2004). All these findings are leading back to research on AF. MT on the other hand also have been associated with stromules in orientation and positioning (Kwok and Hanson 2004a). In a study in which MT were inhibited with cytochalasin, stromule movement has stopped. Others have used MT inhibitors like oryzalin and amiprophosmethyl that did not stop stromule movement, rather reducing the stromule length by 75 % (Kwok and Hanson 2003).

1.3.1 Formation

Stromule formation is still highly hypothesized. There are two possibilities on how they form.

The first hypothesis is that stromules are formed via forces. AF could be anchored to the plastid envelope and either the AF or the plastid move away. This anchored part of the membrane then is extended and is referred to as a stromule. Alternatively, there could be a system working around pressure inside the plastid that might cause the membrane to swell in a direction, forming a stromule in this process.

The second hypothesis covers the usage of altering the peptides in the plastid membrane. It has been observed that mixing short peptides in a membrane can result in tubular formation (Domanov and Kinnunen 2006). Studies on *Arabidopsis thaliana* and *Nicotiana benthamiana* mutants have shown that the overexpression of plastid envelope proteins results in the induction of long tubular structures. After checking the structures with electron microscopy, it was confirmed that these structures were stromules because they consisted of an inner and outer membrane (Machettira et al. 2011). Because these studies were made on chloroplasts, it is comprehensible to assume that stromules might act differently in plants with non-green plastids. All these findings however did not answer the question, what structures stromules need to form in the first place.

1.3.2 Function

Stromule function is yet to be clarified, but there are probable hypothesizes that seem reasonable. The use of stromules for intra- and inter-organelle trafficking is assumed to be one of the likeliest functions. As mentioned above, stromules have a limited diameter that prevents molecules larger than 560 kDa to flow inside. Structures like DNA and proteins have been successfully been falsified to be transported within stromules (Schattat et al. 2012). A study has shown that RuBisCO on the other hand is moving inside of stromules. During these transfers, only stroma is found to be located in the stromule. Other plastid components like thylakoids have been observed to stay in the main plastid body (Bourett et al. 1999). Plastids are part of the jasmonic acid (JA) synthesis. 12-oxo-phytodienoic acid (OPDA) is an intermediate that is transported out of plastids and moved to peroxisomes. As there have been studies that showed that OPDA is not only an intermediate of JA, rather it is also its own signal. Since stromules are formed after triggering the stress response of plants there is the possibility that they are connections in signaling with OPDA or transporting OPDA into peroxisomes. Recently there have also been discoveries that there are proteins located in the plastid stroma, which are

involved in pathogen sensing (Krenz et al. 2010). Because stromules are observed to be formed at various stress inductions, they may be forming on this occasion to detect pathogens.

Another hypothesis for stromule function is that they increase the plastids' surface by up to half of its previous size. Extensions increase the surface area of envelope membranes like for example the cerebral furrows of brains or the microvilli of the small intestine. In the plastid envelope membrane, this could be used for facilitated transport of substances in and out of the plastid body. A relation between the number of plastids and the frequency of stromules has been already discovered. Fewer plastids result in more stromules (Waters et al. 2004). Therefore, stromules would compensate for the lack of plastids and their missing surface area. It has also been observed that stromules assumingly reach to other organelles like the nucleus, mitochondria, peroxisomes or the endoplasmic reticulum (Kwok and Hanson 2004b). This can be explained by either that they are exchanging signal molecules and synthesis intermediates or that the organelle structures both are ruled by the same cytoskeleton microstructure.

1.4 Mitochondrial ROS

Many different stresses induce the stress response in plants. One of them is oxidative stress that has its origin in the accumulation of mitochondrial reactive oxygen species (mtROS). ROS include ozone, singlet oxygen, superoxide, H_2O_2 and hydroxyl radicals. Superoxide and H_2O_2 are best to investigate because they are of high regulatory significance in plant cells (Huang et al. 2016). Superoxide ($O_2^{\cdot-}$) is produced via single electron reduction of O_2 (Murphy 2009). The mitochondrial respiratory complexes I, II and III produce $O_2^{\cdot-}$ normally during the process of the respiratory chain. This production strongly increases when respiratory production is slowed down, by, for example, respiratory chain inhibitors. This leads to a reduced state of mitochondrial electron transport chain (mtETC) components and in the accumulation of $O_2^{\cdot-}$ in mitochondria (Moller 2001). In research on ROS produced by chloroplasts, there has been confirmed stromule formation after inhibiting the photosynthetic electron transport chain (pETC). These findings suggested that stromule formation is a response to light-sensitive redox signals (Brunkard et al. 2015). It is to assume that this study could be reproduced to investigate mtROS.

It has already been established that there is a relation between mtROS and phytohormones. It is also hypothesized that mtROS may serve as intermediates (Berkowitz et al. 2016). With this in mind, it is right to assume that stromules may play their own part in this relation between mtROS and phytohormones.

1.5 Stress Response

1.5.1 Signal Transmission

The plant's stress response is activated by signals that originate from mechanically produced wounds or other adjacent plants. These transmissions can be categorized into short- and long-distance signal transmissions. These signals are part of large regulatory network complexes and metabolic processes that are still partly unknown. Studies have shown that phytohormones play a role by interacting with JA, its precursors and derivates for adapting to the environment (Ryan and Moura 2002).

1.5.1.1 Short-Distance Signal Transmission

If a plant cell is damaged, a rapid accumulation of jasmonates can be determined at the location of the injury. This results in the activation of genes that furthermore result in local defense response (Truman et al. 2007). Jasmonoyl-L-isoleucine (JA-Ile), 12-oxo-phytodienoic acid (cis-OPDA) and arabidopsides are key substances named oxylipins, that activate these defense responsive genes.

1.5.1.2 Long-Distance Signal Transmission

The long-distance transmission can be subcategorized into vascular bundle transmission and airborne transmission. As *BY-2* is a cell line that produces plant calli, only airborne transmission is appliable, because calli have no vascular bundles.

Studies have shown that large plants, that had been injured, show an accumulation of jasmonates in the whole plant and not just around the wound's location (Malone M. 1996). It has been confirmed that many higher plants, as *Nicotiana tabacum*, transmit methyl jasmonate (MeJA) to facilitate communication between damaged and undamaged plant parts via airborne transmission. JA has difficulties penetrating cell membranes without carrier proteins. The volatile phytohormone MeJA, in comparison, does not. Airborne transmission is not limited to the same organism, because it can also activate the accumulation of jasmonates in adjacent plants (Park et al. 2007).

1.5.2 Jasmonates

In this work, stromules are formed with the aid of the phytohormones. They are signal molecules that mostly occur in very small concentrations. In comparison to animals, plant hormones are produced by every cell in the organism, unlike in animals, which produce theirs in specialized glands (Davies 2010). MeJA, a derivate of JA is the main focus of this exposition. Every precursor and derivate of JA are referred to as jasmonates. These phytohormones play a role in the regulation of many physiological processes. By being an endogenous growth-regulating substance found in higher plants, they take part in plant growth and development. Its most crucial usage is in the plant stress response to biotic and abiotic stress conditions. Examples of biotic stresses are pathogenic infestations and mechanical injuries from herbivores. Abiotic stresses consist for instance of salinity and drought (Riemann et al. 2015). Jasmonates actively respond to environmental changes by altering the plants' gene expression. They activate signal pathways that result in triggering JA responsive genes, for example (Ruan et al. 2019) or cis-OPDA-specific response genes (Dave and Graham 2012).

1.5.2.1 Biosynthesis

The research of the JA biosynthesis in monocotyledonous and dicotyledonous plants has come far in the last decades. The octadecane pathway starting from α-linolenic acid and the hexadecane pathway starting from hexadecatrienoic acid are the most common and most explored jasmonate syntheses (Chini et al. 2018). In *Nicotiana tabacum BY-2* the first step starts in the protoplast. Here, the acids mentioned above are converted through the enzymes 13-lipoxygenase, 13-allene oxide synthase and allene oxide cyclase to cis-OPDA and dinor-12-oxo-phytodienoic acid (dn-OPDA) (Dave and Graham 2012). The transport from the protoplast to the next biosynthesis step in the peroxisome is executed by the COMATOSE ATP-binding cassette transporter (Footitt et al. 2007). There, cis-OPDA and dn-OPDA are reduced by the OPDA reductase to 8-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid (OPC-8) and 6-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid (OPC-6). These intermediates are subsequently oxygenized to JA by two and three cycles of β-oxidation (Ruan et al. 2019). The end product is then released via the JA transfer protein into the cytoplasm where it can be modified to its derivates. The volatile derivate MeJA is synthesized by a modification through the enzyme JA carboxyl methyltransferase and the derivate JA-Ile is synthesized by the enzyme jasmonate resistant-1. MeJA then has the ability to induce the JA biosynthesis in other cells of the same plant or other adjacent plants.

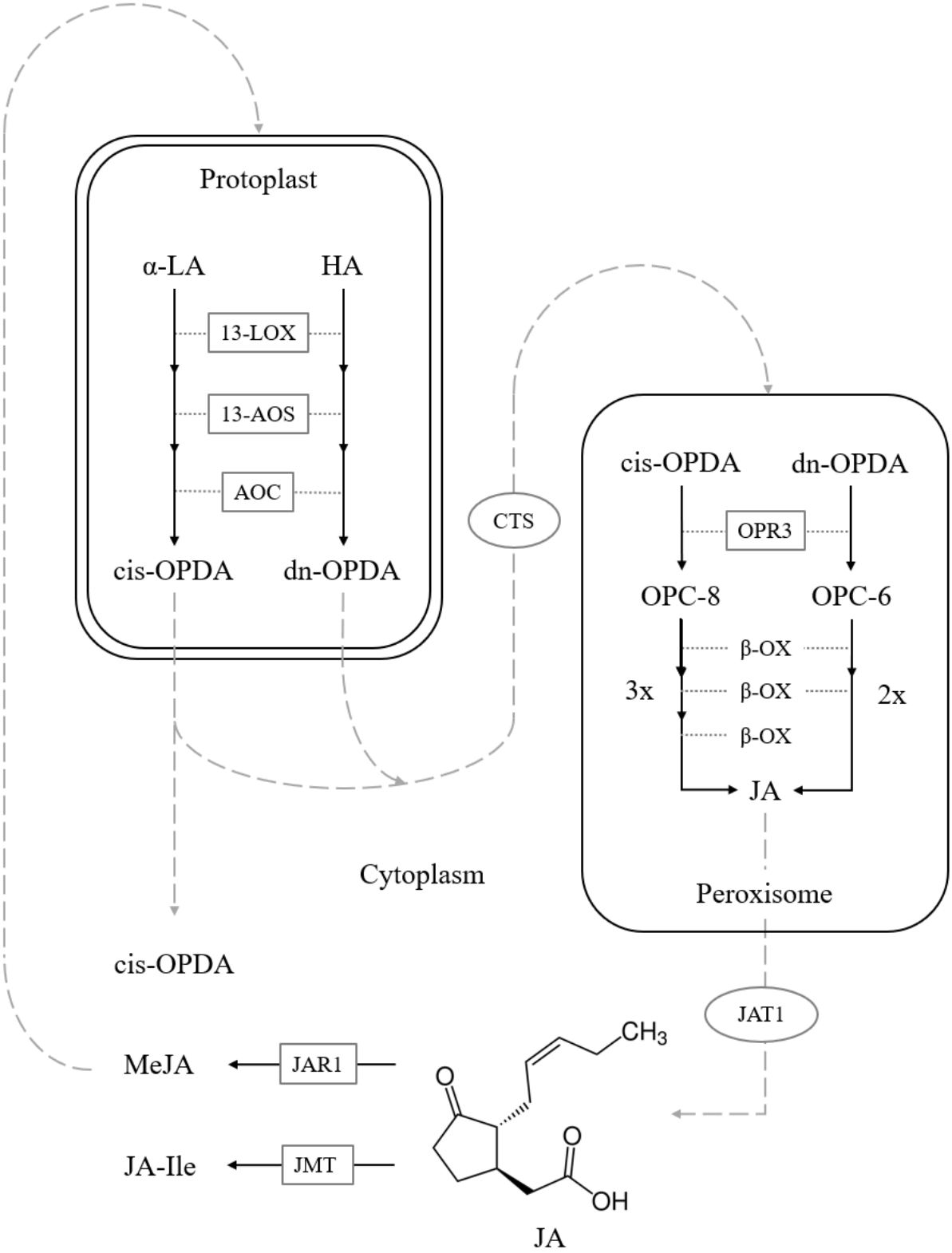


Figure 1: Biosynthesis of Jasmonates. The enzymes and the intermediates are indicated as follows: α-LA for α-linolenic acid, HA for hexadecatrienoic acid, 13-LOX for 13-lipoxygenase, 13-AOS for 13-allene oxide synthase, AOC for allene oxide cyclase, cis-OPDA for 12-oxo-phytodienoic acid, dn-OPDA for dinor-12-oxo-phytodienoic acid, CTS for COMATOSE, OPR3 for OPDA reductase, OPC-8 for 8-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid, OPC-6 for 6-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid, β-OX for β-oxidation, JA for (+)-7-iso-jasmonic acid, JAT1 for jasmonic acid transfer protein 1, JMT for jasmonic acid carboxyl methyltransferase, MeJA for (+)-methyl jasmonate, JAR1 for jasmonate resistant 1, JA-Ile for jasmonoyl-L-isoleucine. Figure inspired by Ruan et al. (2019) and Dave and Graham (2012).

1.5.2.2 Jasmonic Acid Responsive Gene Pathway

JA-Ile has a crucial role in transcriptional control. If this phytohormone accumulates, it will be transported via JAT1 into the nucleus of the plant cell. There, JA-Ile binds to its receptor SCF^{COI1}. This binding triggers the ubiquitination of JAZ-proteins and their depletion through the 26S proteosome. The corepressor TOPLESS, which was connected to JAZ with NINJA now, is no longer able to repress the transcription factors. This results in the activation of JA-responsive genes. These consist of genes that code for example for the production of AOS and AOC, which take part in the JA synthesis. This results in positive feedback. On the other hand, some genes also promote the production of JAZ-proteins that inhibit the gene expression in combination with NINJA and TPL. This is called negative feedback. All these interactions between those genes make this complex network so adaptable to specific situations arising from the environment (Ruan et al. 2019).

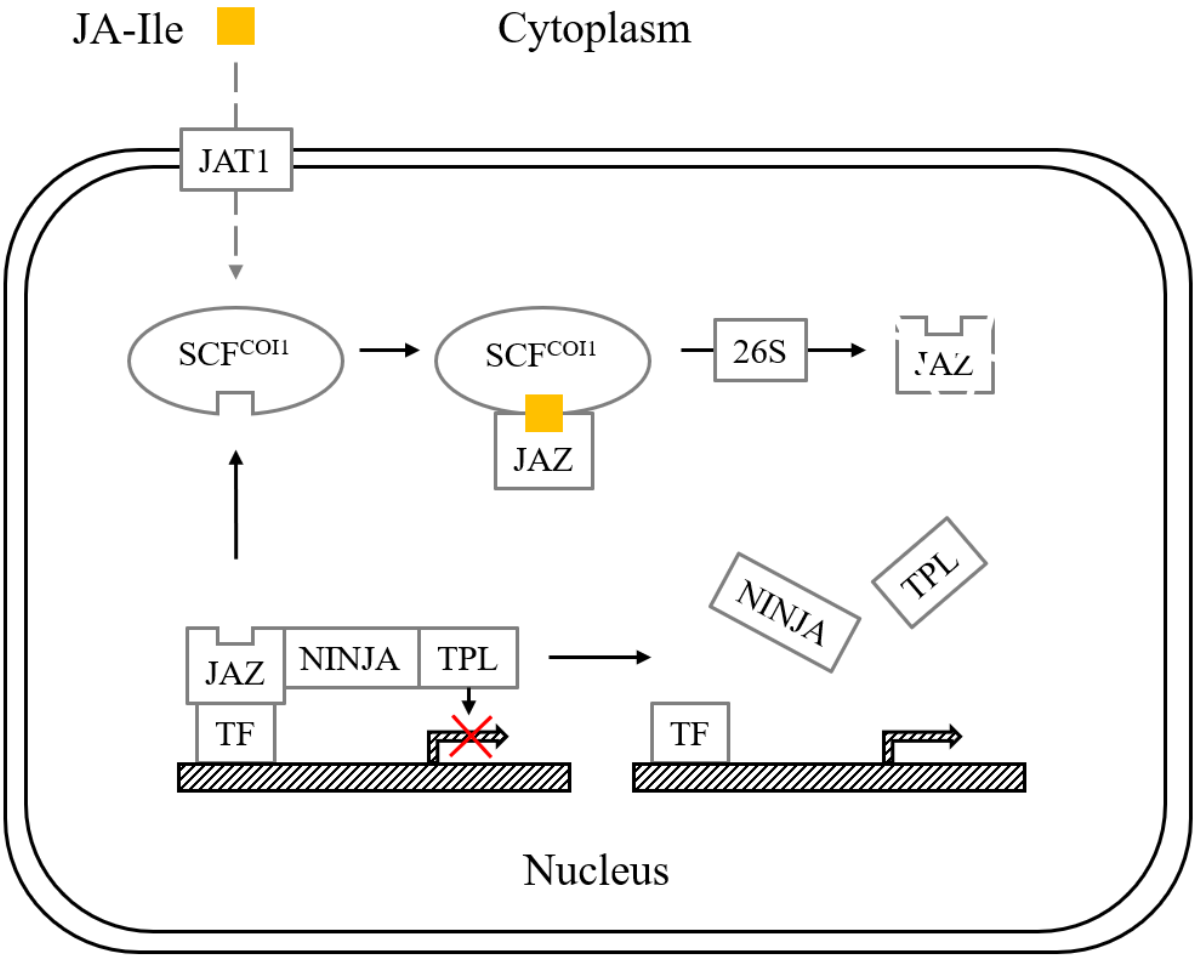


Figure 2: Jasmonic acid signal pathway. JA-Ile for jasmonoyl-L-isoleucine; JAT1 for jasmonic acid transfer protein 1; SCF for Skp1, Cullin and F-box proteins; COI1 for coronatine insensitive 1; JAZ for jasmonate ZIM-domain protein; TF for transcription factor; NINJA for NOVEL INTERACTOR OF JAZ; TPL for TOPLESS protein; 26S for 26S proteasome. Figure inspired by (Ruan et al. 2019).

