Movements of Plant Organs: From Root Skototropism to Leaf Mimicking

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1. List of publications

The following chapters of this thesis have been published in different peer-reviewed scientific journals or books, summarizing this cumulative thesis.

1. **Yamashita, F.,** Njimona, I., Yan, X., & Baluška, F. Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions. *Preprints 2023, 2023080291.* https://doi.org/10.20944/preprints202308.0291.v1

2. Yan, X., Liang, Y., **Yamashita, F**., & Baluška, F. Investigation of *Arabidopsis* root skototropism with different distance settings. *Plant Signaling & Behavior*, 19,1 (2024). https://doi.org/10.1080/15592324.2024.2348917

3. Yan, X., **Yamashita, F**., Njimona, I., & Baluška, F. "Root and hypocotyl growth of Arabidopsis seedlings grown under different light conditions and influence of TOR kinase inhibitor AZD." *International Journal of Biotechnology and Molecular Biology Research* 12,2 (2022). https://doi.org/10.5897/IJBMBR2022.0330

4. **Yamashita, F**, and Baluška, F. "Algal Ocelloids and Plant Ocelli." *Plants* 12.61 (2023). https://doi.org/10.3390/plants12010061

5. White, J, and **Yamashita, F**. "*Boquila trifoliolata* mimics leaves of an artificial plastic host plant." *Plant signaling & behavior* 17,1 (2022). https://doi.org/10.1080/15592324.2021.1977530

6. Baluška, F., **Yamashita, F.**, and Mancuso, S. "Root apex cognition: from neuronal molecules to root-fungal networks." In: Baluška F, Mukherjee S. *Rhizobiology: Molecular Physiology of Plant Roots*, Cham: Springer International Publishing: 1-24 (2021). https://doi.org/10.1007/978-3-030-84985-6_25

7. **Yamashita, F**., Rodrigues, A. L., Rodrigues, T. M., Palermo, F. H., Baluška, F., & Almeida, L. F. R. D. “Potential plant–plant communication induced by infochemical methyl jasmonate in sorghum (*Sorghum bicolor*)”. *Plants*, 10,3 (2021). https://doi.org/10.3390/plants10030485

2. Summary

Despite being sessile organisms, plants constantly move their vegetative and reproductive structures to adapt to changes in their environment, such as water deficiency, allelopathic activity, and herbivory. Plants can move and adapt morphologically to their environment and the root apex can control the rest of the plant, however, this is not something new. In 1880 Charles Darwin and his son, Francis Darwin, already mentioned that the root apex is endowed with sensitivity, having the power to direct the movements of other parts of the plant such as the shoots with leaves and tendrils. Roots grow in the soil, in semi-darkness or complete darkness. When they encounter sunlight in the top-soil layers, they move deeper into the soil, away from the light source to seek darkness.

This Thesis is based on five research papers, one review paper, and one book chapter published during my Ph.D. In our first paper, we investigate the skototropic behavior of roots, which promotes their fitness and survival. Light escape tropism of roots could be defined as the combination of negative root phototropism and increased root growth. Shoot skototropism was discovered in *Monstera gigantea* searching for its potential host plant as the shoot apex tropism towards the dark area by Donald Strong and Thomas Ray in 1975. Skototropism is active plant tropism that represents the directional search behavior performed by the shoot apex to detect a potential host tree for support, or the root apex to navigate towards darkness. We have discovered root apex skototropism as an active recognition of a dark space within the darkened Petri dish and the root apex tropism towards this dark part of the Petri dish. Root skototropism was found to be accelerated by the Target of Rapamycin (TOR) complex. The TOR complex, a vital protein complex, is instrumental in regulating cellular growth, proliferation, metabolism, and survival in response to diverse environmental stimuli, including nutrient availability and stressors. Inhibition of the TOR complex can impede the cellular tropism from growing towards light.

We have used AZD, a TOR inhibitor, to investigate the root and hypocotyl growth of *Arabidopsis thaliana*. We set up six light conditions: (1) Total light (TL): Round Petri dishes with Arabidopsis seedlings were placed under the light of the growth chamber with the intensity of 100 μmol s^{-1} m^{-2}. (2) Total dark (TD): Plants were kept in total darkness (covered with aluminum foil) for 96 h. (3) Gradient light (GL): Plants in the Petri dish were introduced in a black box, where the roots were inside the box and the hypocotyl outside, resulting in a slight gradient with a value of 39.74 μmol s^{-1} m^{-2}. (4) Light blocker (LB): A light blocker strip was placed inside the medium, perpendicular to the Petri dish, preventing light from reaching below

the blocker. Subsequently, they were introduced into a black box, resulting in a light intensity of 7.27 μmol s^{-1} m^{-2}. (5) Shoot dark (SD): The hypocotyls of *A. thaliana* were covered resulting in light intensity of 7.91 μmol s^{-1} m^{-2}. (6) Shoot dark with light blocker (SDB): The light blocker strip (same as LB) was placed on medium in a round Petri dish and then the hypocotyls of the seedlings were covered, resulting in light intensity of 2.03 μmol s^{-1} m^{-2}.

These conclusions were drawn from this study: (1) The root growth speeds up if it grows within light-dark gradients and AZD shows a clear inhibitory role in root growth. (2) The length of hypocotyls under total dark conditions was much larger than that under other illumination conditions and the length of hypocotyls under complete illumination was always the shortest. AZD has no significant influence on hypocotyl growth under the studied condition. (3) Root development of shoot dark (SD) condition was faster than shoot dark with light blocker (SDB) condition. Also, after 96 h of development, the root growth length in the AZD treatment group was less than 0.2 cm under different light conditions, far less than the control group (4). For AZD treatments, the hypocotyl growth in the SD condition is faster than that in the SDB condition. This study shows that AZD, a TOR inhibitor, drastically reduced root and hypocotyl length. The findings are consistent with previous research, which indicated that at the whole plant level, AZD treatments of *A. thaliana* delayed cotyledon and leaf development while also shortening root length.

Continuing the root skototropism investigations, in our second paper, we have organized a second experiment with basically the same setup, however this time we used another inhibitor, the 3-mpercaptopropionic acid (3-MPA), a γ-aminobutyric acid (GABA) inhibitor. GABA is a non-protein amino acid, also found in humans, regulates plant growth, and can accumulate in plants in response to stressful situations. Similarly to the first paper, we established six types of light conditions (TL, GL, LB, SD, SDB) to investigate changes in *A. thaliana* hypocotyl and root development. *A. thaliana* seedlings developed under absolute darkness (TD) with shorter roots and longer hypocotyls. Shoots were shaded in SD and SDB conditions, and seedlings were unable to carry out photosynthesis, resulting in insufficient stored nutrients for root development. In the three groups of different light intensities on the root (TL, GL, LB), light causes stress in the entire plant under total light, the length of the root and hypocotyl in TL condition was shorter than GL and LB conditions. The stimulated natural condition, LB, had a bigger root and longer hypocotyl than the GL condition. Different light treatments did significantly affect root growth and hypocotyl growth. We developed three treatment groups 3-MPA [25 μM], 3-MPA [50 μM], and 3-MPA [100 μM]. Root and hypocotyl growth was

promoted at the concentration of 3-MPA [25 μM], and the development of root and hypocotyl was suppressed gradually as the 3-MPA concentration increased.

In our third paper, we have used mutant lines of Arabidopsis. We have examined the differences in root skototropic behavior in the different expression lines: *atglr3.7 ko*, *AtGLR3.7 OE*, and *pin2* knockout, to better understand their role in root skototropism. The mutant plants have changes in glutamate-like channels 3.7. Glutamate is a neurotransmitter that is present in humans and is approximately 25 years has also been found in plants. In humans, glutamate receptors are responsible for cell-cell signaling and communication, acting as calcium channels. In plants, glutamate channels also act as calcium channels in cell-cell communication and long-distance signaling. The PIN2 protein serves as an efflux carrier responsible for the directional movement of auxin between cells. Our results revealed that as the distance between the roots and darkness increases, the root's positive skototropism becomes significantly weaker. Our findings suggest that GLR3.7 and PIN2 are involved in root skototropism. Summarily, this study provides valuable insights into the skototropic behavior of Arabidopsis roots and the potential involvement of *AtGLR3.7* and *AtPIN2* in mediating this response. Further studies are needed to elucidate better the underlying molecular mechanisms and signaling pathways involved in root skototropism. Understanding these mechanisms could have implications for improving plant growth and development in various environmental conditions.

A fourth experiment was performed to investigate root behavior, in our fourth paper. However, this one also checks the plant memory and plant communication via root exudates, because plants maintain communication with neighboring plants, herbivores, and predators through the emission of diverse chemical compounds by their shoots and roots. These infochemicals modify the occupied by plants and often also induce morphophysiological changes in neighboring plants. We have used methyl-jasmonate (MeJa), a plant natural infochemical, to trigger communication between emitters and receivers of *Sorghum bicolor* plants. The roots of two plants were split and allocated to three different pots, with the middle pot containing the roots of both plants and the side pots containing only the roots of one plant. A randomized block design with four groups was used. The groups were delineated as follows: (1) Mock (M): Contact with the mock solution (without the addition of MeJa), with its root separated into two parts, where half remained in pot 1 and the second half was allocated to pot 2. (2) Mock neighbor (MN): Without contact with any solution, with its root also separated into two parts, where the first half was in pot 2, allowing direct contact with the roots of the mock group, while the second half was in pot 3. (3) Treated (T): Contact with the MeJa solution, with its root separated into two parts, where half remained in pot 1 and the second half was allocated

to pot 2. (4) Treated neighbor (TN): Without contact with the MeJa solution, with its root also separated into two parts, where half was in pot 2, allowing direct contact with the roots of the treated group, while the second half was in pot 3.

The evaluated parameters were the CO_2 net assimilation (A, μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}), maximum fluorescence adapted to light (F_M). In the first exposure to MeJa, we observed a smaller CO_2 net assimilation rate (A) in the treated (T) plants in comparison to the mock (M) plants. In the T group, the A was smaller than that of the M group by 23.5% at 5 h and 20.4% at 7 h after application (HAA). Five days after the first contact, we applied MeJa for a second time and observed that A did not differ between the T and M groups. In contrast, by just comparing the A of the plants that received the infochemical (T) between the first and second contact, we observed that the A was greater during the second contact than in the first contact by 44.31% at 5 and 31.67% at 7 HAA. Similarly to A, stomatal conductance (g_S) decreased after MeJa contact. Just 3 HAA of MeJa, we observed smaller g_S in the plants of the T group compared to those of the M group. This pattern continued until at 7 HAA, only equaling out at 9 HAA. We observed a 68% higher stomatal conductance of the plants in the T group during the second contact when we compared it to the first contact at 5 HAA.

The signaling led to changes in the physiological patterns of the stages in the photochemical phase of photosynthesis. During the second contact, we observed that the plants of the treated neighbor (TN) group had a higher maximum fluorescence adapted to light (F_M) compared to the other groups. This difference was found in neighboring plants (mock neighbor (MN) × TN) at 5, 7, and 9 HAA during the second contact with MeJa. We also recorded the same difference in patterns at the same hours between the T and TN groups, being that the maximum fluorescence of TN was higher by 75.4% at 5 HAA, 57.3% at 7 HAA, and 39.9% at 9 HAA.

The morphological analysis of the *S. bicolor* adventitious roots after going through two rounds of contact with MeJa showed variations regarding the intercellular space in the cortex and the area occupied by the stele. Roots with smaller intercellular spaces were observed in the plants of the T and TN groups. The roots of the M group had a cortical intercellular space area that was 45.9% greater than that of the T group. The plants in the MN group had a cortical intercellular space that was 25.2% greater compared to that of the plants in the TN group. In contrast, the plants of the T and TN groups had larger steles. The plant roots in the T group showed twice the area occupied by the stele (101.6%) concerning those of the M group. The plants of the TN group showed roots with an area occupied by the stele that was 41.17% greater than those of the MN group.

During the first contact with MeJa, the plants of the treated (T) group showed changes in their physiological parameters. However, during the second contact, their responses did not differ from those of the mock (M) group, indicating that sorghum plants became less sensitive to MeJa after the first treatment. We also observed that the plants from the T group may have signaled their sensory information through their roots to their neighboring plants (i.e., the TN group). Nevertheless, our data do not exclude the contribution of shoot volatiles in this plant–plant communication, since some studies have already demonstrated that it has an impact on gene expression and stomatal opening. Altogether, MeJa may have led to plant–plant communication and altered the physiological and morphological patterns of the neighboring plants. In the future, it will be important to study plant-plant communication from the perspective of critical physiological parameters of plant responses to environmental challenges, anticipating responses and increasing the chances of tolerating a possible future stress event.

Finally, in a fifth paper, continuing along the line of research into plants interacting with their environment, we analyzed another plant movement, using the *Boquila trifoliolata*, a unique South American vine plant, that can adapt its leaf shape according to the neighboring plants. After discovering that the Boquila is capable of flexible leaf mimicry, the question of the mechanism behind this ability has been unanswered. Here, we demonstrate that plant vision possibly via plant-specific ocelli is a plausible hypothesis.

The majority Boquila leaves have three lobes with blunted tips. Variation of the number of lobes can be seen with some leaves having multiple lobes and others having less than three. Some leaves showed a similar pattern to the fake leaves concerning lobe variation. As the vine grows toward the artificial plant, the leaves of *B. trifoliolata* take a much different shape. The plants show obvious mimicry attempts to the closest artificial leaves of plastic model plants, though some leaves still maintain a single lobe, however, all leaves showed more longitudinal shapes. An interesting aspect was observed about the venation pattern when we analyzed the leaves under binocular microscopy. It was observed that non-mimic leaves had more free-ending veinlets, represented by tiny veinlets having their extremities ending freely in the leaf mesophyll. Greater amounts of free-ending veinlets were observed in non-mimic leaves in young leaves as well as middle-aged and old leaves.

Prior to this experiment, the main explanation for leaf mimicry in *B. trifoliolata* was volatile signaling and horizontal gene transfer. However, after Boquila mimicked plastic leaves, the two main explanations did not hold up completely. Therefore, a third explanation emerged, suggesting that plants may be able to perceive via some kind of plant-specific vision what is

around. Experimental testing of the ocelli-based *plant vision*, as it was done by Harold Wager, would be the logical next step in our quest for understanding plant sensory complexity.

3. Introduction

During evolution, roughly 450 million years ago, plants successfully colonized land by undergoing a process of co-evolution with symbiotic fungi, which involved a close and mutually beneficial relationship between the two organisms. Specifically with root-fungal co-evolution when the first primordial plants survive the tricky process of moving from sea to land (Redecker et al., 2000; Taylor et al., 1995). It was only possible to discover this symbiosis through paleontological records and also because it was found in the first lineages of evolutionarily ancient plants (Rimington et al., 2020). The root evolution was therefore shaped gradually, with several progressive changes culminating in the generation of complex root systems found among present-day flowering (Fujinami et al., 2020; Hetherington et al., 2016; Hetherington & Dolan, 2017, 2018; Kenrick & Strullu-Derrien, 2014).

In 1880, Charles and Francis Darwin concluded in their book "The Power of Movement in Plants" that the root apex acts as a brain-like organ, "It is hardly an exaggeration to say that the tip of the radicle thus endowed, and having the power of directing the movements of the adjoining parts, acts like the brain of one of the lower animals; the brain seated within the anterior end of the body, receiving impressions from the sense-organs, and directing the several movements." (Darwin & Darwin, 1880). In other words, they meant that the root apex receives information from its surroundings and guides root growth.