1.5.2.3 Cis-OPDA Specific Responsive Gene Pathway

There has been researched on mutants where the OPDA reductase (OPR3) was defective, and therefore cis-OPDA was not converted into the intermediate OPC-8, which finally would get transformed into JA. In this mutant, the plant still undergoes a defense response (Stintzi et al. 2001). This means that there has to be a different pathway that is independent of JA-Ile binding to SCF^{COI1}. The volatile derivate cis-OPDA-methyl ester has been observed to act faster and requires lower concentration than MeJA to achieve the same response (Weiler et al. 1993). Other studies have shown that there are genes that are induced by cis-OPDA and neither JA nor MeJA. Half of these genes were also SCF^{COI1} independent (Taki et al. 2005). Next to cis-OPDA, there is dn-OPDA, which has yet to be confirmed to have its own signaling functions (Dave and Graham 2012).

1.5.3 Arabidopsides

Arabidopsides are galactolipids containing esterified cis-OPDA and dn-OPDA. To describe them further, they are monogalactosyl diacylglycerides containing 12-oxo-phytodienoic acid and/or dinor-12-oxo-phytodienoic acid. First, they were discovered in *Arabidopsis thaliana*, but later they have been found in minuscule amounts in other species as well. Arabidopsides have been observed to accumulate after mechanical cell wounding and bacterial pathogenesis (Buseman et al. 2006). They are, other than cis-OPDA, dependent on JA signaling. It has been shown that in mutants, where JAR1 and SCF^{COI1} are absent, the arabidopsides concentration was significantly reduced. It is hypothesized that arabidopsides function as a cis-OPDA and dn-OPDA storage. When this happens to be confirmed, they could be used for direct signaling of cis-OPDA or as the substrate for JA and its derivates (Ibrahim et al. 2011).

1.6 Main Objective

In this thesis, I investigate which parts of the cytoskeleton are necessary for stromule formation. It has already been identified that AF play a major role in plastid and stromule movement. But which structure is needed to form and maintain stromules is yet to be discovered. This is yet to be discovered. The absence of MT in these relations may assume that they play a part in this undiscovered topic. For this, I will induce stromule formation with the phytohormone MeJA, but with a previous inhibition of AF and MT.

Furthermore, I study the connection of stromules with mtROS. The fundamental question is if stromules behave the same with mtROS as with photosynthetic reactive oxygen species (pROS). For this, I will induce stromule formation with the phytohormone MeJA and fluorescently stain mitochondria and mtROS to observe their relation to one another.

Lastly, I investigate the stromule function. Do they have a role to play in the gene expression of JA responsive genes? For this, I will compare the gene expression of JAZ1, 2 and 3 once with stromules and once without. If there is a change, then there can be made assumptions about stromules inhibiting or supporting the JA responsive genes.

But before starting these experiments, there has to be made some groundwork. This will cover how much time after MeJA induction is needed to see stromules that are high in numbers and length. This is very important so that the experiments have approximately the same stromule quality and quantity.

2 Materials and Methods

2.1 Materials

2.1.1 Organism

This thesis worked on *Nicotiana tabacum*. For research on gene expression, the cultivar *BY-2* of its wildtype was used. For research on fluorescent microscopy, another cell line was being used. For this a vector containing tpFNR:mEOSFP was transformed stably via *Agrobacterium tumefaciens* into the wildtype. The transgenic line has its stroma marked with mEOS and uses hygromycin resistance as a selection marker. The transformation is part of the preliminary work.

2.1.1.1 Cultivation

The cell lines were cultivated in autoclaved 50 ml Erlenmeyer flasks with 30 ml MS-medium. 30 µl of chilled Hygromycin30 were added in the transgenic line's flask as a selection marker. After seven days 1 ml of the cultivating cell line was transferred into a flask with fresh MS-medium and antibiotics were added into the flask with the transgenic cell line. The cells grew in the dark at 30 °C on a shaker KS 260 control (IKA-Werke GmbH & CO. KG, Staufen) at 150 rpm. Two separate flasks for each cell line were retained, one was sub-cultured Mondays the other Fridays. This has been done to assure a cell age of three to four days on every given day.

2.1.1.2 Treatment

The best cell age for researching on stromule forming was three to four days after recultivation. For research on gene expression, 1 ml of wildtype cell culture was added into an Eppendorf-Tube and MeJA was added into it. In some treatments, oryzalin was also added, so there would be different conditions that will be compared to each other.

For research with fluorescent microscopy, 1 ml of mEOS-fluorescent cell culture was added into an Eppendorf-Tube and MeJA was added into it. After that, the cells have formed stromules and are ready to start experiments on. These experiments consisted of fluorescently staining or disrupting other subcellular structures and observing their relation to stromules under a spinning disc microscope.

The specific concentration and duration of incubation are listed in more detail in their specific chapter for this thesis.

2.1.2 Instruments

2.1.2.1 Shaker, Heat Block

Schüttler KS 260 control	IKA-Werke GmbH & CO. KG, Staufen
Thermomixer 5436	Eppendorf AG, Hamburg

2.1.2.2 Thermocyclers

CFX Connect Real-Time PCR Cycler	Bio-Rad Laboratories GmbH, München
Primus 96 advanced	PEQLAB Biotechnologie GmbH, Erlangen
T100 Thermal Cycler	Bio-Rad Laboratories GmbH, München

2.1.2.3 Centrifuges

Micro Star 17 Mikro-Zentrifuge	VWR International GmbH, Darmstadt
Mini-Zentrifuge Rotilabo	Carl Roth GmbH + Co. KG, Karlsruhe
Thermo PICO 17 Zentrifuge	Thermo Fisher Scientific Inc., Waltham, USA

2.1.2.4 Microscope

AxioImager.Z1 Mikroskop	Carl Zeiss Microscopy GmbH, Jena
digitale CCD Kamera AxioCam MRm	Carl Zeiss Microscopy GmbH, Jena
Filtersatz 38 HE	Carl Zeiss Microscopy GmbH, Jena

2.1.2.5 Miscellaneous

Autoklav Systec VE-95	Systec GmbH, Linden
Feinwaage Analytical Plus AP110	Ohaus Europe GmbH, Greifensee, Schweiz
Gelelektrophoresekammer Mupid-One	Advance, Mupid CO., Tokio, Japan
Magnetrührer IKAMAG RCT	IKA-Werke GmbH & CO. KG, Staufen
Membranvakuumpumpe CVC2	Vacuubrand GmbH & CO., Wertheim
NanoDrop Spectrophotometer ND 1000	PEQLAB Biotechnologie GmbH, Erlangen
pH Meter pHenomenal pH 1000L	VWR International GmbH, Darmstadt
Präzisionswaage Kern PCB 350-3	KERN & Sohn GmbH, Balingen
Rührwerk RW 20 DZM	IKA-Werke GmbH & CO. KG, Staufen
Sicherheitswerkbank KR-105 Safety	KOJAIR TECH OY, Vilppula, Finnland
TissueLyser	QIAGEN GmbH, Hilden
Vortex Genie 2 G-560E	Scientific Industries, Bohemia, New York, USA
VWR PCR Plate Spinners	VWR International GmbH, Darmstadt

2.1.3 Chemicals

0,5x Tris-Acetate-EDTA (TAE) -Buffer	Sigma-Aldrich Chemie GmbH, München
100 bp DNA Ladder	New England Biolabs, Frankfurt am Main
2,4-Dichlorophenoxyacetic acid	Sigma-Aldrich Chemie GmbH, München
Agar-Agar Kobe I	Carl Roth GmbH + Co. KG, Karlsruhe
Bovine serum albumin (BSA)	Carl Roth GmbH + Co. KG, Karlsruhe
Cycloheximide	Carl Roth GmbH + Co. KG, Karlsruhe
Disodium phosphate (Na_2HPO_4)	Carl Roth GmbH + Co. KG, Karlsruhe
dNTPs (10 mM)	New England Biolabs, Frankfurt am Main
Ethanol	Carl Roth GmbH + Co. KG, Karlsruhe
Egtazic acid (EGTA)	SERVA Electrophoresis GmbH, Heidelberg
Glycerol 99 %	Carl Roth GmbH + Co. KG, Karlsruhe
GoTaq Reaction buffer (5x)	Promega GmbH, Mannheim
Latrunculin B from red sea sponge	Sigma-Aldrich Chemie GmbH, München
Magnesium Chloride ($MgCl_2$)	Carl Roth GmbH + Co. KG, Karlsruhe
Magnesium Sulfate ($MgSO_4$)	Carl Roth GmbH + Co. KG, Karlsruhe
Methyl jasmonate 95 %	Sigma-Aldrich Chemie GmbH, München
Midori Green Advance DNA stain	Biozym Scientific GmbH, Hessisch Oldendorf
Monopotassium phosphate (KH_2PO_4)	Carl Roth GmbH + Co. KG, Karlsruhe
Murashige und Skoog (MS) salts	DUCHEFA Biochemie B. V., Niederlande
Murine Reverse Transcriptase buffer	New England Biolabs, Frankfurt am Main
Nuclease free water	Biozym Scientific GmbH, Hessisch Oldendorf
Oryzalin	Chem Service, Inc., West Chester, USA
Paraformaldehyde (PFA)	Carl Roth GmbH + Co. KG, Karlsruhe
PIPES pufferan 99 %	Carl Roth GmbH + Co. KG, Karlsruhe
Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, München
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG, Karlsruhe
SYBR Green I	Thermo Fisher Scientific Inc., Waltham, USA
Taq DNA Polymerase buffer	New England Biolabs, Frankfurt am Main
Thiamin Hydrochlorid	Sigma-Aldrich Chemie GmbH, München
Triton X-100	Sigma-Aldrich Chemie GmbH, München

2.1.4 Fluorescent Microscopy

2.1.4.1 Fluorescent Dyes

MitoSOX Red	Thermo Fisher Scientific Inc., Waltham, USA
MitoTracker Red CMXRos	Thermo Fisher Scientific Inc., Waltham, USA
Texas Red-X Phalloidin	Thermo Fisher Scientific Inc., Waltham, USA

2.1.4.2 Antibodies

Goat anti-Mouse IgG, TexasRed-X	Sigma-Aldrich Chemie GmbH, München
Mouse Anti-alpha Tubulin (ATT)	Sigma-Aldrich Chemie GmbH, München

2.1.5 Enzymes

GoTaq DNA Polymerase	Promega GmbH, Mannheim
M-MuLV Reverse Transcriptase	New England Biolabs, Frankfurt am Main
Taq DNA Polymerase	New England Biolabs, Frankfurt am Main
Pectolyase from *Aspergillus japonicus*	DUCHEFA Biochemie B. V., Niederlande
Macerozym R-10	DUCHEFA Biochemie B. V., Niederlande

2.1.6 Kits

innuPREP Plant RNA Kit	Analytik Jena AG, Jena
RNase-Free Dnase Set	QIAGEN GmbH, Hilden

2.1.7 Special Equipment

Cell Culture Plate (24 Wells)	Thermo Fisher Scientific Inc., Waltham, USA
SafeSeal-Reaction Tubes (2 ml)	Sarstedt AG & Co, Nürnbrecht

2.1.8 Softwares

AxioVision 4.8.2	Carl Zeiss Microscopy GmbH, Jena
Bio-Rad CFX Manager 3.1	Bio-Rad Laboratories GmbH, München
Zen 3.4 (blue edition)	Carl Zeiss Microscopy GmbH, Jena

2.1.9 Buffers and Solutions

Blocking solution in PBS (0.5 %):	0.5 %	BSA
Fixing solution in SB (1.8 %):	1.8 %	PFA
	heat up to 60 °C	
Fixing solution in MSB (3.7 %):	3.7 %	PFA
	heat up to 60 °C	
Glycerol solution in SB:	1 %	Glycerol
Loading buffer (5x):	50 mg	Bromo Phenolblue
	50 ml	Glycerin
	50 ml	Nuclease free water
	50 mg	Xylene Cyanol
Microtubule-stabilization buffer (MSB):	2 mM	EGTA *pH: 8*
	2 mM	$MgSO_4$
	0.05 M	PIPES *pH: 7*
	0.1 %	Triton X-100
	adjust pH with NaOH	
Phosphate buffered saline (PBS):	2.7 mM	KCl
	1.2 mM	KH_2PO_4
	0.15 M	NaCl
	6.5 mM	Na_2HPO_4
	pH: 7.2; adjust with NaOH	
Standard buffer (SB):	10 mM	EGTA *pH: 8*
	5 mM	$MgSO_4$
	0.1 M	PIPES *pH: 7*
	adjust pH with NaOH	
TAE-Gel:	40 ml	0,5x TAE Buffer
	1.1%	Agarose
	2.4 µl	Midori Green Advance

2.1.10 Medium

MS-medium:	0,2 mg/L	2,4-Dichlorophenoxyacetic acid
	200 mg/L	KH_2PO_4
	4,3 g/L	MS salts
	100 mg/L	Myo-Inositol
	30 g/L	Sucrose
	1 mg/L	Thiamine
	pH: 5.8; adjust with KOH	

2.1.11 Additives

Table 1: List of used antibiotics. Stated below are the dissolvent of the antibiotic, the concentration of the antibiotic-stock solution and the working concentration of the antibiotic in MS-medium.

Antibiotic	Dissolvent	Conc. Of stock	Working conc.
Hygromycin B	H_2O	30 mg/ml	30 µg/ml

Table 2: List of additives other than antibiotics. These substances have different usages and constitute different conditions, which are the fundamental conditions in this stromule research.

Substance	Dissolvent	Conc. Of stock	Usage
Methyl jasmonate	Ethanol	100 µM	Phytohormone: Stromule Formation
Oryzalin	Acetone	10 mM	MT depolymerization
Latrunculin B	DMSO	1 mM	Actin polymerization inhibitor
Cycloheximide	DMSO	10 mg/ml	Translation inhibitor

2.1.12 Primers

Table 3: List of PCR primers. The primers were ordered with the purification "desalted" from Sigma-Aldrich Chemie GmbH, München. The table below contains the forward and reverse primers for the genes JAZ1, JAZ2, JAZ3 and for the housekeeping gene GAPDH. They arrived in dry form and were hydrated with the right amount of Nuclease free water stated on the package insert. The primer sequences were acquired from their respective source.

Oligo Name	Sequence (5'-3')
NtJAZ1_forward[1]	CCAATTGCGAGACGAAATTCACTTAC
NtJAZ1_reverse[1]	CCAAGCCATGCCTTATTTTCCTCATTC
NtJAZ2_forward[1]	GCAGCACCTGCTCAACTGACC
NtJAZ2_reverse[1]	GCACCACATTAGGAGGAACGCAACC
NtJAZ3_forward[1]	GGATTCCGGTCGATTCGCCG
NtJAZ3_reverse[1]	CCAAGGCTGAGATCTCCAAAGGAAC
GAPDH_forward[2]	AGCTCAAGGGAATTCTCGATG
GAPDH_reverse[2]	AACCTTAACCATGTCATCTCCC

[1] Asfaw et al. (2020)
[2] Liu et al. (2012)

For the cDNA Synthesis, the Oligo-dt Primer (Thermo Fisher Scientific Inc., Waltham, USA) was used, which bind only specifically to mRNA, so it can be transcribed into cDNA.

2.2 Methods

2.2.1 Time Course

To determine the best time for MeJA incubation in stromule research, the method of a time course was used. Plastid fluorescent *BY-2* cells were treated with 100 µM of MeJA and observed at different times after phytohormone induction. The most significant times were, when stromules first arose and when stromules are objectively developed enough that they could be referred to as fully established. The second timestamp will be fundamental for the other experiments in this research. Therefore, it is recommended that the results of the time course (chapter 3.1) had been read and realized.

2.2.2 Translation Inhibition

After the time course, one can act on the assumption that stromules rather form quickly than slowly. First stromules can be seen after already 15 minutes, therefore this raises the question if they are dependent on transcription of the DNA. In molecular biology, transcription is defined as transcribing DNA into RNA in the nucleus. Subsequent the translation occurs, which consists of the synthesis of proteins after the coding of RNA. mRNA is decoded in ribosomes that are located in the cytoplasm or endoplasmic reticulum. This process takes roughly about one day in eukaryotes (Milo and Phillips 2015). The short time for stromule formation tends to assume that it actually does not need translation. To ensure that this hypothesis is correct, plastid fluorescent *BY-2* cells were treated with 100 µg/ml of cycloheximide for 2 hours. This chemical inhibits protein synthesis in eukaryotes. It has been shown to inhibit translation elongation by binding to the E-site of the 60S ribosomal unit and then interfering with tRNA (Schneider-Poetsch et al. 2010). This will be utilized to track if stromules are still forming under these conditions. Although cycloheximide does not interfere in transcription, it still disrupts translation, which is sufficient to either negate or affirm the hypothesis.