The root apex of Arabidopsis is typically divided into four distinct zones (Figure 1) (Verbelen et al., 2006). The root cap is located at the most apical part of the root, protecting the root, and receiving information and signals from the outside. The second zone is the meristematic zone, in which cells divide mitotically and whose cell division activity provides a permanent stock of new cells. The third zone is the transition zone and is composed of square-shaped cells that perform physiological changes in preparation for their rapid cell elongation. In this zone, cells stop their mitosis activity and begin to develop a central vacuole, a polarized cytoskeleton, and the cell wall is remodeled, stretching longitudinally (Verbelen et al., 2006). Cells in the elongation zone, expand rapidly along the root axis, drastically altering the composition of the cell wall thus allowing rapid cell elongation driven by turgor. When they reach their final sizes (end of the elongation zone), these cells differentiate into the different cell types found in the root (Baluška & Mancuso, 2013c).

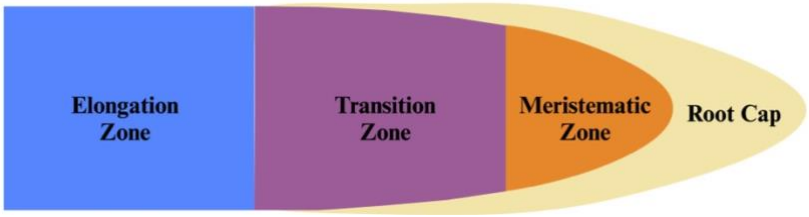

Figure 1 - Schematic view root apex zones anatomy. The root is divided into four zones. The root cap, which protects the root against mechanical impacts and facilitates passage through the soil; Meristematic zone, a region that is in constant mitotic activity, forming all the cells of the root; Transition zone, reduced mitotic activity and preparation for rapid cell elongation; Elongation zone, a region where cell accomplish rapid cell elongation and also where undifferentiated cells undergo maturation and differentiate into the different cells types of the root.

Many important characteristics suggest that the root apex, more specifically the transition zone, represents the " root brain", as originally proposed by Charles and Francis Darwin. Cells in this unique root apex zone are not in charge of any other tasks, such as elongation or cell division, focusing only on sensory activities. These cells are localized nearby the phloem, indicating that they receive plenty of sucrose (Complainville et al., 2003; Ross-Elliott et al., 2017). High levels of sucrose in the apoplast lead to osmotic stress, which is reduced by inducing endocytosis of the fluid phase in cells close to the phloem discharge sites (Baluška et al., 2004); and also, by synthesizing starch grains within the amyloplasts of the cells in the transition zone of the ray apex (Baluška et al., 1993a; Baluška et al., 1993b).

In order to test the Darwinian hypothesis that the root apex controls the plant as a whole, an Italian research group from Padova University carried out experiments with pea, *Pisum sativum* (Fabaceae), under normal conditions their shoot tendrils circumnavigate in search of potential support so that they can continue to grow. In their experimental setup, they cut off the root apices of their hydroponically grown pea seedlings and observed that their shoot tendrils were unable to recognize and contact potential support (unpublished data from Yamashita & Baluska; Guerra et al., 2021, 2022). Similar experiments were performed by Czech scientist Milos Spurný already in the sixties of the last century (Spurný, 1966, 1968, 1973). In addition, other experiments have been conducted in which there was a group of peas with support in contact with the root and another group with support not in contact with the root (Guerra et al., 2021). Shoots of the plants that did not have their roots in the vicinity to with the support had difficulty finding the support. In addition, it was shown that the thickness of the support matters, when thinner supports were offered, the peas tended not to grab the support, when thicker supports were offered, the peas grabbed the support (Ceccarini et al., 2021; Guerra et al., 2021, 2022; Simonetti et al., 2021). So, the root apex is closely linked to the shoot, sending

information and controlling the decision-making of the plant as a whole. These results support Darwin's theory of the root apex acting as a kind of *brain* of lower animals controlling the movements of roots and shoots.

This unique status of transition zone cells enables them to primarily focus on cognitive tasks, similar to the neurons of the central nervous system (CNS) in animal brains. Furthermore, similar to CNS neurons, cells in the root apex transition zone cells require high levels of nutrients and oxygen (Baluška & Mancuso, 2013c, 2013a, 2013b) to produce ATP and support electrical activity, similar to CNS neurons (Masi et al., 2009, 2015). High cytosolic phosphate (Pi) concentrations are critical for ATP synthesis and membrane phospholipid synthesis. Root caps act as the sensing organ and stop root growth under low Pi levels (Kanno et al., 2016; Sahu et al., 2020; Svistoonoff et al., 2007).

There are more interesting similarities between animal brains and the brains found in the roots of plants. Both types of brains are located in protected and privileged locations within their respective bodies. Animal brains are protected by the skull and receive preferential access to nutrition and oxygen. They are responsible for controlling the cognitive behavior of animals. Correspondingly, the Darwinian *brains* in plant root-apex are located between dividing and elongating cells that push the root apex forward (Abdullahi et al., 2018; Hagan & Ben-Zvi, 2015; Madangarli et al., 2019; Nian et al., 2020; Righy et al., 2016; Segarra et al., 2021). Many features that are typically associated with neurons are also present in plant cells, particularly in the transition zone of root apices. Intriguingly, the term "neuron" originates etymologically from the ancient Greek word for "vegetal fiber" (Brenner et al., 2006; Mehta et al., 2020). Both nerves in animals and vascular bundles in plants play similar roles in conducting rapid electric signals (Stahlberg, 2006b, 2006a).

In living organisms, it is well known that rapid electrical signaling is an efficient way to achieve cell-to-cell communication over long distances. In plants, the phloem acts as a "green cable" that allows the transmission of action potentials (APs) caused by stimuli such as wounding or cold (Hedrich et al., 2016). All these stimuli can be sensed and shared via roots with neighboring plants (Venturi & Keel, 2016; Yamashita et al., 2021). However, the APs are not the only way that plants can communicate with neighboring plants via volatile chemical substances (Baluška & Mancuso, 2018). It is well known that plants generate many different volatile compounds, from their shoots and their roots (Landi, 2020), called root exudates (Dicke, 2003). These compounds assist plants in their ability to communicate with herbivores, predators, and parasites of their herbivores, and even with neighboring plants, and can help in their defense strategy. As mentioned before, roots are an essential part of plants, and they play

an essential role for a wide range of reasons such as fixing the plant body in the soil, providing water and nutrients, responding to environmental signals and stimuli such as gravity, mechanical impedance, humidity, oxygen, and light (Baluška et al., 2010a; Verbelen et al., 2006).

Light is one of the most important environmental factors for plant growth and development throughout their life cycle. For example, light controls plant flowering, seed germination, plant development, and most importantly, light provides energy for photomorphogenesis and photosynthesis, two mechanisms that determine plant growth (Bloomfield et al., 2014). Light sensing plays a vital role in the growth of plants, and any changes in the intensity and quality of light can significantly alter their morphological traits (Yadav et al., 2020).

Plant roots, usually an underground organ of terrestrial plants, typically grow within the soil, a medium that is often low in light, receiving much less light than the above-ground organs. Even though the area below the soil surface is not completely darkness, light can penetrate the root cells via two possible paths. It either reaches up to several centimeters into the soil or is directed through the vascular tissues (Mo et al., 2015). However, this amount of light is not significantly stressful for the plant roots. One common plant used in research laboratories around the world is *Arabidopsis thaliana*. For easy visualization and manipulation of the roots, these plants are grown in transparent Petri dishes with a culture medium, providing all the necessary sucrose and nutrients for the first few weeks of the plant's development. Transparent Petri dishes leave the roots exposed to light, something that would not happen in their natural habitat (Yokawa et al., 2011).

Roots of plants growing in normal conditions, dark soil, or any other artificial darkening condition, have a root-shoot ratio of 1:1. However, this is not what happens when the root is illuminated, as within Petri dishes placed in growth chambers. When the roots grow under the constant influence of light, a stressful situation immediately accelerates root growth as these try to escape the light (Yokawa et al., 2011), meanwhile, hypocotyl growth is inhibited (Novák et al., 2015a). The shoot-root growth ratio does not remain around 1:1, but changed to 1:5 ratio with the root growing much more than the shoot (Yokawa et al., 2011). Besides that, illuminated Arabidopsis roots generate bursts of reactive oxygens species (ROS), showing different responses to salt stress (Yokawa et al., 2011; Yokawa et al., 2014b), growth of lateral roots, root hair formation, and root gravitropic and phototropic bending (Burbach et al., 2012; Hopkins & Kiss, 2012; Wan et al., 2012a).

Gravitropic root growth is promoted by specialized cells in the root cap, called statocytes. Statocytes are highly specialized sensory cells containing amyloplasts, plastids filled with

starch, which settle on the underside of the cells, thus allowing the root apex to perceive the gravity field and reorient itself within it. A key growth-related phytohormone is auxin, which is essential for cell elongation and lateral root growth (Petricka et al., 2012) and light has a fundamental role to play in the production and transport of auxin (Suzuki et al., 2016; Yokawa, et al., 2014a).

The asymmetrical allocation of auxin leads to the elongation of the cells on the darker side of the plant, causing it to lean towards the light source. Under normal physiological conditions, a significant proportion of apoplastic auxin exists in its protonated form, indole-3-acetic acid (IAAH), which can freely permeate cell membranes. This process is facilitated by members of the AUXIN/LIKE AUX1 (AUX/LAX) family of auxin importers. Upon entering the cell, where the intracellular pH is neutral, the weak acid form of auxin, indole-3-acetate (IAA-), becomes trapped and requires the activity of efflux transporters for extrusion, allowing intercellular transport (Sakai & Haga, 2012). The long integral membrane proteins (PINs), including PIN1-4 and PIN7 in *A. thaliana*, serve as efflux transporters responsible for the directional movement of auxin between cells. In addition, the ATP binding cassette B (ABCB) class, which includes several multidrug resistance transporters, also participates in auxin efflux and facilitates intercellular auxin transport (Christie et al., 2011).

Another important receptor is the phototropin blue light (PHOT) (Kutschera & Briggs, 2012), which interacts with PIN2 in light-induced root responses (Wan et al., 2012b). In Arabidopsis and other flowering plants, there are two PHOTs present, namely *phot1* and *phot2*. *phot1* acts mainly as a photoreceptor for root phototropism and hypocotyl phototropism over a wide range of blue light intensities. In contrast, the involvement of *phot2* in hypocotyl phototropism is limited to high light intensities. This restriction is mainly attributed to the increase in *phot2* protein abundance mediated by light exposure (Christie et al., 2015). Interestingly, this phototropic response is observed over a wide range of light intensities, ranging from very low light levels to the intensity of blue light experienced on a sunny day (Vandenbussche et al., 2014).

Normally in nature, all roots grow underground, i.e. in darkness, and the photoreceptors are localized in the root apices (Mo et al., 2015). Low amounts of light may not mean oxidative stress for the roots, but high amounts of light can become a stress factor (Burbach et al., 2012b; Yokawa et al., 2011; Yokawa et al., 2014b). In routine laboratory experiments, transparent Petri dishes are used, but the ideal would be to use partially darkened dishes (Novák et al., 2015b; Xu et al., 2013; Yokawa et al., 2013). One study suggested an alternative, using the D-Roots system. This system consists of dark chambers that allow the shoot part in a light

environment and the root part in a shaded environment (Lacek et al., 2021; Miotto et al., 2021; Silva-Navas et al., 2015). However, contrary to what was expected, the roots grew faster when they were grown in the D-Root system, revealing that this may have occurred due to the steep light-darkness gradient, i.e. the roots perceiving this gradient grew faster trying to escape the light and get as close to dark places as possible (Qu et al., 2017; Yan et al., 2022). This process of accelerated root growth is based on the activity of the Target of Rapamycin (TOR) complex (Yan et al., 2022).

The TOR complex is a protein complex that plays a central role in regulating cell growth, proliferation, metabolism, and survival rate in response to various environmental factors, such as nutrient availability and stressful conditions, and inhibition of the TOR complex can block the tropism to grow against light (Yan et al., 2022). Another study showed that roots placed under the light, but at different distances from the area where the light gradient began, were able to recognize the dark part. The seedlings were placed at the edge (0 cm), 1 cm, and 2 cm away from the start of the dark area, as shown in Figure 2 (Yan et al., 2022, 2024). This experiment may indicate that some kind of vision is possible at the root apex, based on the blue light photoreceptor *phot1*. In contrast to the ocelli located in the epidermis of leaves, which are distributed diffusely, root ocelli are distributed locally (Wan et al., 2012c; Wan et al., 2008) in the transition zone of the root apex (Baluška et al., 2010b; Baluška & Mancuso, 2013c), an ideal position for orienting the root apex towards darkness (Baluška et al., 2021).

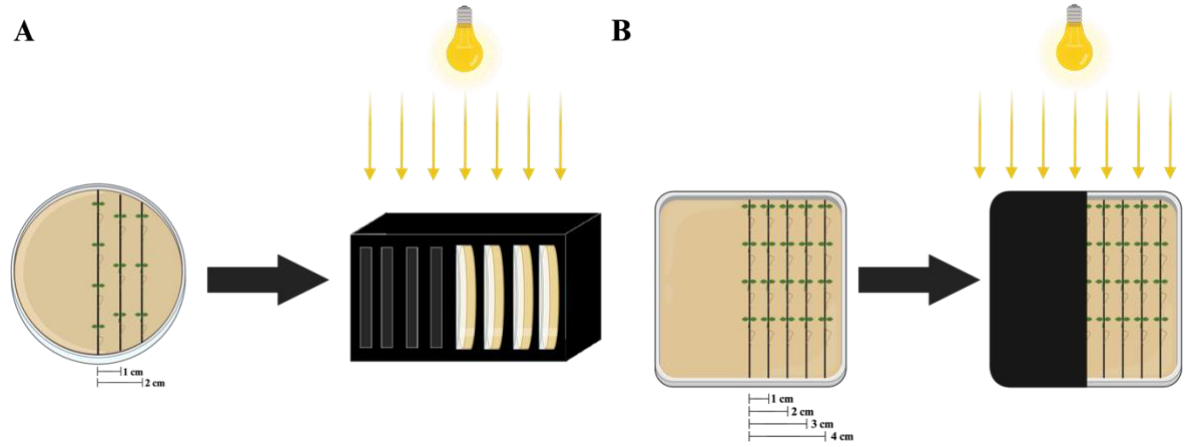

Figure 2 - Experimental setup in Yan et al. 2024. Examples of different shade approaches we adopted: (A) treatment of round Petri dishes. within black boxes with a light source at the growth chamber ceiling. (B) Treatment of l square Petri dishes darkened with black covers with a light source at the growth chamber ceiling. Three rows of Arabidopsis seedlings were positioned in A and five rows in B, respectively. Each column was spaced 1 cm (10 mm) apart from each other as the label. To ensure consistent positioning, the inner row of seedlings was aligned with the border of the covers.

In contrast to phototropism, there is another root tropism, not as well-known as negative phototropisms. The combination of negative root phototropism and increased root growth rate represents to root escape tropism and the active growth towards darkness is root skototropism. This tropism is the directional search behavior which was discovered in 1975 by *Monstera gigantea* (Araceae) shoot apex searching for a host tree for support (Strong & Ray, 1975). *M. gigantea* is an arboreal vine, which is commonly found in tropical regions where the permanent conditions of heat and humidity allow the large, voluminous leaves to be viable. Researchers have observed that seedlings of this plant grow towards the trunks of surrounding trees and also toward dark surfaces (Strong & Ray, 1975). In general, climbing plants need adequate support to obtain the amount of light they need to carry out photosynthesis (Rodriguez-Quintero et al., 2022; Wyka, 2023), since close to the ground in tropical forests, they would receive very little of the necessary light rays.