2.2.3 Cytoskeleton Disruption

For later experiments, it is beneficial to identify on which structures and processes stromules are dependent on. Because of the high association of plastids with the cytoskeleton, it can be assumed that stromules have this in common. In plants, two different structures are part of the cytoskeleton: AF and MT. These will be separately disrupted by 2 µM of latrunculin B and 10 µM of oryzalin for each 1 hour, to identify if there is any dependency for stromule formation.

2.2.4 Fluorescent Cell Staining

Fluorescent microscopy is a key method that allows the user to visualize structures and organelles in organisms. Fluorescent proteins get excited by a specific wavelength. These proteins then emit light with a longer wavelength that conveniently has a different visual color than before. By using antibodies with different fluorophores that target their related antigen, it is possible to visualize a wide range of targets at once. Some structures like AF can also be visualized through a phallotoxin named phalloidin. By binding a fluorescent protein to this toxin, the use of an antibody is made unnecessary.

As preparation for all staining methods, plastid fluorescent *BY-2* cells were incubated in 100 µM MeJA for one hour before starting the procedures. This guaranteed that stromules were existent in the cells and were able to get fixated by their respected fixing solution.

2.2.4.1 Actin Filament Staining

AF were visualized by the same method established by Kakimoto and Shibaoka (1987). This method has been modified according to Olyslaegers and Verbelen (1998). 0.5 ml of suspended *BY-2* cells have been fixed for 10 minutes with 1 ml of a 1.8 % paraformaldehyde solution. After a subsequent 10-minute fixation with 1 ml of a 1 % glycerol solution, the cells were washed twice for 10 minutes in standard buffer. Then, they were incubated in the dark for 35 minutes in 0.5 ml of 0.66 µM TexasRed-Phalloidin (Sigma-Aldrich Chemie GmbH, München). At the end, the cells have been washed trice for 10 minutes in phosphate-buffered saline. After that, they were ready to investigate under the spinning disc microscope at 63x oil magnification.

2.2.4.2 Microtubule Immunofluorescence Staining

When trying to stain MT, one should be very careful with the handling of the cells. This cytoskeleton structure is highly fragile so that any shock can disturb their location or break them entirely. In this work, MT were visualized by the method of Kühn (2014). 0.25 ml of suspended *BY-2* cells had been fixed for 30 minutes with 0.5 ml of a 3.7 % paraformaldehyde solution. After the cells were washed trice for 5 minutes in microtubule-stabilization buffer (MSB), they were digested for 5 minutes with 0.5 ml of MSB containing 1 % macerozyme and 0.2 % pectolyase (DUCHEFA Biochemie B. V., Niederlande). After subsequently washing the cells three times for 5 minutes in MSB, they were blocked for 20 minutes in 0.5 ml of an 0.5 % BSA

solution. Then, they have incubated in 0.5 ml anti-alpha tubulin (ATT) antibodies from the mouse. The antibodies were diluted in phosphate-buffered saline (PBS) in the ratio of 1:500, and the cells were left overnight at 4 °C. The next day the cells were washed trice for 5 minutes in PBS and incubated for 45 minutes at 37 °C in 0.5 ml of the second antibody. It was an anti-mouse IgG antibody from goat, which was linked to TexasRed-X and diluted in PBS (1:250). Finally, the cells were washed again three times for 5 minutes with PBS. After that, they were ready to investigate under the spinning disc microscope at 63x oil magnification.

2.2.4.3 Mitochondria and mitochondrial ROS Staining

Mitochondria and mitochondrial ROS were visualized with reagents from Thermo Fisher Scientific Inc. (Waltham, USA). MitoTracker Red CMXRos and MitoSOX Red both bind to their respective counterpart without the need of fixing the cells before. Both are able to permeate the cell membrane without any help from digesting solutions. Therefore, the cells are still alive and can be monitored for changes in mitochondria functions. These reagents are also compatible with other staining that require fixation or immunofluorescence.

Two Eppendorf-Tubes with 0.5 ml of resuspended *BY-2* cells were centrifuged to obtain a cell pellet. The liquid supernatant was discarded. One pellet was resuspended in 1 ml of 5 µM MitoSOX Red and incubated for 10 minutes at 37 °C. The other pellet was resuspended in 1 ml of 0.5 µM MitoTracker Red CMXRos and incubated for 45 minutes at 37 °C. Afterwards, both were washed three times for 5 minutes with PBS.

After that, the differently stained cells were ready to investigate under the spinning disc microscope at 63x oil magnification.

2.2.5 Gene Expression Analysis

2.2.5.1 RNA-Extraction

The RNA was extracted by using the innuPREP Plant RNA Kit (Analytik AG, Jena). In total nine flasks with three days old cell cultures were used, i.e., for every condition, a biological triplet was prepared. The conditions consisted of a control with nothing added, of a treatment with MeJA and of a treatment with MeJA and oryzalin. After treating the cell cultures, the liquid MS-medium was vacuumed off the cells through the membrane vacuum pump CVC2 (Vacuubrand GmbH & CO., Wertheim). 300 mg of the plant material were loaded into 2 ml SafeSeal-Microtubes, which already contained a steel ball. Those tubes were directly frozen in liquid nitrogen and then pulverized twice by a TissueLyser for 20 seconds at 20 hertz. Further procedures were taken as described by the instructions of the manufacturer. After the application of the samples to the second column, they were digested with DNase I (RNase-Free DNase Set, QIAGEN GmbH, Hilden) for 15 minutes at 37 °C. The samples were eluted with 50 µl Nuclease-free water.

The concentration of the extracted RNA was determined by using the NanoDrop Spectrophotometer ND 1000 (PEQLAB Biotechnologie GmbH, Erlangen). After that, the RNA was stored at -26 °C until it was used for the cDNA-Synthesis.

2.2.5.2 cDNA-Synthesis

The cDNA was synthesized by following the reaction steps in the table below. The synthesis has been made possible through the Primus 96 advanced (PEQLAB Biotechnologie GmbH, Erlangen) by using the pre-set program named “P.CYC”, which is visualized in Table 4.

The Oligo-dT Primer specifically only transcribes mRNA into cDNA, by binding to its poly-A tail at the 3’ end of the mRNA. The transcribed cDNA then was stored at -26 °C until it was used for gene expression analysis.

Table 4: Reaction approach and steps of the cDNA-Synthesis. Below there are the concentrations and volumes of the used components for one reaction of the cDNA-Synthesis. Temperatures and incubation times are also listed.

Component	Concentration/Volume
dNTPs (10 mM)	1 µl
extracted RNA	1 µg
Oligo-dT Primer (100 µM)	0.4 µl
Nuclease-free water	add up to 14.6 µl
Incubation: 5 minutes at 65 °C	
M-MuLV Reverse Transcriptase	0.25 µl
Reverse Transcriptase buffer	2 µl
RNA free water	1.25 µl
Rnase Inhibitor	0.5 µl
Incubation: 1 hour at 42 °C and then 10 minutes at 90 °C	

2.2.5.3 Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method to duplicate specific DNA ranges. For this, specifically designed primers are used to determine the amplification range of this reaction.

A PCR is often used to figure out if a specific area of genes or a gene of interest is present in the DNA that is investigated. Like in this work, the PCR is also used to check if the used primers are binding to the right area of the DNA and therefore checking if the primers are working in a quantitative real-time PCR (qPCR) as desired. The primers in question were those that are used to track JAZ1, 2 and 3 (Table 5).

The reaction approach per sample is visible in the table below. For every primer, there has been prepared a negative control, which contained nuclease-free water instead of cDNA. This negative control is necessary to exclude impurities in the samples.

Table 5: Reaction approach of the PCR. Below there are the volumes of the used components for one sample of the PCR. One sample has a volume of 20 µl.

Component	Volume
cDNA (1:50)	5 µl
dNTPs (10 mM)	0.4 µl
Forward Primer (10 µM)	0.8 µl
Reverse Primer (10 µM)	0.8 µl
Taq DNA Polymerase (5 U/µl)	0.1 µl
Taq DNA Polymerase buffer (10x)	2 µl
Nuclease-free water	10.9 µl

Table 6: Reaction steps of the PCR. Below there is the PCR program with its reaction steps. Listed are their respective temperature, duration and cycle count.

Reaction Step	Temperature (°C)	Duration (min:sec)	Cycles
Initiation	94	1:00	1
Denaturation	94	0:30	
Annealing	60	0:30	35
Elongation	68	0:30	
Final Elongation	68	2:00	1
Final Hold	12	∞	1

2.2.5.4 Agarose Gel Electrophoresis

The gel electrophoresis is needed to separate nucleic acids from each other according to their size. They will get separated in an agarose gel equivalent to their base-pair length. The parting is performed by using an electric field inside the electrophoresis chamber.

5 µl of 5x Loading buffer were added to the 20 µl of amplified PCR products. 10 µl of this mixture were loaded into each slot of a 1,1 % TAE-gel. The first and last slots were loaded with 2 µl of a 100 bp DNA ladder (New England Biolabs, Frankfurt am Main). The gel was covered with 0,5x TAE buffer and after loading the gel with all samples, the electrophoresis ran for 20 minutes at 100 volts. Afterwards, the gel was observed under UV light and captured on film.

2.2.5.5 Quantitative real-time PCR

The difference in gene expression of *BY-2* with different conditions was analyzed with the quantitative real-time polymerase chain reaction (qPCR). Three independent biological replicates were prepared for each condition there is. The cDNA that will be used for the qPCR, is the same as for the PCR.

The qPCR was realized with the CFX Connect Real-Time PCR Cycler (Bio-Rad Laboratories GmbH, München). Each reaction approach contains the volume for three technical replicates. For every primer, there has been prepared a negative control, which contained nuclease-free water instead of cDNA. The primers in question are once again the primers that are used to track JAZ1, 2 and 3 but also one to track a housekeeping gene named GAPDH. This housekeeping gene is necessary because it shows a constant and uninfluenced gene expression, which will be useful later to determine how the genes of interest are expressing compared to such a constant. In the following tables, there is the reaction approach for three technical replicates and the reaction steps.

Table 7: Reaction approach of the qPCR. Below there are the volumes of the used components for three technical replicates of one sample of the qPCR. Sampling errors are added. First, the reaction mix will be made. Then 61.8 µl of that mix will be transferred to a reaction tube. 3.25 µl of cDNA will be added. One replicate has a volume of 20 µl, therefore this volume will be taken out of the mix three times.

Component	**Volume**
dNTPs (10 mM)	1.4 µl
Forward Primer (10 µM)	1.4 µl
Reverse Primer (10 µM)	1.4 µl
GoTaq Reaction buffer	14 µl
GoTaq DNA Polymerase (5 U/µl)	0.35 µl
$MgCl_2$ (50 mM)	3.5 µl
SYBR Green I (10x)	3.3 µl
Nuclease-free water	41.15 µl
61.8 µl transferred to new reaction tube	
cDNA (1:10)	3.25 µL
Three replicate samples at 20 µl will be put into qPCR plate	

Table 8: Reaction steps of the qPCR. Below there is the qPCR program with its reaction steps. Listed are their respective temperature, duration and cycle count. The melting curve analysis was realized by increasing for 0.5 °C steps each cycle.

Reaction Step	Temp. (°C)	Duration (min:sec)	Cycles
Initiation	95	3:00	1
Denaturation	95	0:15	40
Annealing and Elongation	60	0:40	
1st Preparation step for melting curve	95	0:10	1
2nd Preparation step for melting curve	65	0:30	1
Melting curve	65-95	0:05 each	50

After the qPCR stops running, the results contain Ct-values. This value indicates the number of cycles needed for the fluorescent SYBR Green I to exceed a certain threshold value in each sample. First, the average Ct value was calculated by averaging the values from the technical triplet. Then ΔCt was calculated by subtracting the average endogenous control gene Ct from the average target gene Ct. Then, the relative expression is calculated with $2^{-\Delta Ct}$.

There had been run three separate qPCRs to be able to state a significant result. A first problem was that the relative expression differed highly, and a second problem was that the values had the same ratio to each other. Because of that, the ratio of the conditions oryzalin + MeJA and MeJA was calculated and compared.

To be sure that the results were statistically significant, the Chi-squared test was used. This test was chosen because the results contained proportions and not simple values. If the test result was $P < 0.01$ the null hypothesis will be rejected.

3 Results

3.1 Time Course

The results of the time course determine the best time to incubate MeJA. Plastid fluorescent *BY-2* cells were treated with 100 µM of MeJA and observed at different times after its induction. The time stamps were 0, 15, 30, 45, 60, 120, 180, 240 and 300 minutes. Meaning the cells were observed for a total of five hours. The most meaningful time stamps are shown below.

Directly at the time of MeJA induction, only round and partly loose but also partly clustered plastids are visible. 15 minutes later there is already a change in plastid morphology. In Figure 3 the forming of slight excrescences is visible in (B). These emerging structures in (C) develop into long and thin tubular extensions. In (D) there are dark spots within the stromule. Because the fluorescent signal of this cell line only occurs in the plastid stroma, these dark spots can be identified as the envelope membrane of the plastid. Two possibilities explain these dark spots. First, the stromule curls itself, meaning it wriggles in and outside of the depths the microscope can register, meaning it symbolically pokes itself through the layer which is observed. The dark spots then are the areas that cross the layer. The second explanation would be that there is not only one stromule, rather multiple that are connected via plastid bodies. Plastids are round to oval structures that fit the shape of these dark spots. According to this explanation, stromules would connect plastids via a plastid chain, in which these plastids serve as a kind of checkpoint or anchor for the stromules.

As mentioned above, the plastids are not particularly spread nor clustered at the start. After the stress response is induced the position of plastids change to a more clustered phase that surrounds the nucleus (B). After 30 minutes though, this arrangement is disbanded itself and plastids begin to move away from the nucleus (C). In later observations, plastids are highly distributed in the cell (C). It suggests that stromules may play a role in plastid distribution or are both structures are moved by the same microfilaments.

Later time stamps of the time course are not shown in the following figure, because the alignment and distribution of plastids and stromule do not significantly change. The time course was performed to identify the time after stress response induction, in which stromules are highly formed. This time happens to be around 1 hour after MeJA induction. This will be the groundwork for further experiments in this thesis.

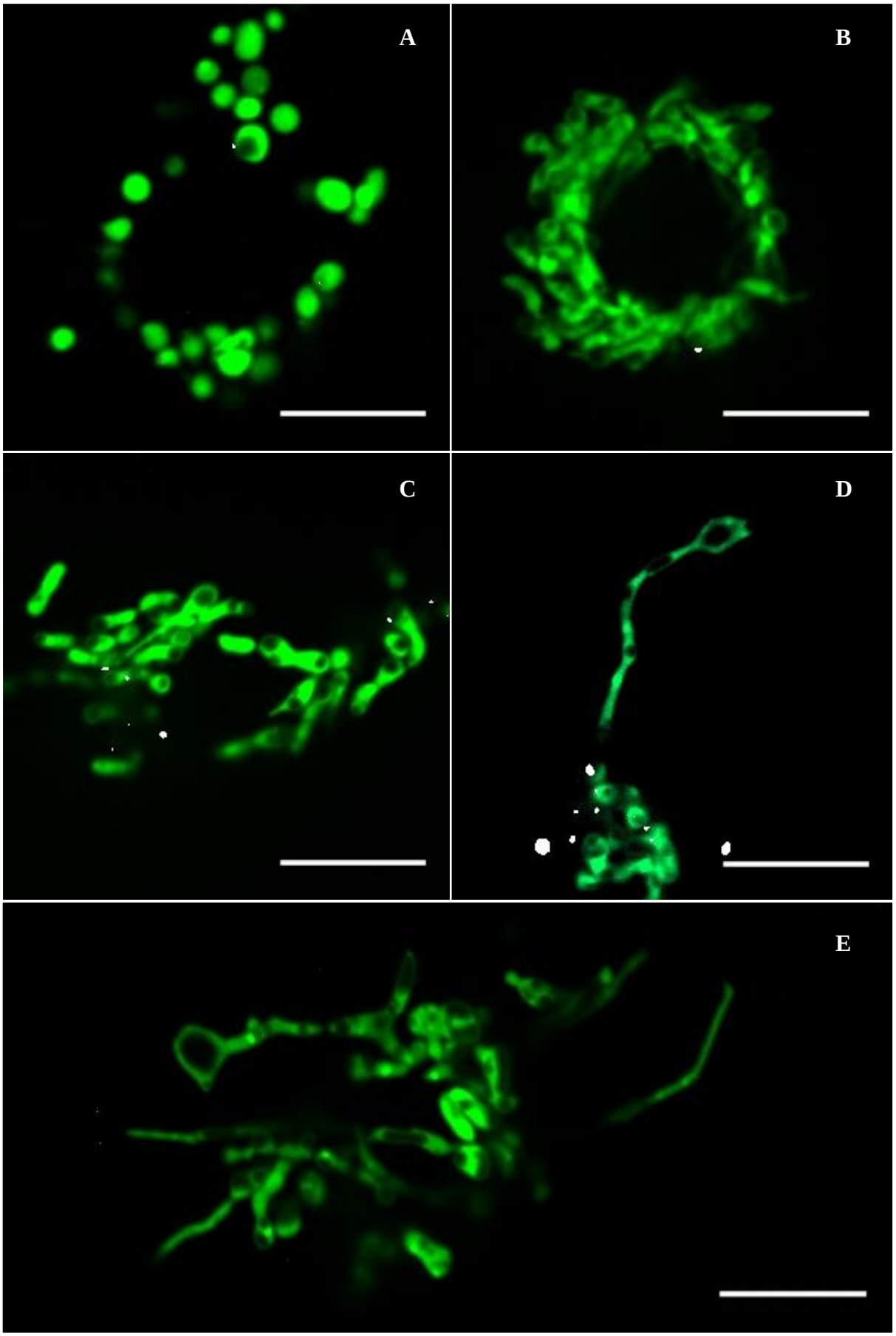


Figure 3: Time Course with important Time Stamps. Stromule formation after 0 minutes (A), 15 minutes (B), 30 minutes (C), 45 minutes (D) and 60 minutes (C) of triggering stress response with MeJA. Scale is 10 µm.