But Strong and Ray were not the first to observe growth against the light gradient and skototropism. Darwin and his son carried out a brief experiment with *Bignonia capreolata*, observing that its tendrils moved away from the window (Darwin & Darwin, 1880). In the laboratory, the roots of several plants (*Schizophragma hydrangeoides, Trachelospermum asiaticum* and *Hedera rhombea*) actively grew away from the artificial light source, as well as the tendril of Parthenocissus quinquefolia, which showed the same behavior (Kato et al., 2011; Kato et al., 2012; Kato et al., 2012). The skototropic behavior may be an adaptive mechanism in that it also allows roots to avoid potentially unfavorable light conditions as explained by Yan et al. 2024. Furthermore, as shown in Figure 3, while an Arabidopsis seedling has a diameter of 100 µM and is 20 mm (2 cm) away from the dark area, this would correspond to a person with a diameter of 0.7 m being able to recognize the darkness from 140 m away.

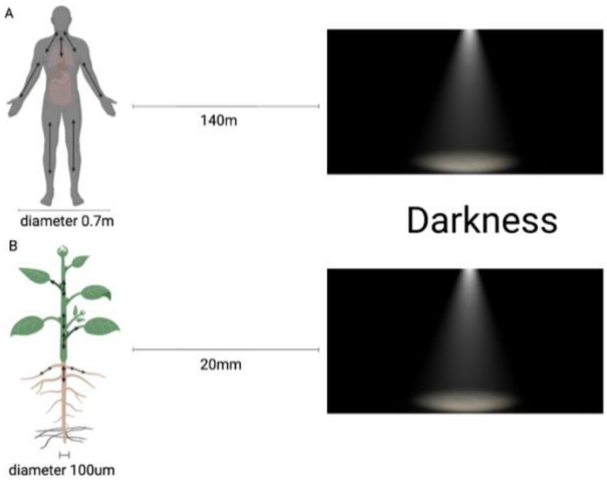

Figure 3 - How far a plant can recognize darkness - Comparative perception of darkness in Arabidopsis and human beings. The *Arabidopsis thaliana* seedling (about 100 µM in diameter) is positioned at 20 mm from. Figure from Yan et al. 2024.

Nearly all reports of skototropism have used climbing plants, which depend on support to reach the canopy of neighboring trees and thus capture sunlight. A perfect example of this is a climbing plant called *Hydrangea serratifolia* (Hydrangeaceae), found in a temperate rainforest in South America, more specifically in Chile. This plant has shown an active, patterned, and directional foraging process in locating trees to use as support. Young shoots of this plant, reddish pink in color, grow against the light gradient, i.e. towards dark areas of the forest in order to reach tree trunks on which they can support themselves (Rodriguez-Quintero et al., 2022). When it finds a potential host, the shoots change color and turn green, i.e. acquiring chlorophyll and ready to develop (Rodriguez-Quintero et al., 2022; Schlanger, 2024).

The other quite interesting and unusual plant, also found in the Chilean rainforest, is *Boquila trifoliolata*. This plant was discovered in 1817 and is a common perennial climbing plant that can reach up to 6 meters in height, classified as a liana, or climber, due to the shape that it rests on neighboring plants. During favorable seasons, it can grow rapidly and reach the canopy of trees, always leaning on the trunks and branches of neighboring plants, known as host plants. If Boquila does not find host plants or adequate support, its growth or reproduction can be drastically affected (Marticorena et al., 2010).

Boquila is part of the Lardizabalaceae family, with 8 genera and 45 species. Six of these genera are found in East Asia (from the Himalayas to Japan) and only two genera are found in South America, in Chile. The two species found in Chile are generally called "voqui" or Pil-Pil by the locals. This name is derived from the indigenous peoples and means "vine" in the local language. The only two Chilean species are Lardizabala biternata and *B. trifoliolata* (Cárdenas & Villagrán, 2005; Christenhusz, 2012).

Boquila is an endemic plant of temperate rainforest in southern South America, more specifically south-central Chile, and can also be found in some areas of Argentina (Christenhusz, 2012). This forest, where it is found, has a mild climate and grows well in shady and humid places in evergreen forests, between 100 and 600 m above sea level (Christenhusz, 2012). During the winter it can reach temperatures of -8º C and snow, but the Boquila can withstand these adverse conditions without any problems (Christenhusz, 2012). It is rarely possible to grow it outside of its natural habitat, so there are no reports on where else Boquila can be found. It can be said that the plant is relatively isolated geographically and phylogenetically (Christenhusz, 2012).

As for its morphological characteristics, it is a perennial or partially deciduous plant, with alternate leaves made up of three leaflets, the central leaflet being the largest and with the

presence of pulvinus, so it can change the direction of its leaves, with a petiole that can be 2-6 cm long. It has an oval leaf surface with a lobed or emarginate leaf apex and lobed leaf sides, forming a trilobe. The adaxial side is green and the adaxial side is grayish green (Marticorena et al., 2010). It has a pubescent stem, thin branches that are hardly more than 1 cm in diameter, with reddish-brown bark covered in elliptical lenticels. As for its flowers, they are dioecious, i.e. male, and female plants are needed to produce fruit, which are arranged in small clusters (Christenhusz, 2012; Gay et al., 2010). The fruit is a creamy white berry, round in shape, 4.3 to 6.5 mm in diameter with 2 to 5 seeds inside. In the southern hemisphere, where they are native, they flower from October to November and bear fruit from December to January (Marticorena et al., 2010).

Local indigenous peoples used Boquila for various purposes, depending on which part of the plant was used. The fruit was squeezed, and its juice was used medicinally to treat eye diseases, i.e. as eye drops (Marticorena et al., 2010). The flexible stems are used for handicrafts, i.e. making baskets or ropes, which are still used today.

Boquila used to be a common local plant, used only for making baskets or ropes, or as an ornamental plant. But all that changed with a publication in 2014 by a Peruvian ecologist called Ernesto Gianoli, a professor at the Universidad de La Sirena in Chile. During one of his expeditions into the Chilean forest with his students, they observed that the leaves of the Boquila mimicked the leaves of the host trees in size, color, orientation, shape, and venation pattern (Gianoli & Carrasco-Urra, 2014). However, the scientific literature did not acquire the mimicry characteristics of the Boquila. In addition, previous records of mimicry in plants only existed of one species mimicking another species, something different from what was found by Gianoli (Gianoli & Carrasco-Urra, 2014). Furthermore, it was observed that a single individual of Boquila extended under three host plants of three different species. What was most surprising was that this same individual of Boquila mimicked leaves from the three different host plants, i.e. the mimicry of Boquila was not limited to just one species, something never found in science before (Gianoli & Carrasco-Urra, 2014).

Mimesis is not very common among plants and why the plant species did it is hardly ever reported. Some examples are succulent plants of the *Lithops sp* species, native to South Africa and resembling local rocks because, without an arid climate, the plants have a rocky appearance (Hammer, 2005). Another example of a plant used on a large scale is *Secale cereale*, or rye, which used to be treated as a weed in wheat and barley plantations. The rye plant was very similar to wheat and barley, and farmers had to analyze their seeds in search of the weed carefully. After the worldwide expansion of wheat and barley cultivation, rye caught on and

was also expanded worldwide, widening its distribution area (McElroy, 2014). However, the best-known case is Australian mistletoe, a group of semi-parasitic plants that have leaves that simulate the leaves of host tree species (Barlow & Wiens, 1977).

However, how, and why Boquila is able to mimic different host plants is still uncertain. Why it does this, Gianoli and Carrasco-Urra tried to answer in their research. According to their publication, they found three pieces of evidence that the Boquila mimics host plant leaves to avoid predation, other words, herbivory. The first evidence cited was the rate of leaf damage. Boquila and the host plants had the same rate of herbivory on the leaves. The second evidence was that the herbivory rate of the Boquilas that did not use a host plant and remained close to the ground was significantly higher than that of the individuals that used host plants and remained above ground. The third evidence was the higher herbivory rate of the Boquilas that used a support to stay away from the ground, but this support had no leaves, in other words, the Boquila did not mimic leaves because they had no leaves to mimic as the support was the trunk and branches of leafless trees. Therefore, these three pieces of evidence support the researchers' conclusion that the Boquila obtains protection from herbivores by climbing and avoiding being close to the ground, as well as its leaves being confused with the leaves of host plants (Gianoli & Carrasco-Urra, 2014).

How the Boquila mimics the leaves of host trees was not concluded in this research. In the article, the researchers proposed two hypotheses. The first hypothesis is that the Boquila changes the shape of its leaves because it has received volatile compounds from the host plant, as leaf mimicry was observed even when the Boquila was not in direct contact with the host plant. However, there are no reports of aerial plant-plant communication resulting in leaf morphology changes. The second hypothesis is that a vector, i.e. microorganisms present in the air around the leaf of the host plant, can carry genetic material from one plant to another (Gianoli & Carrasco-Urra, 2014).

After seven years, we published a paper with a third hypothesis in a publication called "*Boquila trifoliolata* mimics leaves of an artificial plastic host plant". We were not convinced by Gianoli's hypothesis, and they decided to test a different one. This different hypothesis would have to deny the two previous hypotheses, so there could be no traces of volatile chemical compounds or the presence of genetic material from the host plant for some microorganism to carry from one individual to another. It was therefore decided to use an artificial plant, a plant made of plastic material. Consequently, it would be impossible for Boquila to receive any volatile chemical compound that would induce the modification of its

leaves, nor would it be able to receive genetic material, as the plastic plant has no genetic material at all (White & Yamashita, 2022).

Because it is difficult to find such a plant anywhere else on the planet, as they are phylogenetically and geographically isolated (Christenhusz, 2012), only four individuals were used in the experiment, which consisted of placing the four Boquila plants in a row and their opaque shelves above them. As they are lianas, which means climbing plants, a wooden support was placed so that the plants could have a support to grow on. Plastic plants were placed above the first shelf so that new Boquila leaves would come into contact with the artificial plastic leaves. After a while, the Boquila leaves that grew next to the plastic leaves stopped showing a three-lobed shape and grew into a more elongated shape, similar to the plastic plants. After measuring leaf parameters (such as leaf area, leaf perimeter, width, and length) it was observed that the Boquila leaves resembled the artificial plastic leaves (White & Yamashita, 2022).

Consequently, if the Boquila copied the plastic leaves, Gianoli's two hypotheses ("mimicry by the release of volatile chemical substances or by genetic material from the host plant transferred by microorganisms") no longer worked. There had to be a third hypothesis. It was then that the hypothesis arose that the Boquila could, in a way, be able to see what it was around, what the host was around it. And what would it be like to observe what was around it?

A research study from 2014 suggested that young *A. thaliana* plants can differentiate between their neighbors through recognition by photodetectors based on the shape of their bodies. For this recognition to take place, Arabidopsis would have to possess some kind of specific vision, as it would have to perceive the body shape of the plants around it. It would also have to see and decode the images it receives (Baluška & Mancuso, 2016; Crepy & Casal, 2015; Mancuso & Baluška, 2017). As different and sometimes even absurd as it may sound, this idea of a plant having some kind of vision was proposed over 100 years ago.

In 1905, an Austrian botanist Gottlieb Haberlandt proposed a revolutionary concept at the time and even today, the idea of the plant ocelli. This theory proposes that the upper cells of the leaf epidermis act as convex or planoconvex lenses, thus capable of converging light rays towards the cells below the epidermis, where light-sensitive structures would be present (Haberlandt, 1905). This proposal that leaf epidermis cells act as a kind of lens was confirmed by a series of papers from the group of Thomas Vogelmann, some 90 years later (Vogelmann, 1993; Vogelmann et al., 1989, 1991).

Five years after Haberlandt proposed the ocelli theory, Harold Waner put it into practice. In his paper, Waner explains that the perception of light by plants is mediated by specialized photoreceptor proteins that allow them to perceive different wavelengths and light intensities.

After a light stimulus, these photoreceptor proteins initiate a signaling cascade that leads to different physiological responses in the plant, allowing plants to adjust their growth and development according to the light conditions captured by the photoreceptor proteins (Batschauer, 1998; Wager, 1909).

These photoreceptors, in turn, are classified into different types, depending on the region of the electromagnetic spectrum they are capable of receiving. The most widely studied photoreceptors are UV-A, UV-B, phototropins, cryptochromes and phytochromes. Phytochromes are responsible for detecting red light and far-red light, playing a fundamental role in the regulation and germination of seeds, seedling development, and also responsible for flowering. Cryptochromes are responsible for detecting blue and UV-A light, which play essential roles in photomorphogenesis and the regulation of the circadian clock. UV-B photoreceptors are proteins responsible for capturing, as the name implies, UV-B, attenuating and triggering protective responses to this spectrum of light. Phototropins detect blue light and control phototropic responses, such as directed growth and the opening of stomata (Batschauer, 1998; Kong & Okajima, 2016; Wager, 1909).

After Wager's research in 1909, few studies were done on how plant cells can focus light into the cell. However, an American research group studied the leaves of *Medicago sativa* and realized that light intensity generally decreases as light penetrates the inner layers of the leaf mesophyll, influencing photosynthetic efficiency (Vogelmann et al., 1989, 1991, 1996). It was reported that epidermal cells focus light on specific inner layers of the leaves, increasing photosynthetic efficiency in low light conditions (Martin et al., 1989; Poulson & Vogelmann, 1990), indicating an evolutionary adaptation to maximize light capture in environmental conditions that are not so favorable to photosynthesis (Brodersen & Vogelmann, 2007). After analysis of the isolated epidermal layer, it was proven that these cells improve the focusing of light and optimize the efficiency of photosynthesis (Martin et al., 1991). It is therefore clear that light interacts differently with different parts of plants, altering the absorption and transmission of light, and the epidermis acts as a lens, converging light rays to more internal layers that can be captured by photoreceptors (Vogelmann, 1993). In this way, plant leaves show that they have all the cellular apparatus to be able to see around them.

The theory of ocelli in plants is not so surprising if you consider that several evolutionary older organisms such as bacteria, fungi, and algae have light-sensitive cells similar to those found in plants. A great example of this is the green alga *Chlamydomonas reinhardtii*, which has a subcellular apparatus of eyespots. These eyespots are attached to the side of the cell by bundles of D4 microtubules, anchored to the basal body (Figure 4). In addition, an important

feature of Chlamydomonas that is often neglected is the rhizoplast, which is a contractile centrin-based structure connecting the basal bodies of the flagella with the nuclear surface (Salisbury et al., 1984, 1988; Wright et al., 1989). These structures, known as rhizoplasts or fibrous flagellar roots, attach nuclei to the basal bodies of flagella or cilia (Dutcher, 2003; Geimer & Melkonian, 2005; Lechtreck & Melkonian, 1991; Mahen, 2021; Owa et al., 2014; Salisbury, 1998).