3.2 Translation Inhibition

In the chapter “3.1 Time Course” it has been observed that stromules start forming within shorter than 15 minutes after induction of the plant stress response. This raises the question if stromule formation is dependent on the transcription of DNA. The process of the start of transcription to the end of protein synthesis takes roughly one day in eukaryotes (Milo and Phillips 2015). To investigate this further, plastid fluorescent *BY-2* cells were treated with 100 µg/ml of cycloheximide for 2 hours, to prevent protein synthesis and translation. After that the stress response was initiated by 100 µM of MeJA. Samples with cycloheximide without any MeJA were also prepared as a control.

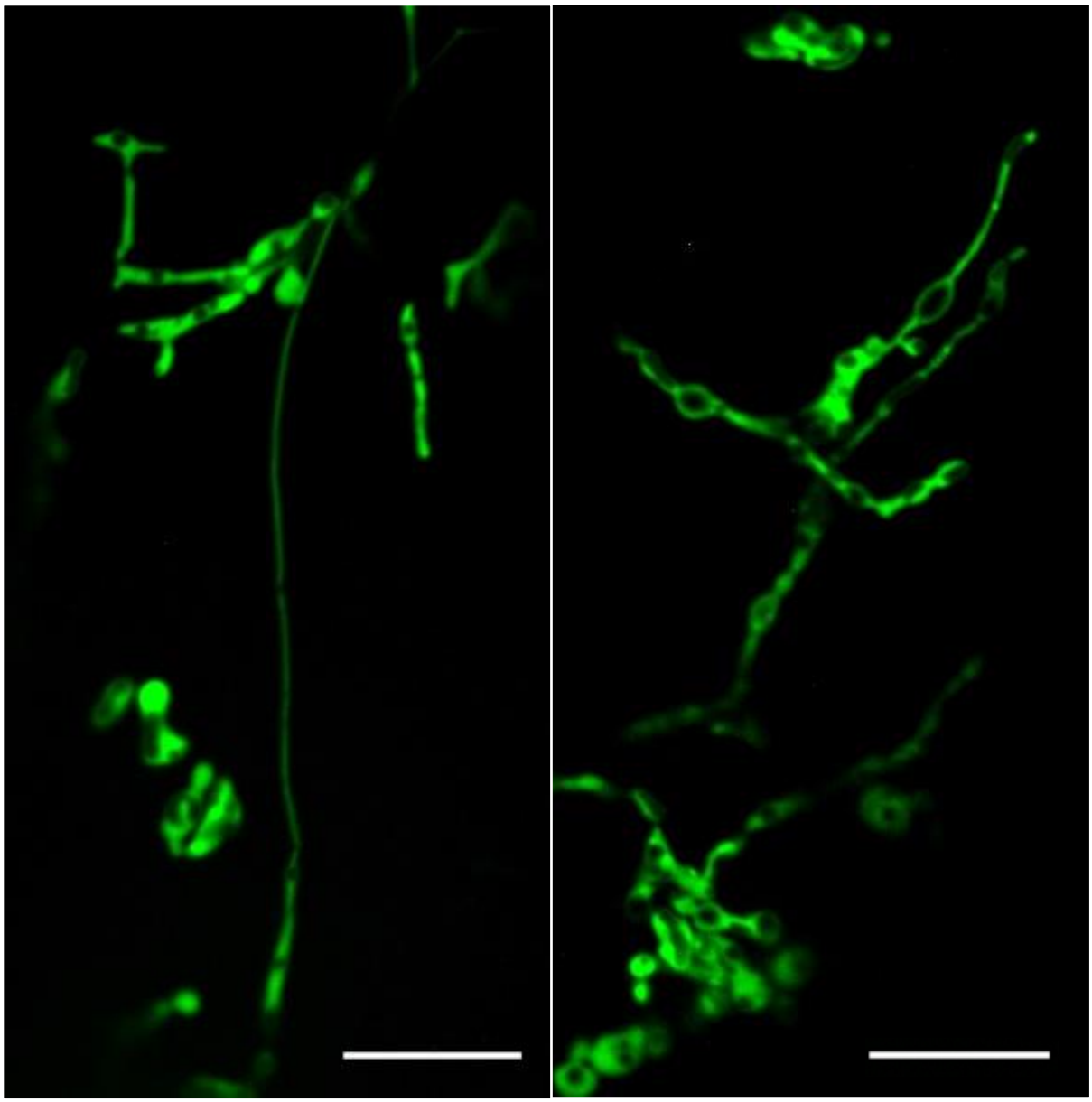


Figure 4: Stress Response with Translation Inhibitor. Stromule formation after triggering the stress response with MeJA and simultaneous inhibiting the translation of RNA by cycloheximide. Scale is 10 µm.

This short experiment confirmed the hypothesis stated at the end of chapter “3.1 Time Course”. Comparing the sample with MeJA added to the control sample without MeJA, there was a clear result. In Figure 4 there are multiple long and highly distributed stromules and in the control sample (shown in Figure 13 in chapter 8 Appendix) there were none.

3.3 Cytoskeleton Disruption

To determine the dependency of stromules on the cytoskeleton it was observed if stromules form when AF and MT are inhibited. First, two plastid fluorescent *BY-2* cell samples were treated with 2 µM of latrunculin B for in (A) and 10 µM of oryzalin in (B). Each treatment lasted for 1 hour. Then, both samples got treated with 100 µM MeJA for 1 hour. This resulted in a trigger of the plant stress response in cells in which actin polymerization was inhibited (A) and which MTs got depolymerized (B).

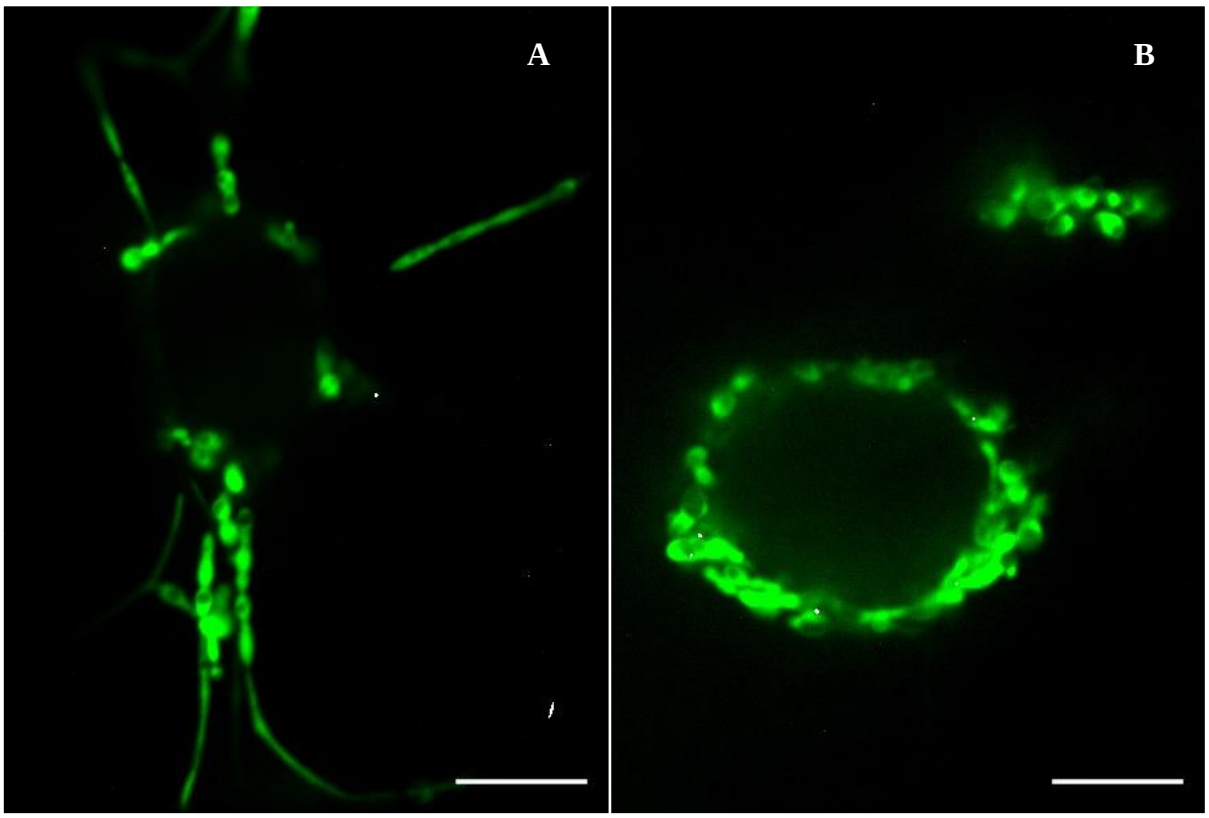


Figure 5: Stress Response with Cytoskeleton Inhibitors. Stromule formation after triggering the stress response with MeJA and inhibiting the actin polymerization with latrunculin B (A) and depolymerizing MTs with oryzalin (B). Scale is 10 µm.

The experiment was quite clear. In the sample where AFs were absent (A), stromules still formed in high numbers. In the sample in which MTs were disrupted (B) only very small excrescences could be observed. Nevertheless, these could not be labelled as stromules. It seems that stromule formation is highly dependent on MTs.

Another approach was made by fluorescently staining AF and MT and observing their relation to stromules via fluorescent microscopy. For that, plastid fluorescent *BY-2* cells were treated with 100 µM MeJA for 1 hour, to trigger stromule formation. Then, AFs were stained with TexasRed-Phalloidin after the method of Kakimoto and Shibaoka (1987) which was modified by Olyslaegers and Verbelen (1998).

MTs on the other hand were visualized with the method of (Kühn 2014). The method covered immunofluorescence staining. The secondary antibody here was fused to TexasRed. This staining approach did not work as desired. Because of that and reasons of time no analysis of the visualization could be made. The staining is visible in Figure 14 of chapter 8 Appendix.

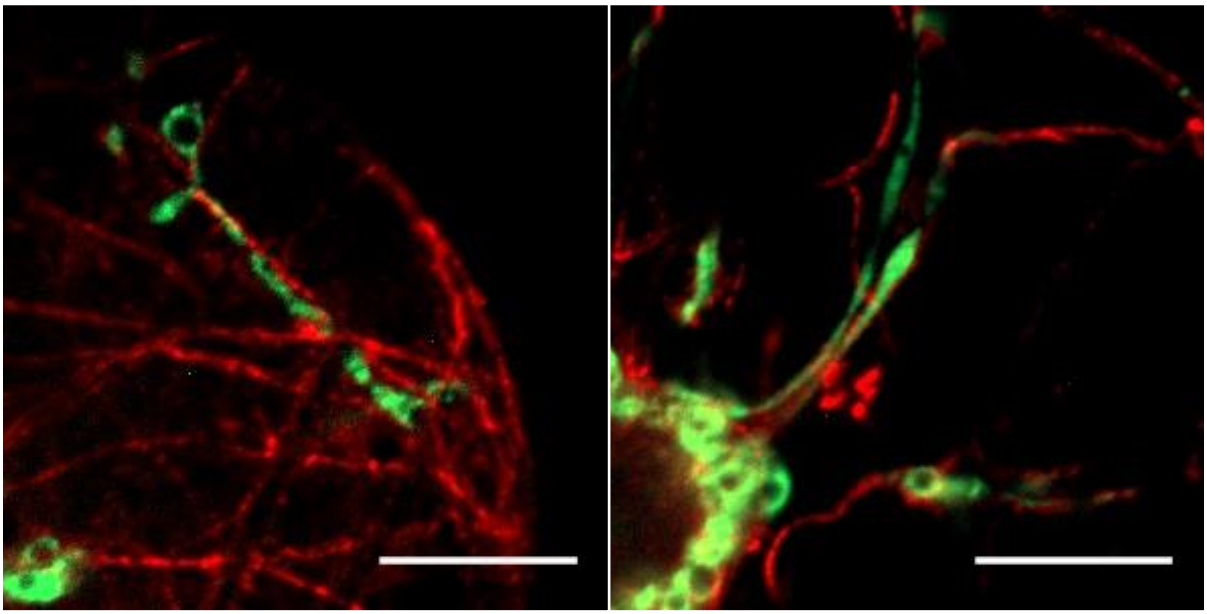


Figure 6: Stress Response with stained Actin Filaments 1. Stromule formation after triggering the stress response with MeJA and AF after staining with TexasRed-Phalloidin. Scale is 10 µm.

In some areas, there had been stromules that were very near to AFs. This suggests that stromules are reliant on AF for motility. In Figure 6 some stromules partially conform to AFs. When green and red fluorescent signals cover each other directly, they are seen as a yellow-orange color instead of their previous. This has not been observed on this occasion, meaning stromules and AF are not in the closest proximity. For the most part, however, some stromules were in strict opposite directions than AFs. In Figure 7 you can see that some stromules even cross AFs vertically. It suggests that stromules are not reliant on AFs for formation and stability, because if they needed AFs, they would be surrounded and guided by AFs. The observations in Figure 6 could only apply to motility. But because *Nicotiana tabacum* is a highly vacuolated plant, the two structures had no other way to align in the same area, since there is sometimes no other way for structures to move in.

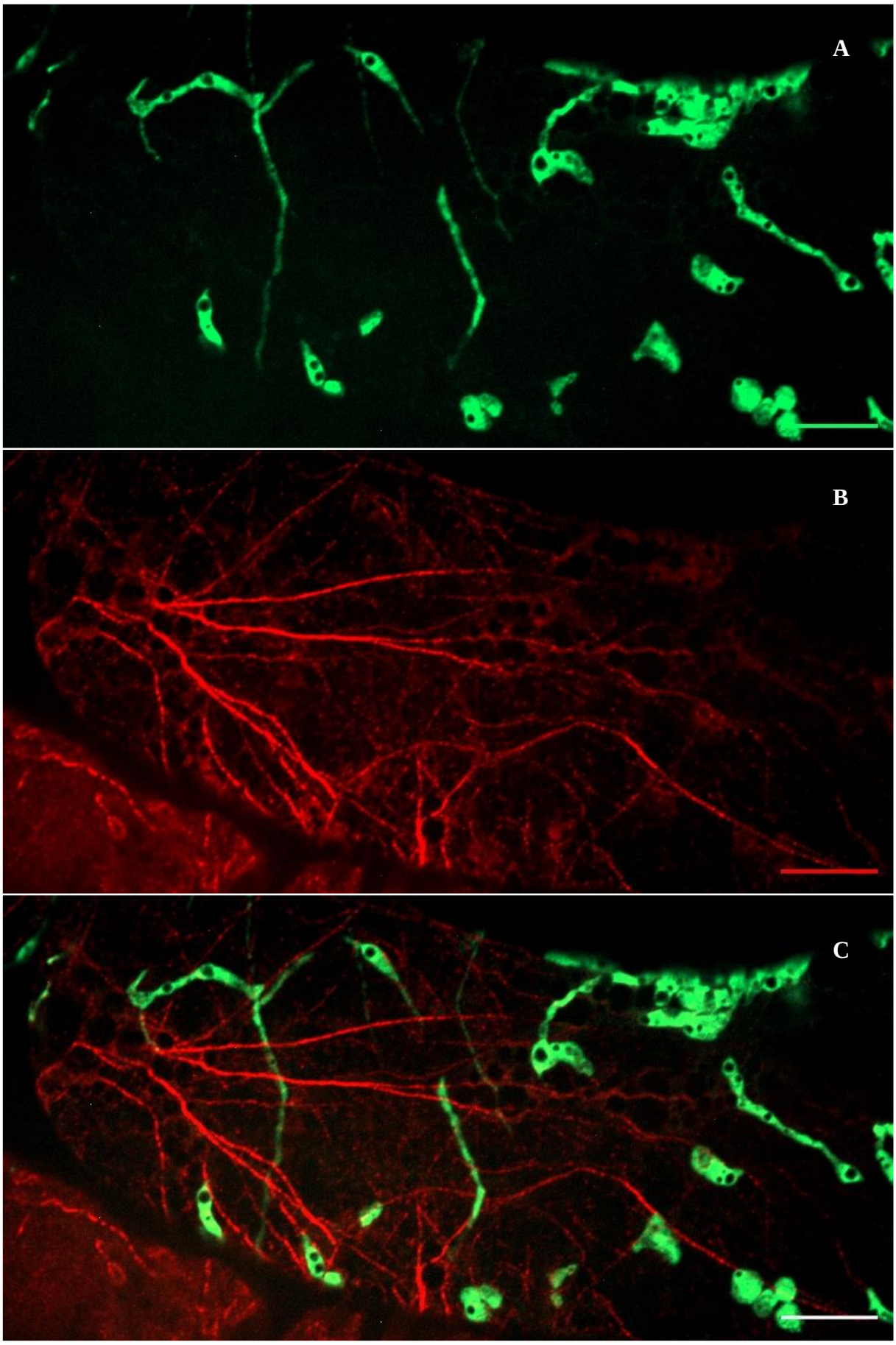


Figure 7: Stress Response with stained Actin Filaments 2. Stromule formation after triggering the stress response with MeJA and AF after staining with TexasRed-Phalloidin. (A) mEOS signal, (B) TexasRed signal, (C) combined. Scale is 10 µm.