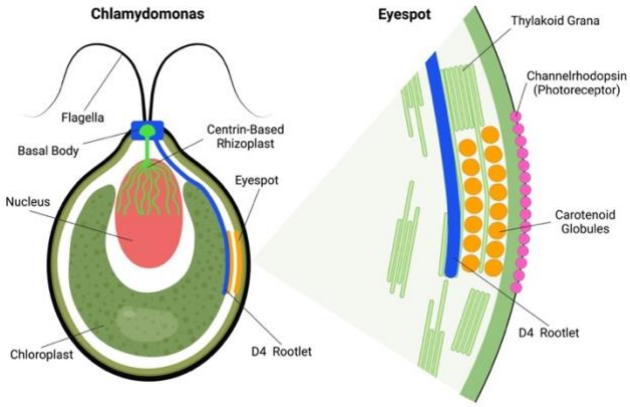

Figure 4 - Algal Eyespot of Chlamydomonas - Chlamydomonas alga with two flagella associated with the basal bodies which intracellularly organize intracellular bundles of microtubules (known as rootlets) of which the D4 bundle anchors the eyespot. This eyespot is constructed from chloroplast thylakoid membranes and carotenoid globules aligned under the plasma membrane which is enriched with photoreceptor channelrhodopsin. Basal body organizes besides the bundles of microtubules also the centrin-based contractile nucleo-basal body connector anchoring the nucleus. M4, M2 and D2 rootlets are not shown in this simplified scheme.

However, the responses of green algae can be of two types: swimming toward the light or fleeing away from the light rays, called phototaxis, which depends on the concentration of reactive oxygen species (ROS) inside the cell (Morishita et al., 2021; Wakabayashi et al., 2011); the second response is after receiving a light stimulus, the green algae remain motionless for seconds, swim in the opposite direction to the light ray, and then swim in a random direction. This second response is called photo-shock because the alga is paralyzed for a few seconds (Schmidt et al., 2006; Wakabayashi et al., 2021). Using microscopes, it is relatively easy to find the eyespots of green algae, as they are made up of globules of orange carotenoids, located in the plasma membrane, filled with photoreceptor proteins, channel rhodopsins ChR1 and ChR2 (Nagel et al., 2002). In addition, some species of green algae can have two layers of carotenoid globules, such as *Chlamydomonas reinhardtii*, positioned between the thylakoid and chloroplast membranes (Kreimer, 2009; Schmidt et al., 2006; Ueki et al., 2016). An important detail is the electrical currents in the eyespot induced by light, which activate and control the flagella currents, a process similar to the electrical action potential (Hegemann, 1997, 2008; Holland et al., 1997; Sineshchekov & Spudich, 2005).

Besides *C. reinhardtii*, another algae with a photosensitive apparatus is *Euglena gracilis*. Adapted for unicellular vision, it has two types of photo movement, phototopic and phototatic behavior. Carotenoids are also important for their movements in response to light stimuli and their plastids do not develop into chloroplasts due to the lack of chlorophyll (Kato et al., 2020; Tamaki et al., 2020). Some recent research has shown that Euglena without carotenoids have lost their ability to respond to light, meaning that carotenoids are essential for light detection (Kato et al., 2020).

As well as green algae, an eyespot has been found in a dinoflagellate. In 1967, researcher David Francis studied the dinoflagellate *Nematodinium armatum* and observed the presence of an eyespot. This eyespot had a lens capable of focusing light rays into a single point, called a pigment cup. This structure, which receives the focused light rays, may be a light-sensitive retinoid with an image-forming function (Francis, 1967). Another study in 2015 discovered the presence of ocelloids in warnoiid dinoflagellates. In this case, the dinoflagellates use cellular organelles that have been obtained through symbiosis. For example, the mitochondria generated a cornea-like surface, and the plastids formed the body of the retina (Gavelis et al., 2015; Nilsson & Marshall, 2020). To prove that these eye structures came from cell organelles, scientists sequenced the DNA of the warnoid's retinal body, which showed a higher percentage of DNA originating from plastids than samples from other cells (Gavelis et al., 2015). These dinoflagellates are the only microorganisms that have a camera-like apparatus for vision, similar to that found in animals. (Colley & Nilsson, 2016; Francis, 1967; Gavelis et al., 2015; Nilsson & Marshall, 2020; Richards & Gomes, 2015).

A year after the discovery of ocelli in dinoflagellates, a type of vision was discovered in bacteria, more specifically in the cyanobacterium *Synechocystis sp*. PCC 6803 (Dieckmann & Mittelmeier, 2016; Nilsson & Colley, 2016; Schuergers et al., 2016, 2017). Unlike previous cases, because it is a relatively smaller organism in size, the entire cell acts as a lens, converging light rays at a single point on the plasma membrane. This has also been found in eukaryotic volvocine algae, where the entire cell is used as a lens to enable the ability to see (Kessler et al., 2015). So, if cyanobacteria, eukaryotic algae, dinoflagellates, and a protist such as Euglena have some kind of ocelli, giving them the ability to see and react to the environment around them, it would not be surprising to find plants have similar version-like ability to perceive their environment. It is important to remember that during biological evolution, elaborate structures and processes that are useful and relevant for their survival tend to remain and not be left behind.

Nonetheless, human eyes and specialized eyes such as those of insects probably evolved from a common predecessor, from some eyespots, something very similar found in

cyanobacteria. It is obvious that biological evolution does not always happen in such a linear way, but many characteristics in all kingdoms have appeared and been left behind over millions of years of evolution never discarding useful traits. Although it has not been proven that plants have ocelli, that does not mean that they cannot be there. Why would plants alone not have a specialized structure for vision, even if it is a primitive structure (Yamashita & Baluška, 2022). Also, complex specialized vision organs such as the eyes of humans and animals represent part of the biological evolutionary process.

Furthermore, recent research in animals has shown that electrical gradients in cells play a fundamental role in embryonic development, tissue regeneration, and morphogenesis (Adams et al., 2007; Beane et al., 2011, 2013; Levin, 2014; Tseng et al., 2010). These electrical gradients are generated through the activity of specific ion channels and transport proteins, influencing the cellular process of migration, differentiation, and cell polarity. By manipulating the electrical gradient, cell behavior can be modulated, altering the direction and speed of cell migration, and changing the final specificity of a totipotent cell, i.e. the authors suggest that it is possible to transform a totipotent cell into any other type of cell (Levin, 2014).

Therefore, the aim of this thesis and the published articles that served as the basis for this thesis was to demonstrate that plants are not passive organisms, but that they actively move their organs and seek to communicate with surrounding plants through chemical compounds released by the root (Yamashita et al., 2021). In addition, we were able to demonstrate that Arabidopsis roots growing in illuminated environments can actively find and seek out darker environments in a growth called skototropism, which in turn may be related to the fact that the root apices also have some aspect of vision. Likewise, Boquila was able to mimic artificial objects such as plastic plants, suggesting a new hypothesis that plants may indeed be able to perceive the environment via plant-specific version of vision. This is not so surprising given that other less complex living organisms such as cyanobacteria and algae have also some kinds of vision.

4. Results

This section is distributed into seven chapters, all of which are published or submitted in different peer-reviewed journals or scientific books. A dedicated Material and Methods section has not been incorporated into this thesis because each publication includes a detailed methodology.

The different chapters are ordered in a non-chronological but content-wise meaningful way.

4.1. Chapter 1

"Root and hypocotyl growth of Arabidopsis seedlings grown under different light conditions and influence of TOR kinase inhibitor AZD"

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DOI: https://doi.org/10.5897/IJBMBR2022.0330

"Root and hypocotyl growth of Arabidopsis seedlings grown under different light conditions and influence of TOR kinase inhibitor AZD" was published in 2022 as an open access article in the International Journal of Biotechnology and Molecular Biology Research. The original publication is attached in the appendix of this dissertation. The abstract below is intended to highlight my personal contribution to this document. As an abstract focusing only on the main important points to provide clarity and brevity, the appropriate references to some statements have been omitted, which can be found in the full article attached (Appendix 1).

This publication, in which I am the second author, the skototropic behavior of roots, which promotes their fitness and survival. Approximately 50% of the experiments were carried out by Xingyu Yan, and the other 50% by me. The data generated was discussed with all the co-authors before a manuscript was prepared. I wrote the first manuscript version and Xingyu Yan and Ibrahim Njimona finalized it. All the co-authors helped to revise its content. All the co-authors approved the final manuscript version before I sent it to the corresponding journal.