3.4 Mitochondrial ROS

The question if mtROS behave in the same way as pROS concerning stromule relation, is still unanswered. To attempt to answer this, mtROS and mitochondria have been stained in plastid fluorescent *BY-2* cells that were undergoing a stress response. For that, the cells were treated with 100 µM MeJA for 1 hour, to trigger stromule formation. Then mtROS were stained with MitoSOX Red and mitochondria were stained with MitoTracker Red CMXRos after the method of Thermo Fisher Scientific Inc. (Waltham, USA).

Stromules and mtROS seem to be connected in a particular manner (Figure 8). At first glance, it looks like that stromules reach for mtROS because mtROS are located at the end and around the stromule. In the control (Figure 15, chapter 8 Appendix) were no mtROS visible.

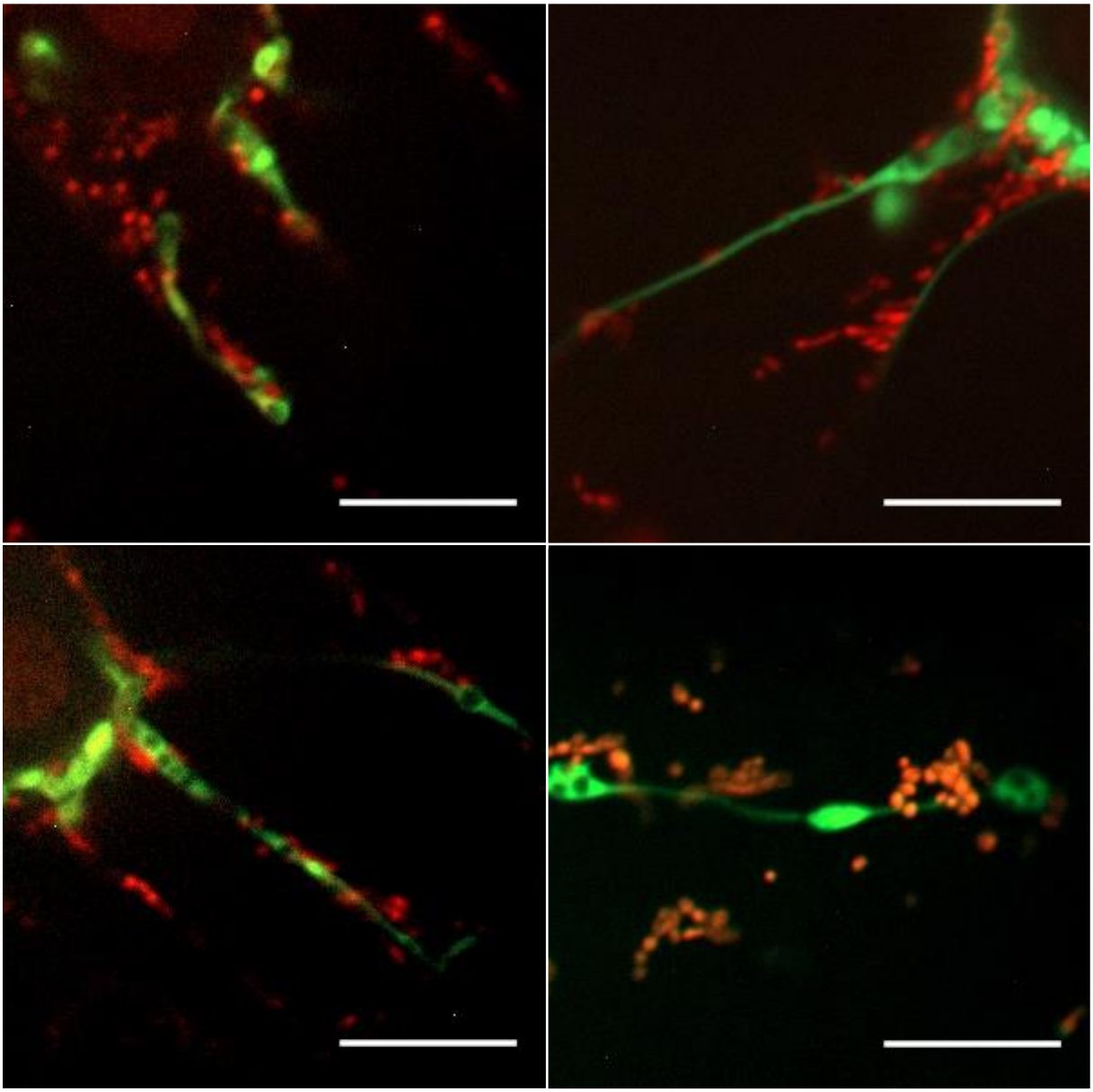


Figure 8: Stress Response with stained mtROS. Stromule formation after triggering the stress response with MeJA and mtROS after staining with MitoSOX Red. Scale is 10 µm.

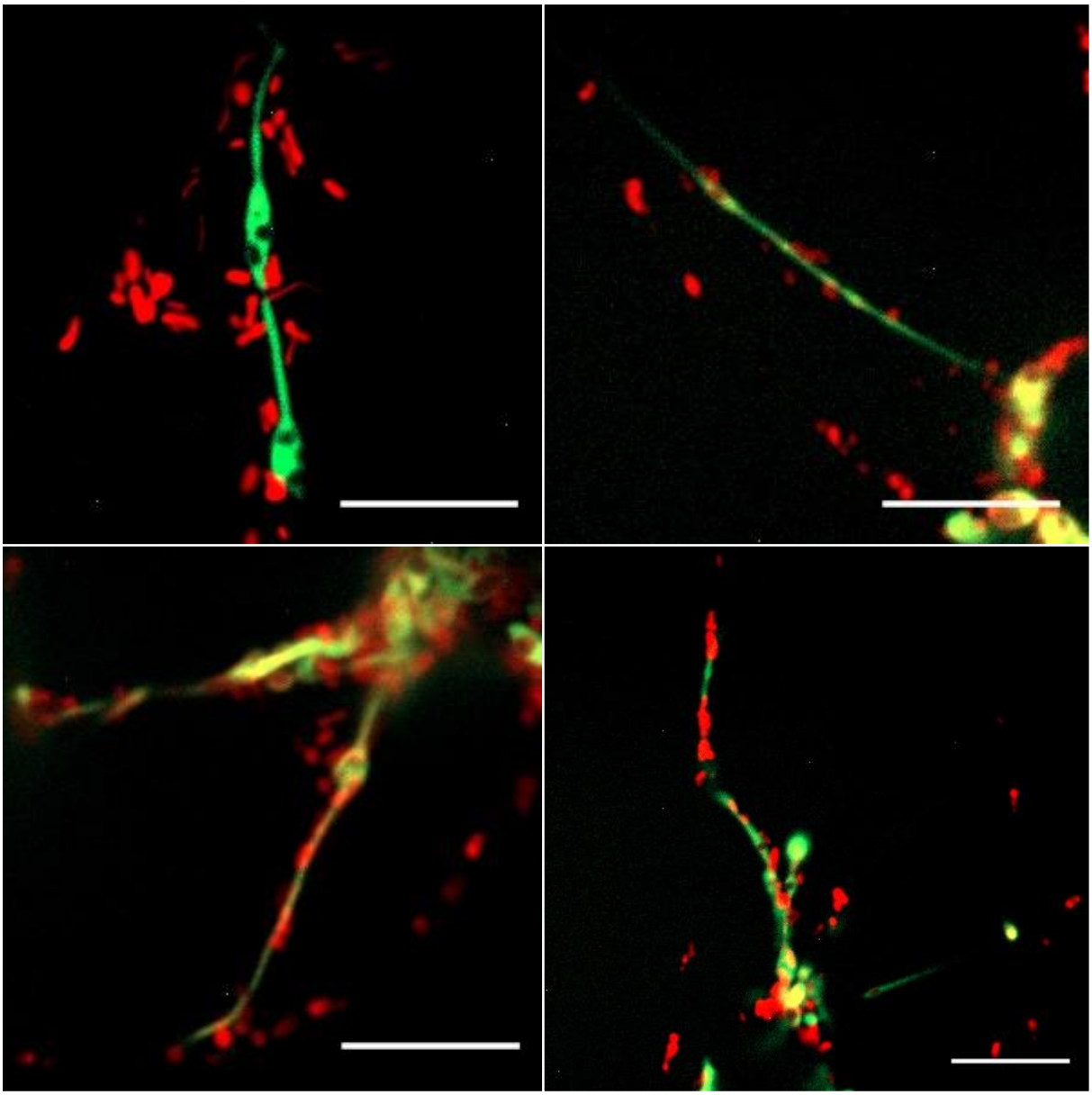


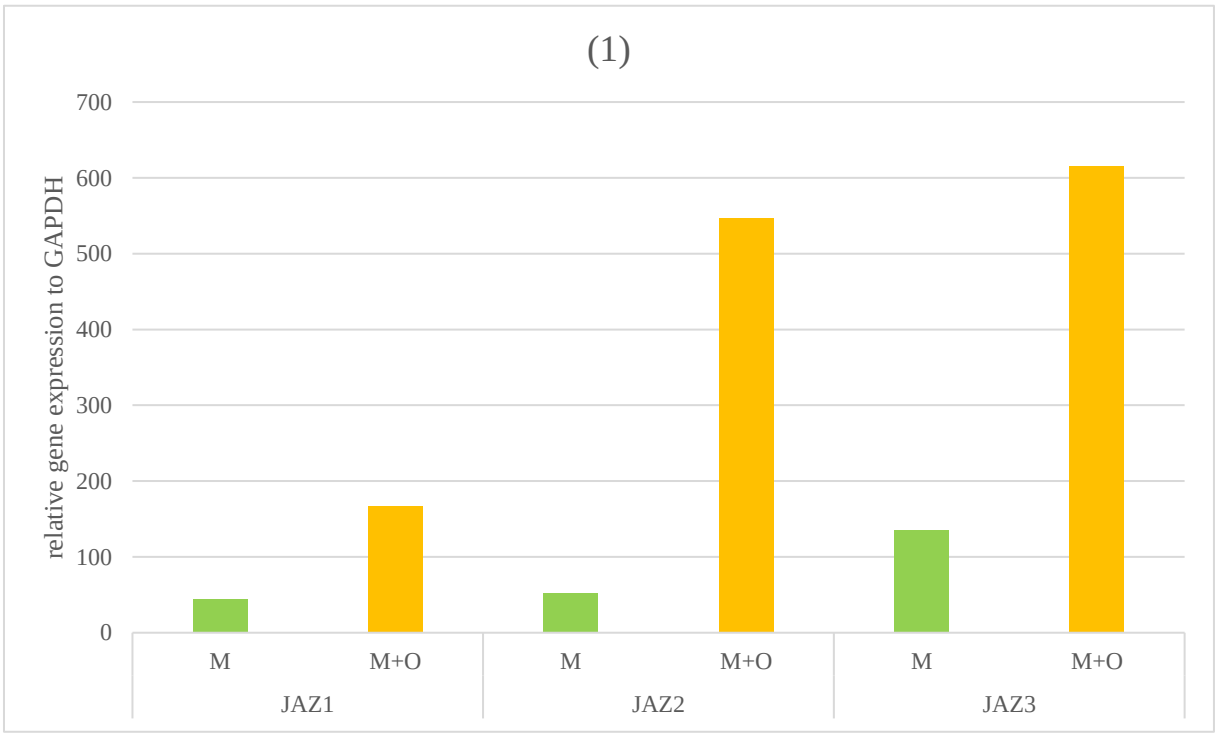
Figure 9: Stress Response with stained Mitochondria. Stromule formation after triggering the stress response with MeJA and mitochondria after staining with MitoTracker Red CMXRos. Scale is 10 µm.

After visualizing mitochondria, not quite the same result has been achieved as after mtROS visualization. In Figure 9, there is also a seemingly high relation between stromules and mitochondria, compared to the control (Figure 16, chapter 8 Appendix) in which it is clear that mitochondria are not interacting with plastids. But at a second glance, mitochondria are also distributed where no stromules are located. This could mean that stromules focus on mtROS rather than mitochondria themselves. Therefore, there have to be mitochondria in Figure 9 that did not produce mtROS yet in a concentration that is recognizable by stromules, meaning that stromules need a specific threshold to act.

3.5 Gene Expression Analysis

Before qPCRs could be realized, the primers (Table 5) needed to be checked. This was done by a standard PCR and a subsequent agarose gel electrophoresis. In Figure 17 (Chapter 8 Appendix) it is visible that the primers worked as desired. The right sequence length has been multiplied. This preliminary work showed that the ordered primers were suitable for further experiments regarding gene expression analysis.

The gene expression analysis was needed to detect whether the presence of stromules make a difference in gene expression of specific genes in cell cultures that were treated with MeJA. At the start of the experiment, biological triplets of three-day old *BY-2* cell cultures were prepared for each treatment. The first treatment was a control culture that has been not treated with any stress response inducing substance. The second treatment was a culture that was treated with 100 µM of MeJA for 1 hour. This guaranteed that the culture was performing a stress response in which stromules are being formed. The third and last treatment was a culture that was also treated with 100 µM of MeJA for 1 hour, but beforehand it was treated for 1 hour with 10 µM of oryzalin that depolymerized MTs and therefore prevented stromule formation. With this setup, there were the treatments “stress response with stromules”, “stress response without stromules” and “no stress response”. The genes selected to observe were JAZ1, JAZ2 and JAZ3 with the inclusion of the housekeeping gene GAPDH. In the following figures, one can see the results of the qPCR relative gene expression to GAPDH of each biological triplet.



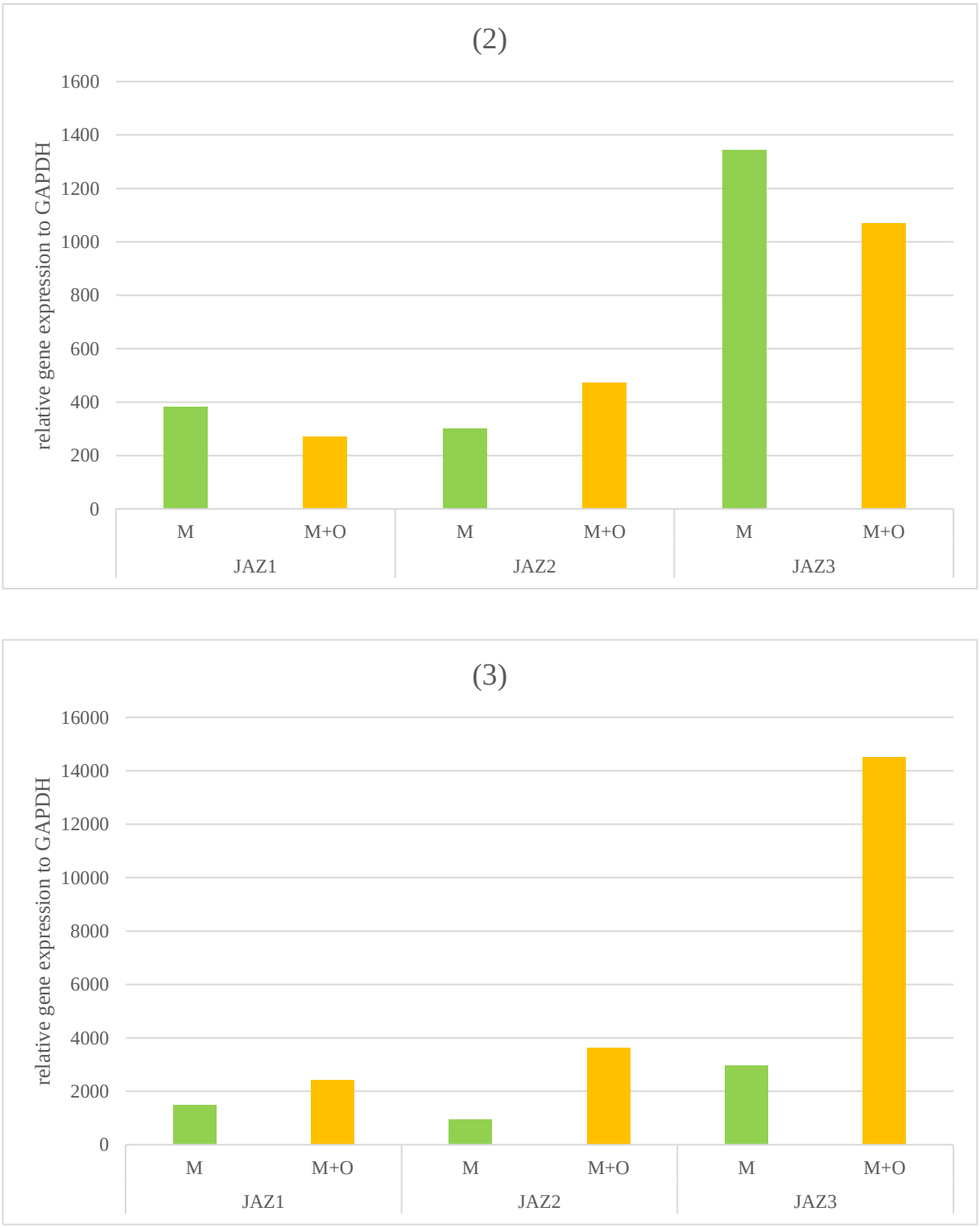


Figure 10: The relative gene expression of JA responsive genes with different treatments. Shown are the three different biological replicates ((1), (2) and (3)) from the results of the qPCR. The genes in question are JAZ1, 2, 3. The gene expression is shown relative to the housekeeping gene GAPDH. M for "MeJA" meaning "stress response with stromules", M+O for "MeJA and oryzalin" meaning "stress response without stromules", C for "control" meaning "no stress response".

Both times in (1) and (3), the relative gene expression to GAPH is higher in the samples with the stromule preventing substance. Meaning regarding those two results, that the JA responsive genes JAZ1, 2 and 3 are more expressed during stress response without stromules than with stromules. Therefore, stromules would inhibit the expression. In (2) on the other hand, the gene expression of JAZ1 and JAZ3 shows the opposite. However, the difference in gene expression between the samples with and without stromules in (2) is minor than in (1) and (3).

The results of the biological triplets show that the gene expression varies a lot between them. An indicator is the scaling of the vertical axis, which has its maxima reaching from 700 to 16000. To overcome this problem, the ratio of the conditions "stress response with stromules" (M) to "stress response without stromules" (M+O) was calculated.

To be sure that the results are statistically significant, the Chi-squared test was used. This test was chosen because the results contain proportions and not simple values. The null hypothesis stated that there is no significant difference in the gene expression between M and M+O. The test results were significantly smaller than 0.01 and therefore the null hypothesis is rejected.

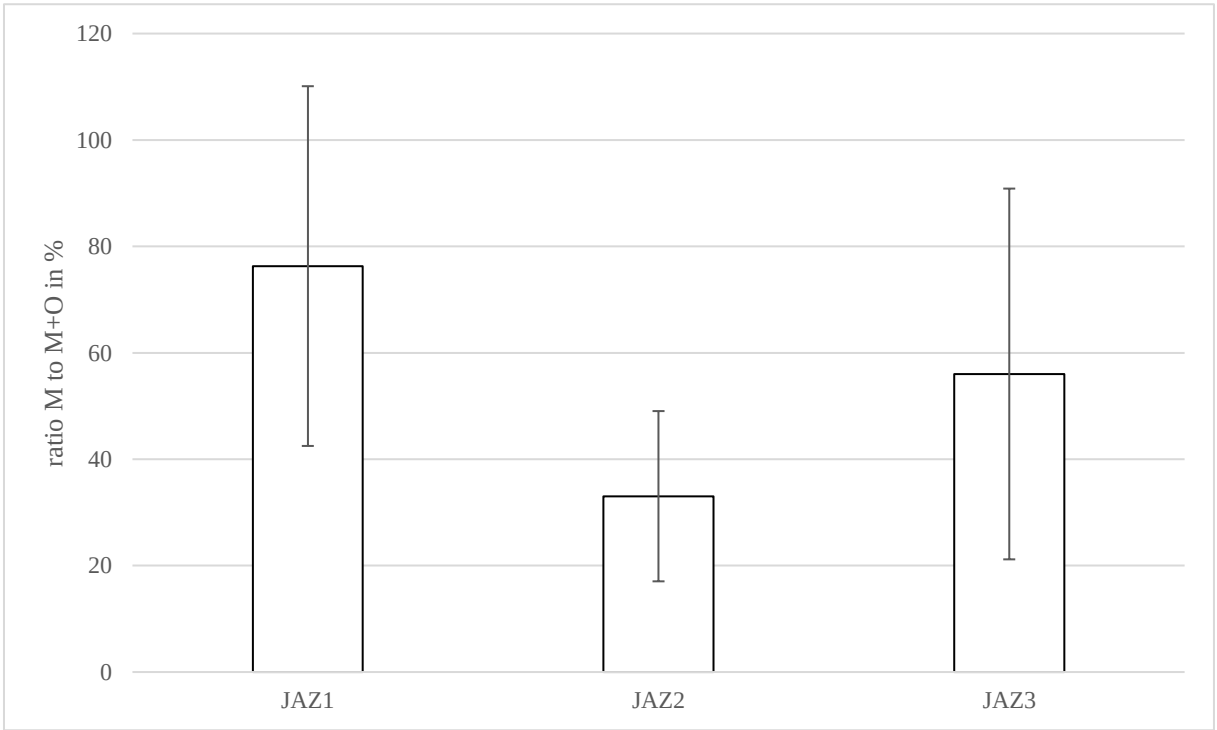


Figure 11: Ratio M to M+O of relative gene expression of JA responsive genes. Shown are ratios of the treatments M to M+O. The genes in question are JAZ1, 2, 3. M for "stress response with stromules", M+O for "stress response without stromules".

In Figure 11 you can see that all genes are higher expressed when the stress response was fulfilled without stromules than with stromules. Although the standard error is objectively high in comparison to the column size, the Chi-squared test still confirms a significant difference.

4 Discussion

4.1 Stromule Dependency

Stromules seem to be independent of transcription or translation. This was observed after inhibiting the translation with cycloheximide. In this approach, the stromule density, as well as the peculiarity, stayed unchanged to the control which remained untreated with the translation inhibiting substance. The reason for this is most probably that stromules are highly appurtenant to the plant stress response. In natural conditions, this stress response has to be executed within minutes, to either grant survival of the plant itself or to warn adjacent plants so that they can initiate their own defense response. The process of DNA transcription, the subsequent translation of RNA and the following protein synthesis takes roughly about one day in eukaryotes (Milo and Phillips 2015). If stromule formation was dependent on this process it would be surely too slow for the plant to react.

Another candidate for factors, on which stromules could depend, is the presence and accumulation of mtROS. In this research, it has been visualized that stromules are highly intertwined with mtROS. Both occur during the stress response, so a relationship between these two seems consequential. mtROS have been observed to surround stromules, this is visible in Figure 8. ROS in general can serve as signaling molecules when present in low concentrations. In higher concentrations, however, for example through oxidative stress, ROS damage subcellular organelles and interfere in cell processes. They have been reported to damage DNA, lipids in cell membranes and amplify initial injuries by enhancing inflammatory responses (Auten and Davis 2009). This biological paradox is only possible if the ROS concentration stays under a certain toxicity threshold. It has been shown that there are special ROS pathways that make use of ROS sensors, which stimulate the production of ROS-specific scavengers (D'Autréaux and Toledano 2007).

When taking this into consideration, stromules may possibly be forming because of these ROS signaling. After triggering the defense response through MeJA, the accumulation of mtROS has been highly increased, which is visible comparing Figures 8 and 15. Figure 15 still has some mtROS visible, but not to the extent as Figure 8. This difference could indicate that the toxicity threshold has been exceeded. Stromules are not forming with low mtROS concentrations, only when they accumulated, and the toxicity threshold has seemingly been surpassed.

To determine stromule dependency on the cytoskeleton two approaches have been made. First, AF and MT were separately inhibited by latrunculin B and oryzalin. Latrunculin B binds actin monomers and prevents them from polymerizing. This results in sequestering of G-actin and prevents the assembly of F-actin and furthermore in blocking actin polymerization (Wakatsuki et al. 2001). Oryzalin on the other hand binds to α-tubules of plants and prevents with this the integration of MT. These will be shortened by the natural uninhibited depolymerization until they are completely gone. To ensure that only the formation and not the maintenance of stability is investigated, the cytoskeleton got disrupted before triggering the plant's defense response. In this thesis, it has been shown that regarding only the formation, MT are the only part of the cytoskeleton that stromules depend on. There was a clear result, in which stromules did not form when the cells were treated with oryzalin. Compared to the samples with latrunculin B no particular change to the normal stromule formation without cytoskeleton inhibitors has been observed. To support this claim, the cytoskeleton also got visualized and its relation to stromules have been observed.

When AF were visualized their relation to stromules occurred in two different ways. On some occasions, both structures aligned with each other (Figure 6), seemingly supporting the claim that stromules are reliant on AF for motility (Gunning 2005; Sattarzadeh et al. 2009). For the most part, however, they did not align, rather showing the exact opposite (Figure 7), meaning stromules and AF were oriented in different directions. The studies that claim that stromules are reliant on AF were realized on plants that carry chloroplasts.

A study that researched on non-green plastids of *Nicotiana tabacum*, showed that there is a difference in stromule formation dependency (Kwok and Hanson 2003) and another study on non-green plastids of *Nicotiana benthamiana* showed that there is also a difference in stromule motility (Erickson et al. 2018). Both studies claim that this task is adopted by MT.

In the study of Erickson et al. (2018) it is shown that, after visualizing stromules and MT, they align. Time-laps movies revealed that stromules can be seen moving along MT as it is extending. Branching has been shown to occur when stromules simultaneously follow different MT after junctions. In some cases, however, stromules were independent of MT. These stromules were different in the pace of formation and movement. Those that were faster had a velocity that was six times higher than the slower ones. Slower moving stromules were dependent and faster moving stromules were independent of MT. Faster stromules did not just form faster, they also were short-lived.

To summarize, stromules of non-green plastids most certainly mainly depend on MT for formation and motility, but partially there are uncontrolled stromules that require presumably AF instead. In this thesis, there have also been sightings of AF aligning with stromules. This could be explained to be short-lived stromules. But to keep in mind that *BY-2* is highly vacuolated, so sometimes there is no other possibility for organelles and subcellular structures than to move in little cytoplasmic strands embedded in large vacuoles and therefore having no particular connection than just being at the same location.

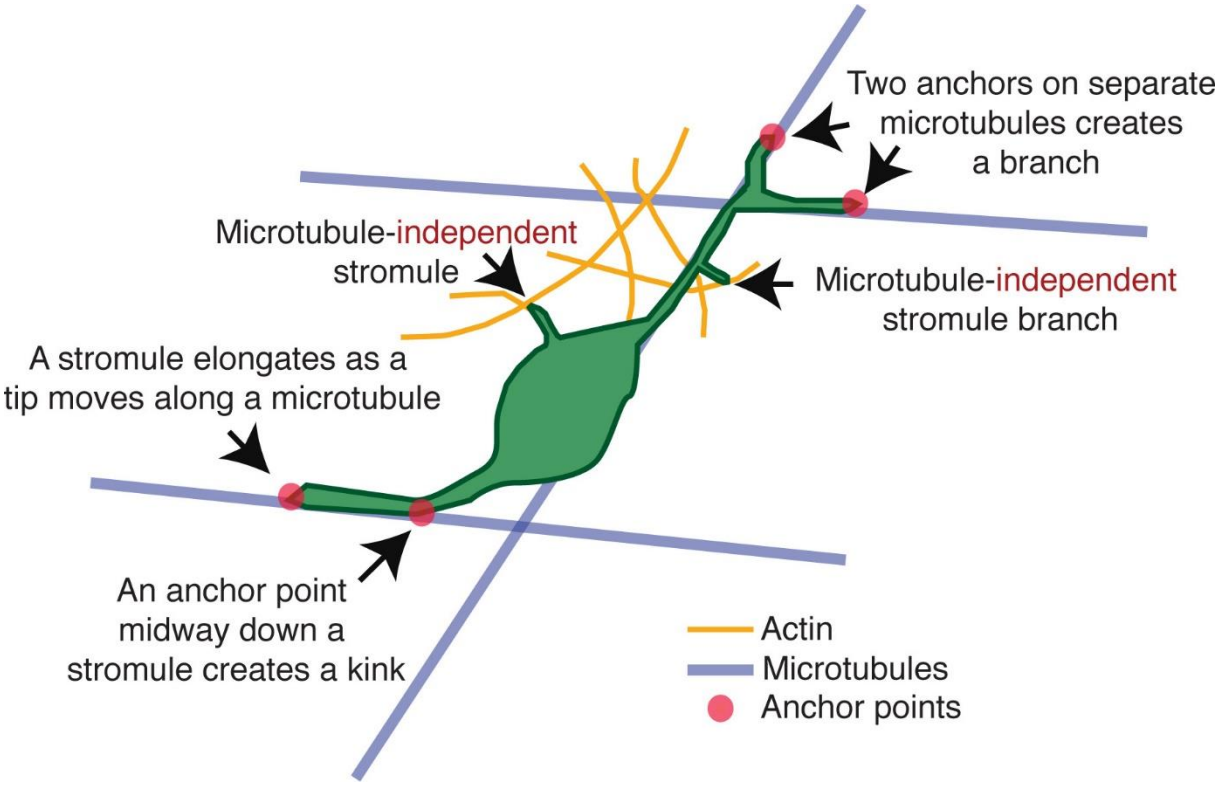


Figure 12: Stromule dependency on the cytoskeleton. Cooperation of AF and MT for different stromule types. AF associated stromules were short-lived and had a high velocity and MT associated stromules were long-lived, had a low velocity and developed branches by anchoring to two different stromules. Figure by Erickson et al. (2018).

Figure 12 shows the above-mentioned cooperation between AF and MT in stromule motility and formation. This figure also mentions the role of anchor points that are responsible for stromule branching and that is exclusively associated with MT dependent stromules.

4.2 Stromule Function

It has already been hypothesized that stromules are dependent on the mtROS accumulation in the plant cell. Likely that there is also a function that stromules have which includes mtROS. Studies in chloroplasts working with pROS have shown that stromules form connections from chloroplasts to the nuclei. This was interpreted as a chloroplast-to-nuclei signal transport. The signal which got transported was H_2O_2, a ROS. Stromules that were reaching to the nuclei got surrounded by nuclear envelope, suggesting a direct membrane-membrane interaction. Presumably to transport ROS through this connection. They observed the ROS concentration in chloroplasts and nuclei and found out that through stromule formation, the concentration in chloroplasts was reducing and, in the nuclei, it was accumulating. This rise in ROS levels could surpass the threshold that is required to induce a defense response, meaning the activation of stress-responsive genes in the nuclei (Caplan et al. 2015). This approach will question the assumption for stromule function. It has been assumed that stromules are the result of the stress response induction, not the force that initiates the stress response in the first place. This approach however does not mean that stromules have only one function, they could be the initiator and the result of the defense response.

Applying this take from pROS to mtROS. The grand difference is that chloroplasts produce their own ROS, meaning that they are directly in the stroma of the plastid and easy for the stromules to transport. mtROS on the other side is produced in a different organelle, the mitochondria. In Figures 8 and 9 there have been made observations that stromules and mtROS are highly associated with each other. The question here would be if these associations have the function of mtROS uptake to the stromule to then later transport them into the nuclei to trigger the stress response. Regarding the research on pROS of Caplan et al. (2015), stromules are capable of membrane-membrane interaction. So, if mtROS can get transported into the plastid, it has to be similar to the stromule-nuclei docking. This docking had been observed to happen in nuclear grooves. These invaginations of the nuclear envelope might be associated with nuclear pores. In the research it was not possible to determine if those nuclear grooves were, in fact, nuclear pores, only a close proximity to them have been detected (Caplan et al. 2015). So, if mitochondria have a way to simulate or reproduce this membrane interaction, it might be possible that stromules first take up mtROS of their origin and then relocate them into the nuclei for defense response induction, like they do with pROS.

The genes that are expressed as a result of ROS signaling, are ROS responsive genes and not JA responsive genes. To investigate those dependent on JA, the gene expression of JAZ1, JAZ2 and JAZ3 were analyzed under different conditions. The result was that in every gene there was a higher gene expression in the samples without stromules. Meaning stromules serve as an inhibiting factor. For JAZ1 the expression was 76.3 % higher, in JAZ2 33.01 % and in JAZ3 56.02 % without stromules than with stromules.

First, it was assumed that stromules could enhance the expression, because they may be facilitating the transport of OPDA to the peroxisomes by developing membrane-membrane interactions, like observed during pROS transport (Caplan et al. 2015). This assumption now has to be questioned. A hypothesis is that stromules are formed during the OPDA synthesis. The plastid could be preventing the molecules to be transported out via the peroxisomal ABC transporter CTS. This accumulation of OPDA in the plastid could result in a physical swelling of the envelope membrane and the extension of excrescences. This however would mean that stromule formation would be arbitrary, which got already falsified. Nevertheless, it still might be possible that stromules are preventing the OPDA transport to the peroxisomes. A reason for that could be that the intermediate dn-OPDA is also its own signal which has its own dn-OPDA specific responsive genes. Stromules may have the function to ration the dn-OPDA amount to different areas to be used in different ways. For example, as its own signal or for the transformation into JA and its derivates. Also, arabidopsides could play a role in this activity. By preventing OPDA from being synthesized into JA, they may get stored in arabidopsides instead.

One factor on the other hand must not be disregarded. To force stromules not to be formed, MT which stromules mainly rely on for formation, got inhibited. This inhabitation could not just result in stromule prevention, but also in more stress for the plant cell. MT are a key structure of the cytoskeleton that manages a lot of things in the cell, like determining the cell shape and guiding the deposition of cellulose microfibrils, which controls the mechanical anisotropy of the cell wall (Mirabet et al. 2018). This could raise the plant stress level, explaining the higher gene expression of the defense response.

Besides all these speculations, it is sure that there is much that has to be discovered to state if these hypotheses happen to become true.

5 Abstract and Perspectives

This thesis studied the general dependency and interaction of stromules with other subcellular structures in the plant cells of *Nicotiana tabacum*. First, it was researched on cytoskeleton and gene expression dependency and afterwards stromule function in and interaction with mtROS from mitochondria and JA responsive gene expression.

It has been discovered which part of the cytoskeleton is necessary for stromule formation. Mainly stromules depend on MT when they are long-lived, but short-lived and spontaneous stromules depend on AF. Special anchors on the cytoskeleton enable stromule branching, by stromules being connected to two anchors on different MT.

Furthermore, it has been discovered that stromules are independent of translation, because of their fast-forming speed and their formation, although being treated with translation inhibitors. This fast-forming speed is needed for the survival of the plant by inducing a fast defense response.

Stromules have been observed to be highly intertwined with mtROS and mitochondria. Concerning stromule function, it might be possible that there is a connection to mtROS like it is with pROS. Stromules may transport mtROS from mitochondria to the nuclei to induce ROS responsive genes. They could potentially also try to transport mtROS to organelles that dispose of ROS, so they do not harm the cell by amplifying initial injuries. Nevertheless, it has to be investigated if mtROS are taken up by stromules from mitochondria with membrane-membrane interaction. Another approach would be to observe ROS levels in stromules and mitochondria simultaneously, to maybe see an increase of accumulation in plastids and a decrease of ROS in mitochondria.

There has been discovered a relation to stromules and JA responsive gene expression. With stromules, the gene expression is inhibited, maybe because stromules prevent OPDA to get synthesized into JA. To further standardize these results, the experiment could be done by using cells of different cell ages, so cells that are and cells that are not in the proliferation phase. This could result in different effects. Furthermore, instead of the gene expression of JA responsive genes, the JA-Ile, such as the OPDA quantity could be detected with a gas chromatography-mass spectrometry. Maybe these values could result in new ideas on how stromules affect not only JA- and OPDA- responsive genes, but also their connection to mtROS and mtROS responsive genes.

6 Tables

6.1 Table of Figures

6.2 Table of Tables

6.3 Table of Abbreviations

Abbreviation	Definition
13-AOS	13-allene oxide synthase
13-LOX	13-lipoxygenase
26S	26S proteasome
AF	actin filament(s)
AOC	allene oxide cyclase
ATT	anti-alpha tubulin
bp	base pair
BSA	bovine serum albumin
BY-2	*Bright-Yellow-2*
cDNA	complementary deoxyribonucleic acid
cis-/OPDA	12-oxo-phytodienoic acid
COI1	coronatine insensitive 1
Ct	cycle threshold
CTS	COMATOSE
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dn-OPDA	dinor-12-oxo-phytodienoic acid
dNTP	deoxyribonucleotide triphosphate
EGTA	egtazic acid
HA	hexadecatrienoic acid
JA	jasmonic acid/(+)-7-iso-jasmonic acid
JA-Ile	jasmonoyl-L-isoleucine
JAR1	jasmonate resistant 1
JAT1	jasmonic acid transfer protein 1
JAZ	jasmonate ZIM-Domain protein
JMT	jasmonic acid carboxyl methyltransferase
kDa	kilodalton
MeJA	methyl jasmonate/ (+)-methyl jasmonate
MS	Murashige and Skoog
MSB	microtubule-stabilization buffer

MT	microtubule(s)
mtETC	mitochondrial electron transport chain
mtROS	mitochondrial reactive oxygen species
NINJA	NOVEL INTERACTOR OF JAZ
$O_2^{\cdot -}$	superoxide
OPC-6	6-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid
OPC-8	8-(3-oxo-2-(pent-2-enyl)cyclopenthyl) octanoic acid
OPR3	12-oxo-phytodienoic acid reductase
P	probability
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pETC	photosynthetic electron transport chain
PFA	paraformaldehyde
pROS	photosynthetic reactive oxygen species
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RuBisCO	ribulose-1,5-biphoosphate carboxylase oxygenase
SB	standard buffer
SCF	Skp1, Cullin and F-box proteins
TAE	tris-acetate-EDTA
TF	transcription factor
TPL	TOPLESS protein
U	unit
UV	ultraviolet
α-LA	α-linolenic acid
β-OX	β-oxidation

7 Bibliography

Asfaw, Kinfemichael Geressu; Liu, Qiong; Xu, Xiaolu; Manz, Christina; Purper, Sabine; Eghbalian, Rose et al. (2020): A mitochondria-targeted coenzyme Q peptoid induces superoxide dismutase and alleviates salinity stress in plant cells. In *Scientific reports* 10 (1), p. 11563. DOI: 10.1038/s41598-020-68491-4.

Auten, Richard L.; Davis, Jonathan M. (2009): Oxygen toxicity and reactive oxygen species: the devil is in the details. In *Pediatric research* 66 (2), pp. 121–127. DOI: 10.1203/PDR.0b013e3181a9eafb.

Berkowitz, Oliver; Clercq, Inge de; van Breusegem, Frank; Whelan, James (2016): Interaction between hormonal and mitochondrial signalling during growth, development and in plant defence responses. In *Plant, Cell & Environment* 39 (5), pp. 1127–1139. DOI: 10.1111/pce.12712.

Bhat, Riyaz A.; Thompson, Richard D. (2004): The Tobacco BY-2 Cell Line as a Model System to Understand in Planta Nuclear Coactivator Interactions. In Toshiyuki Nagata, Horst Lörz, Jack M. Widholm, Seiichiro Hasezawa, Dirk Inzé (Eds.): Tobacco BY-2 Cells, vol. 53. Berlin, Heidelberg: Springer Berlin Heidelberg (Biotechnology in Agriculture and Forestry), pp. 316–331.

Bourett, Timothy M.; Czymmek, Kirk J.; Howard, Richard J. (1999): Ultrastructure of chloroplast protuberances in rice leaves preserved by high-pressure freezing. In *Planta* 208 (4), pp. 472–479. DOI: 10.1007/s004250050584.

Brunkard, Jacob O.; Runkel, Anne M.; Zambryski, Patricia C. (2015): Chloroplasts extend stromules independently and in response to internal redox signals. In *Proceedings of the National Academy of Sciences of the United States of America* 112 (32), pp. 10044–10049. DOI: 10.1073/pnas.1511570112.

Buseman, Christen M.; Tamura, Pamela; Sparks, Alexis A.; Baughman, Ethan J.; Maatta, Sara; Zhao, Jian et al. (2006): Wounding stimulates the accumulation of glycerolipids containing oxophytodienoic acid and dinor-oxophytodienoic acid in Arabidopsis leaves. In *Plant Physiology* 142 (1), pp. 28–39. DOI: 10.1104/pp.106.082115.

Caplan, Jeffrey L.; Kumar, Amutha Sampath; Park, Eunsook; Padmanabhan, Meenu S.; Hoban, Kyle; Modla, Shannon et al. (2015): Chloroplast Stromules Function during Innate Immunity. In *Developmental Cell* 34 (1), pp. 45–57. DOI: 10.1016/j.devcel.2015.05.011.

Chini, Andrea; Monte, Isabel; Zamarreño, Angel M.; Hamberg, Mats; Lassueur, Steve; Reymond, Philippe et al. (2018): An OPR3-independent pathway uses 4,5-didehydrojasmonate for jasmonate synthesis. In *Nat Chem Biol* 14 (2), pp. 171–178. DOI: 10.1038/nchembio.2540.

D'Autréaux, Benoît; Toledano, Michel B. (2007): ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. In *Nature reviews. Molecular cell biology* 8 (10), pp. 813–824. DOI: 10.1038/nrm2256.

Dave, Anuja; Graham, Ian A. (2012): Oxylipin Signaling: A Distinct Role for the Jasmonic Acid Precursor cis-(+)-12-Oxo-Phytodienoic Acid (cis-OPDA). In *Frontiers in plant science* 3, p. 42. DOI: 10.3389/fpls.2012.00042.

Davies, Peter J. (2010): Plant Hormones. Biosynthesis, signal transduction, action! 3., rev. ed. Dordrecht: Springer Netherlands. Available online at http://sub-hh.ciando.com/book/?bok_id=41764.

Domanov, Yegor A.; Kinnunen, Paavo K. J. (2006): Antimicrobial peptides temporins B and L induce formation of tubular lipid protrusions from supported phospholipid bilayers. In *Biophysical journal* 91 (12), pp. 4427–4439. DOI: 10.1529/biophysj.106.091702.

Erickson, Jessica L.; Adlung, Norman; Lampe, Christina; Bonas, Ulla; Schattat, Martin H. (2018): The Xanthomonas effector XopL uncovers the role of microtubules in stromule extension and dynamics in Nicotiana benthamiana. In *The Plant Journal* 93 (5), pp. 856–870. DOI: 10.1111/tpj.13813.

Footitt, Steven; Dietrich, Daniela; Fait, Aaron; Fernie, Alisdair R.; Holdsworth, Michael J.; Baker, Alison; Theodoulou, Frederica L. (2007): The COMATOSE ATP-binding cassette transporter is required for full fertility in Arabidopsis. In *Plant Physiology* 144 (3), pp. 1467–1480. DOI: 10.1104/pp.107.099903.

Gray, John C.; Hansen, Michael R.; Shaw, Daniel J.; Graham, Katie; Dale, Rosemary; Smallman, Philippa et al. (2012): Plastid stromules are induced by stress treatments acting through abscisic acid. In *The Plant journal : for cell and molecular biology* 69 (3), pp. 387–398. DOI: 10.1111/j.1365-313X.2011.04800.x.

Gunning, Brian E. S. (2005): Plastid stromules: video microscopy of their outgrowth, retraction, tensioning, anchoring, branching, bridging, and tip-shedding. In *Protoplasma* 225 (1-2), pp. 33–42. DOI: 10.1007/s00709-004-0073-3.

Haberlandt, Gottlieb (1888): Die Chlorophyllkörper der Selaginellen.

Hanson, Maureen R.; Sattarzadeh, Amirali (2013): Trafficking of proteins through plastid stromules. In *Plant Cell* 25 (8), pp. 2774–2782. DOI: 10.1105/tpc.113.112870.

Herrmann, R. G.; Possingham, J. V. (1980): Plastid DNA-the plastome. In *Results and problems in cell differentiation* 10, pp. 45–96. DOI: 10.1007/978-3-540-38255-3_3.

Hoober, J. K. (2012): Chloroplasts: Springer Science & Business Media.

Huang, Shaobai; van Aken, Olivier; Schwarzländer, Markus; Belt, Katharina; Millar, A. Harvey (2016): The Roles of Mitochondrial Reactive Oxygen Species in Cellular Signaling and Stress Response in Plants. In *Plant Physiology* 171 (3), pp. 1551–1559. DOI: 10.1104/pp.16.00166.

Ibrahim, Amina; Schütz, Anna-Lena; Galano, Jean-Marie; Herrfurth, Cornelia; Feussner, Kirstin; Durand, Thierry et al. (2011): The Alphabet of Galactolipids in Arabidopsis thaliana. In *Frontiers in plant science* 2, p. 95. DOI: 10.3389/fpls.2011.00095.

Kakimoto, T.; Shibaoka, H. (1987): A New Method for Preservation of Actin Filaments in Higher Plant Cells. In *Plant and Cell Physiology*. DOI: 10.1093/oxfordjournals.pcp.a077453.

Kandasamy, Muthugapatti K.; Meagher, Richard B. (1999): Actin-organelle interaction: Association with chloroplast inArabidopsis leaf mesophyll cells. In *Cell Motil. Cytoskeleton* 44 (2), pp. 110–118. DOI: 10.1002/(SICI)1097-0169(199910)44:2<110::AID-CM3>3.0.CO;2-O.

Krenz, Björn; Windeisen, Volker; Wege, Christina; Jeske, Holger; Kleinow, Tatjana (2010): A plastid-targeted heat shock cognate 70kDa protein interacts with the Abutilon mosaic virus movement protein. In *Virology* 401 (1), pp. 6–17. DOI: 10.1016/j.virol.2010.02.011.

Kühn, S. (2014): A Moonlighting Kinesin Modulates Abiotic Sensing.

Kwok, Ernest Y.; Hanson, Maureen R. (2003): Microfilaments and microtubules control the morphology and movement of non-green plastids and stromules in Nicotiana tabacum. In *The Plant journal : for cell and molecular biology* 35 (1), pp. 16–26. DOI: 10.1046/j.1365-313X.2003.01777.x.

Kwok, Ernest Y.; Hanson, Maureen R. (2004a): In vivo analysis of interactions between GFP-labeled microfilaments and plastid stromules. In *BMC Plant Biol* 4 (1), p. 2. DOI: 10.1186/1471-2229-4-2.

Kwok, Ernest Y.; Hanson, Maureen R. (2004b): Plastids and stromules interact with the nucleus and cell membrane in vascular plants. In *Plant Cell Rep* 23 (4), pp. 188–195. DOI: 10.1007/s00299-004-0824-9.

Liu, Deshui; Shi, Lindan; Han, Chenggui; Yu, Jialin; Li, Dawei; Zhang, Yongliang (2012): Validation of reference genes for gene expression studies in virus-infected Nicotiana benthamiana using quantitative real-time PCR. In *PLoS ONE* 7 (9), e46451. DOI: 10.1371/journal.pone.0046451.

Machettira, Anu B.; Groß, Lucia E.; Tillmann, Bodo; Weis, Benjamin L.; Englich, Gisela; Sommer, Maik S. et al. (2011): Protein-induced modulation of chloroplast membrane morphology. In *Frontiers in plant science* 2, p. 118. DOI: 10.3389/fpls.2011.00118.

Malone M. (1996): Rapid, long-distance signal transmision in higher plants: Adv. Bot. Res.

Milo, Ron; Phillips, Rob (2015): Cell Biology by the Numbers: Garland Science.

Mirabet, Vincent; Krupinski, Pawel; Hamant, Olivier; Meyerowitz, Elliot M.; Jönsson, Henrik; Boudaoud, Arezki (2018): The self-organization of plant microtubules inside the cell volume yields their cortical localization, stable alignment, and sensitivity to external cues. In *PLoS Comput Biol* 14 (2), e1006011. DOI: 10.1371/journal.pcbi.1006011.

Moller, Ian M. (2001): Plant Mitochondria and Oxidate Stress: Electron Transport, NADPH Turnover, and Metabolism of Reactive Oxygen Species. In *Annual review of plant physiology and plant molecular biology* 52, pp. 561–591. DOI: 10.1146/annurev.arplant.52.1.561.

Murphy, Michael P. (2009): How mitochondria produce reactive oxygen species. In *Biochemical Journal* 417 (1), pp. 1–13. DOI: 10.1042/BJ20081386.

Olyslaegers, G.; Verbelen, J.-P. (1998): Improved staining of F-actin and co-localization of mitochondria in plant cells. In *J Microsc* 192 (1), pp. 73–77. DOI: 10.1046/j.1365-2818.1998.00398.x.

Oropeza (2005): Between puffs: a history of tobacco: two thousand years of tobacco use. In *Choice Reviews Online* 44 (03), 44-1598-44-1598. DOI: 10.5860/choice.44-1598.

Park, Sang-Wook; Kaimoyo, Evans; Kumar, Dhirendra; Mosher, Stephen; Klessig, Daniel F. (2007): Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. In *Science (New York, N.Y.)* 318 (5847), pp. 113–116. DOI: 10.1126/science.1147113.

Riemann, Michael; Dhakarey, Rohit; Hazman, Mohamed; Miro, Berta; Kohli, Ajay; Nick, Peter (2015): Exploring Jasmonates in the Hormonal Network of Drought and Salinity Responses. In *Frontiers in plant science* 6, p. 1077. DOI: 10.3389/fpls.2015.01077.

Ruan, Jingjun; Zhou, Yuexia; Zhou, Meiliang; Yan, Jun; Khurshid, Muhammad; Weng, Wenfeng et al. (2019): Jasmonic Acid Signaling Pathway in Plants. In *International journal of molecular sciences* 20 (10). DOI: 10.3390/ijms20102479.

Ryan, Clarence A.; Moura, Daniel S. (2002): Systemic wound signaling in plants: a new perception. In *Proceedings of the National Academy of Sciences of the United States of America* 99 (10), pp. 6519–6520. DOI: 10.1073/pnas.112196499.

Sampath Kumar, Amutha; Dinesh-Kumar, Savithramma P.; Caplan, Jeffrey L. (2014): Stromules. In S. M. Theg, Wollman FA (Eds.): Plastid Biology: Springer, New York, NY, pp. 189–207. Available online at https://link.springer.com/chapter/10.1007/978-1-4939-1136-3_7.

Sato, Y.; Wada, M.; Kadota, A. (2001): Choice of tracks, microtubules and/or actin filaments for chloroplast photo-movement is differentially controlled by phytochrome and a blue light receptor. In *Journal of Cell Science* 114 (2), pp. 269–279. DOI: 10.1242/jcs.114.2.269.

Sattarzadeh, Amir; Krahmer, Johanna; Germain, Arnaud D.; Hanson, Maureen R. (2009): A myosin XI tail domain homologous to the yeast myosin vacuole-binding domain interacts with plastids and stromules in Nicotiana benthamiana. In *Molecular Plant* 2 (6), pp. 1351–1358. DOI: 10.1093/mp/ssp094.

Schattat, Martin H.; Griffiths, Sarah; Mathur, Neeta; Barton, Kiah; Wozny, Michael R.; Dunn, Natalie et al. (2012): Differential coloring reveals that plastids do not form networks for exchanging macromolecules. In *Plant Cell* 24 (4), pp. 1465–1477. DOI: 10.1105/tpc.111.095398.

Schneider-Poetsch, Tilman; Ju, Jianhua; Eyler, Daniel E.; Dang, Yongjun; Bhat, Shridhar; Merrick, William C. et al. (2010): Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. In *Nat Chem Biol* 6 (3), pp. 209–217. DOI: 10.1038/nchembio.304.

Senn, Gustav (1908): Gestalts-und Lageveranderung der Pflanzen-Chromatophoren. Available online at https://agris.fao.org/agris-search/search.do?recordid=us201300575596.

Serlin, Bruce S.; Ferrell, Sandra (1989): The involvement of microtubules in chloroplast rotation in the alga Mougeotia. In *Plant Science* 60 (1), pp. 1–8. DOI: 10.1016/0168-9452(89)90037-X.

Stintzi, A.; Weber, H.; Reymond, P.; Browse, J.; Farmer, E. E. (2001): Plant defense in the absence of jasmonic acid: the role of cyclopentenones. In *Proceedings of the National Academy of Sciences of the United States of America* 98 (22), pp. 12837–12842. DOI: 10.1073/pnas.211311098.

Taki, Nozomi; Sasaki-Sekimoto, Yuko; Obayashi, Takeshi; Kikuta, Akihiro; Kobayashi, Koichi; Ainai, Takayuki et al. (2005): 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. In *Plant Physiology* 139 (3), pp. 1268–1283. DOI: 10.1104/pp.105.067058.

Truman, William; Bennett, Mark H.; Kubigsteltig, Ines; Turnbull, Colin; Grant, Murray (2007): Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. In *Proceedings of the National Academy of Sciences of the United States of America* 104 (3), pp. 1075–1080. DOI: 10.1073/pnas.0605423104.

Wada, Masamitsu; Kagawa, Takatoshi; Sato, Yoshikatsu (2003): Chloroplast movement. In *Annual review of plant biology* 54, pp. 455–468. DOI: 10.1146/annurev.arplant.54.031902.135023.

Wakatsuki, T.; Schwab, B.; Thompson, N. C.; Elson, E. L. (2001): Effects of cytochalasin D and latrunculin B on mechanical properties of cells. In *Journal of Cell Science* 114 (Pt 5), pp. 1025–1036. Available online at https://pubmed.ncbi.nlm.nih.gov/11181185/.

Waters, Mark T.; Fray, Rupert G.; Pyke, Kevin A. (2004): Stromule formation is dependent upon plastid size, plastid differentiation status and the density of plastids within the cell. In *The Plant journal : for cell and molecular biology* 39 (4), pp. 655–667. DOI: 10.1111/j.1365-313X.2004.02164.x.

Weiler, Elmar W.; Albrecht, Tanja; Groth, Beate; Xia, Zhi-Qiang; Luxem, Martin; Liß, Harald et al. (1993): Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of Bryonia dioica. In *Phytochemistry* 32 (3), pp. 591–600. DOI: 10.1016/S0031-9422(00)95142-2.

Zimorski, Verena; Ku, Chuan; Martin, William F.; Gould, Sven B. (2014): Endosymbiotic theory for organelle origins. In *Current opinion in microbiology* 22, pp. 38–48. DOI: 10.1016/j.mib.2014.09.008.

8 Appendix

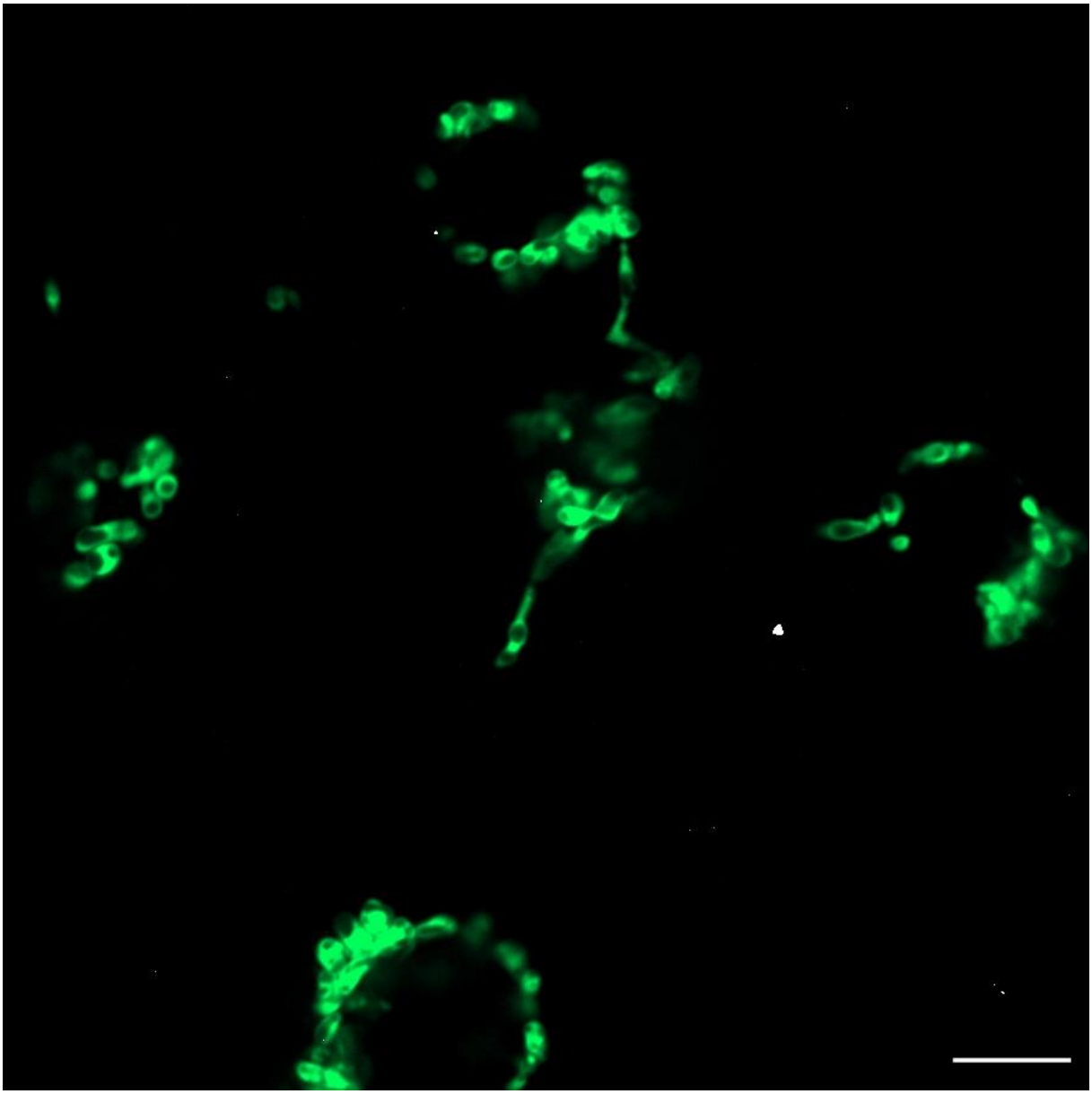


Figure 13: Control of Chapter 3.3 Translation Inhibition. *BY-2* cells with only inhibiting the translation of RNA by cycloheximide and no MeJA added. Scale is 10 µm.

Figure 14: Failed Microtubule staining. Stromule formation after triggering the stress response with MeJA and failed MT staining with immunofluorescence. The secondary antibody contained TexasRed. Scale is 10 µm.

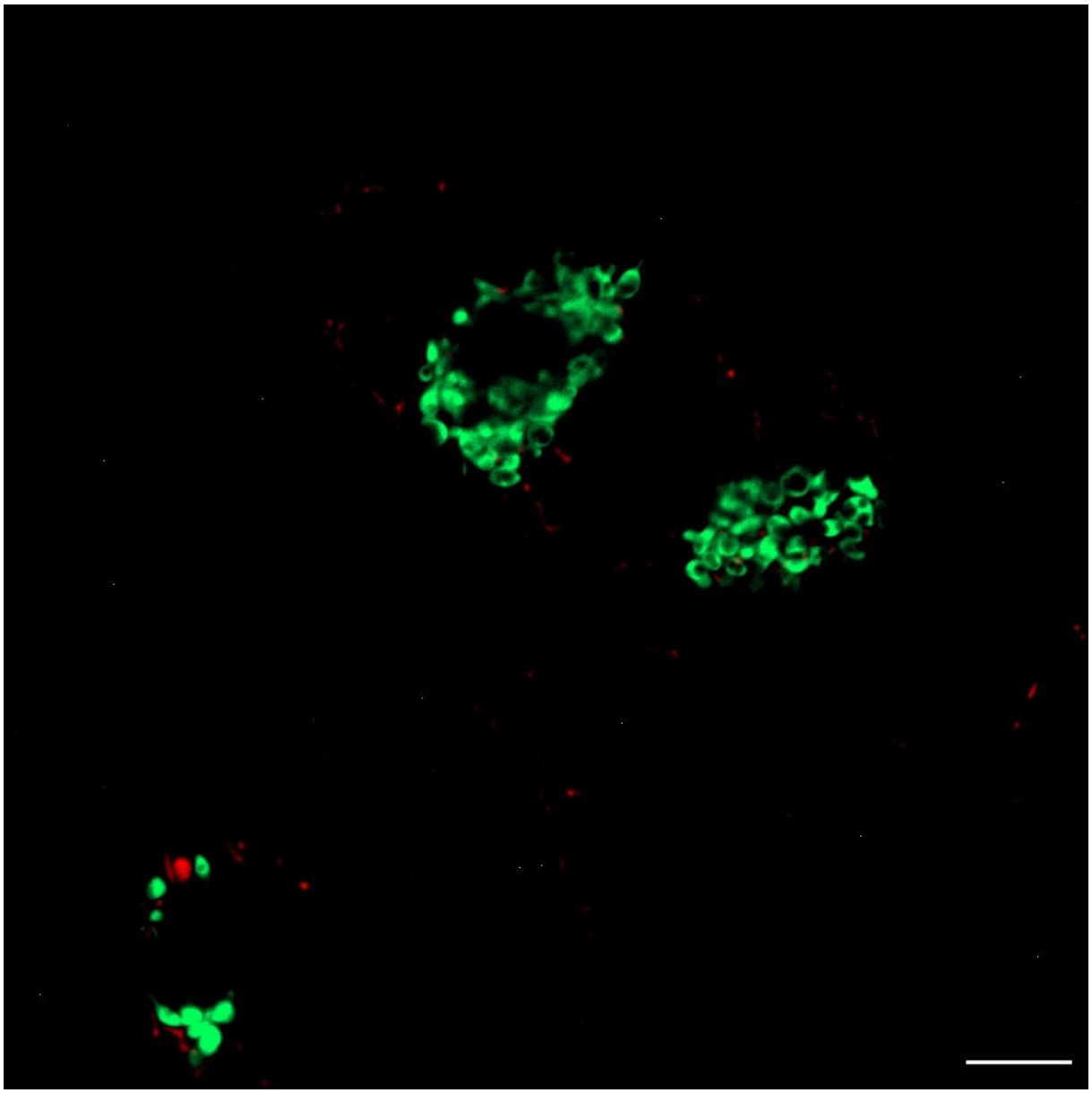


Figure 15: Control of Chapter 3.4 Mitochondrial ROS: mtROS. *BY-2* cells with only mtROS staining with MitoSOX Red and no MeJA added. Scale is 10 µm.

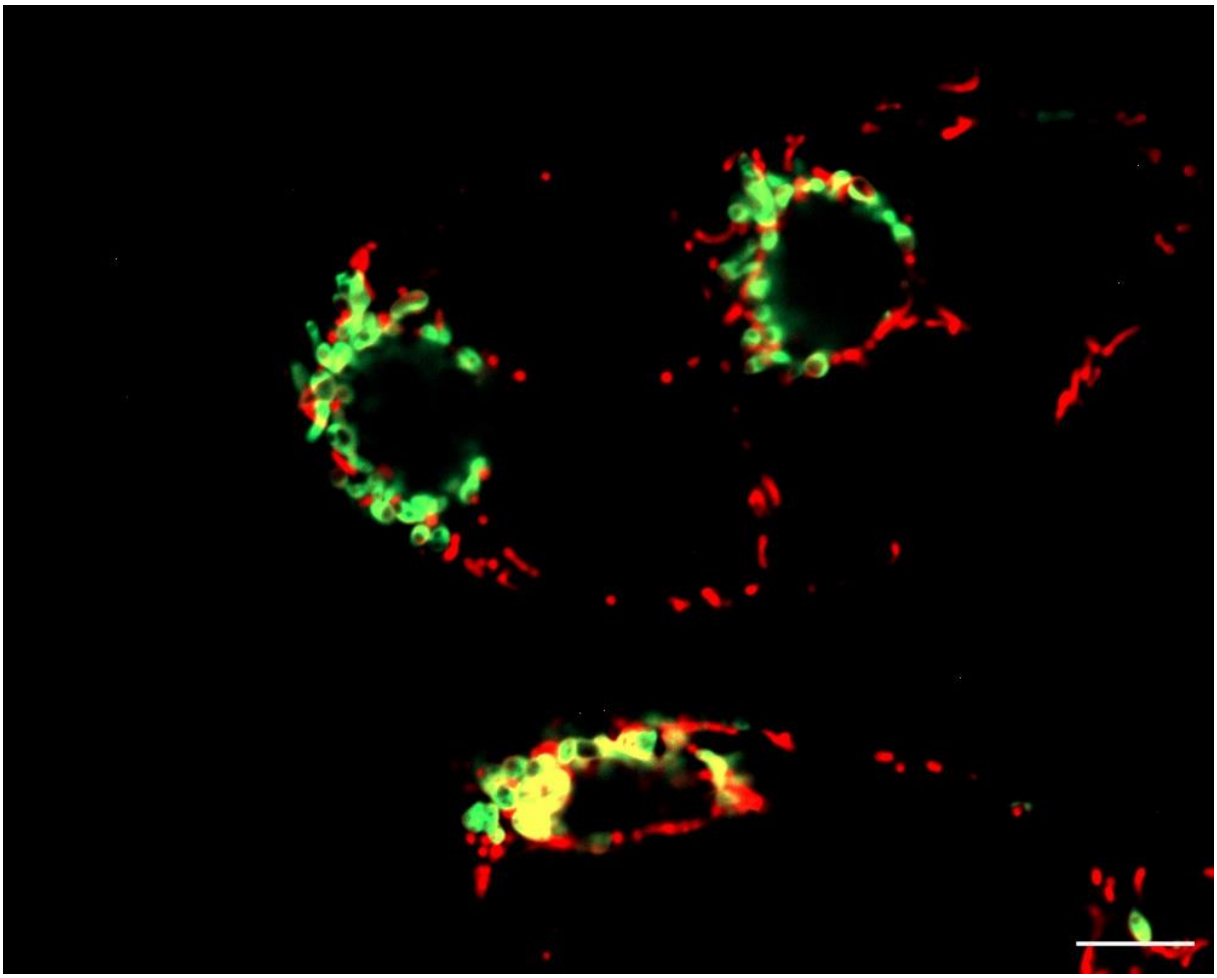


Figure 16: Control of Chapter 3.4 Mitochondrial ROS: Mitochondria. *BY-2* cells with only mitochondria staining with MitoTracker Red CMXRos and no MeJA added. Scale is 10 µm.

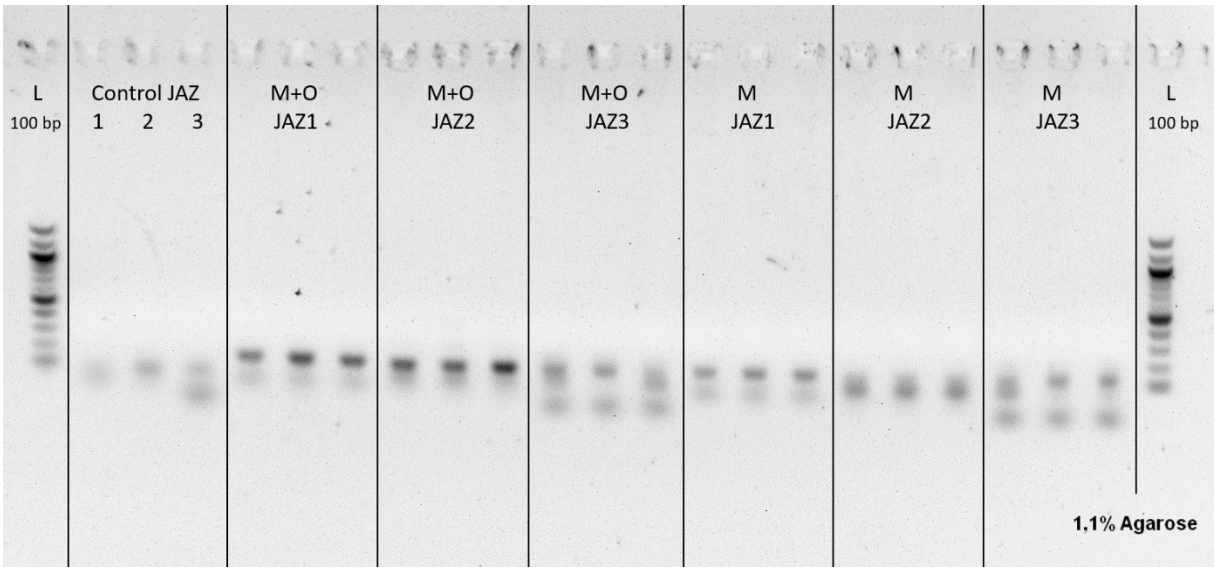


Figure 17:Agarose Gel Electrophoresis for Primer Testing. Visible is one biological triplet from the gene expression analysis. Primers for JAZ1, 2 and 3 were tested. In the control samples in which no cDNA was present, there is still a band visible. This is explained by primer-self- and primer-cross-dimers. Keeping this in mind, there is a significant band in all three genes, meaning the primers worked and helped to quantify the genes.