The ratio of roots to shoots in plants grown in regular conditions, dark soil, or any other simulated dark conditions is 1:1. However, when the roots are exposed to light, such as scientific experiments in Petri dishes placed in growth chambers, the situation shifts. The consistent presence of light on the roots speeds up their growth as they seek to avoid the light, while hypocotyl growth is suppressed. The ratio of shoot to root growth is no longer approximately 1:1 but changes to a 1:5 ratio with the roots growing much more than the shoots.

To avoid this unbalanced ratio, some researchers suggested using an alternative cultivation method to grow plants in Petri dishes. This method is called the D-Root system, which consists of dark chambers that allow the shoot in a light environment and the root part in a dark environment. Therefore, in this publication, we developed a system similar to D-root, which consists of placing half of the Petri dishes in black boxes, thus creating a light gradient, where the roots are allocated, and the other half is allocated to the shoot.

In light gradient conditions, the roots behave by trying to escape the light, which can be called negative phototropism. Combining this phototropism with root growth toward the light represents root escape tropism, the active growth of the root toward the dark, in other words, skototropism. The mechanism behind accelerated root growth relies on the function of the Target of Rapamycin (TOR) complex.

The TOR complex is a large, highly conserved protein that is part of the phosphatidylinositol 3-kinase-related kinases (PIKKs) family. When faced with changes in the surroundings, such as nutrients, energy levels, and growth stimulants, TOR acts as a vital detector of cell development and metabolism.

Recent studies have revealed that the conserved TOR pathway is crucial for coordinating overall plant development. Additionally, it appears that the Arabidopsis genome contains a single important TOR gene, and reducing its expression leads to decreased plant growth, reduced stress resistance, and longer life span. Furthermore, Arabidopsis plants with silenced TOR expression show a notable decrease in polysome abundance, indicating that TOR is involved in regulating plant translation. TOR inhibitors limit the ability of meristematic cells to proliferate by reducing the number of cells in the MZ, primarily through promoting differentiation. One of the most effective TOR inhibitors is AZD. This second-generation mTOR inhibitor, known as an ATP-competitive mTOR kinase inhibitor, is designed not only to target mammalian TOR for cancer treatment but also to inhibit TOR in plants. AZD can bind to the TOR kinase domain within the ATP-binding pocket, deactivating the TOR complex. In this paper, we show when Arabidopsis is treated with AZD and grown under different light conditions, the root and hypocotyl are significantly modulated.

To investigate the influence of AZD on *A. thaliana* root and hypocotyl growth, we set up six light conditions used as follows: Total light (TL): round Petri dishes with *A. thaliana* seedlings were placed under the light of the growth chamber with the intensity of 100 μmol s^{-1} m^{-2}. Total dark (TD): plants were kept in total darkness (covered with aluminum foil). Gradient light (GL): plants in the Petri dish were introduced in a black box, where the roots were inside the box and the hypocotyl outside, resulting in a light gradient with a value of 39.74 μmol s^{-1}

m^{-2}. Light blocker (LB): a light blocker strip was placed inside the medium, perpendicular to the Petri dish, preventing light from reaching below the blocker. Shoot dark (SD): The hypocotyls of *A. thaliana* were covered resulting in a light intensity of 7.91 μmol s^{-1} m^{-2}. Shoot dark with light blocker (SDB): the light blocker strip (same as LB) was placed on medium in a round Petri dish and then the hypocotyls of the seedlings were covered, resulting in a light intensity of 2.03 μmol s^{-1} m^{-2}.

We obtained the following results: (1) Root growth accelerates in the presence of light-dark gradients, while AZD plays a clear inhibitory role in root growth. (2) The length of hypocotyls is significantly greater in total darkness compared to other light conditions, with the shortest length observed under complete illumination. AZD does not have a significant impact on hypocotyl growth under the conditions studied. (3) Root development is faster under shoot dark (SD) conditions compared to shoot dark with light blocker (SDB) conditions. In AZD treatments, hypocotyl growth is faster in the SD condition than in the SDB condition. This research demonstrates that AZD, a TOR inhibitor, markedly decreases root and hypocotyl length. These findings align with previous studies, confirming that in *A. thaliana*, AZD treatments delay cotyledon and leaf development at the whole plant level while also reducing root length.

4.2. Chapter 2

"Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions"

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"Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions" was submitted to Plant Signaling & Behavior in April 2024 and is under review at the time I submit this thesis. The original file is attached in the appendix of this dissertation. The abstract below is intended to highlight my personal contribution to this document. As an abstract focusing only on the main important points to provide clarity and brevity, the appropriate references to some statements have been omitted, which can be found in the full article attached (Appendix 2).

This publication, in which I am the first author, investigates the effects of 3-mercaptopropionic acid (3-MPA), a GABA-transaminase inhibitor, on the growth and physiological responses of *Arabidopsis thaliana* under various light conditions. The experiments were carried out by Xingyu Yan and me, and the data generated was discussed with all the co-authors before a manuscript was prepared. I wrote the first manuscript version and all the co-authors helped to revise its content. All the co-authors approved the final manuscript version before I sent it to the corresponding journal.

GABA (γ-aminobutyric acid) is a critical non-protein amino acid involved in plant stress responses and regulation of physiological processes including growth, development, and stress tolerance. GABA also plays roles in stomatal regulation under water deficiency, modulation of reactive oxygen species (ROS), and plant communication with bacteria. GABA-binding sites on plant cell membranes suggest it functions as a plant signaling molecule. GABA concentrations vary significantly across plant organs and tissues, from low micromolar in the

xylem to high millimolar levels in fruits, indicating its dual function as a signaling molecule and a primary metabolite involved in balancing nitrogen and carbon metabolism. Combined transcriptomics and metabolomics studies suggest GABA's role in plants is predominantly metabolic. Light conditions also influence plant growth, with different intensities and qualities affecting morphological traits and directing growth patterns like phototropism.

The inhibition of GABA-transaminase by 3-MPA was employed to delve into the role of GABA metabolism in plants' adaptive responses to different light environments, providing insights into the mechanisms underlying light-induced stress responses. Under high light intensity, 3-MPA-treated seedlings showed significant reductions in growth, with notable decreases in seedling height, leaf area, and overall biomass. They also exhibited a decline in chlorophyll content and photosynthetic efficiency, alongside increased stomatal conductance, indicating compromised photosynthetic performance. Enhanced levels of ROS and lipid peroxidation in 3-MPA-treated seedlings indicated increased oxidative stress under high light conditions. Under low-light conditions, 3-MPA-treated seedlings maintained growth parameters and photosynthetic efficiency, indicating that GABA metabolism plays a less critical role in managing low-light stress.

To investigate the effects of different light conditions and GABA modulation on *A. thaliana* hypocotyl and root development, we set up six light conditions: total light (TL), gradient light (GL), light blocker (LB), total dark (TD), shoot dark (SD), and shoot dark with blocker (SDB). Three concentrations (25 µM, 50 µM, and 100 µM) were applied to Arabidopsis seedlings. Differences were observed among the control, 25 µM, and 50 µM groups. Seedlings treated with 3-MPA under gradient light (GL) and light blocker (LB) conditions had longer roots than those in the control group. Treatment with 3-MPA did not affect the size of roots fully exposed to light (TL). However, roots that were gradually covered (GL) or shielded from light (LB) exhibited increased size when treated with 25 µM and 50 µM of 3-MPA, compared to the control. For plants kept entirely in the dark (TD), root size increased only at the 25 µM concentration of 3-MPA. There was no difference in root size between the SD and SDB groups, regardless of the 3-MPA treatment. After four days, significant differences in root development were noted between both concentrations of 3-MPA [25 µM] and [50 µM] and the control. It was also found that as light intensity decreased, the rate of hypocotyl development increased.

Hypocotyl length varied in the 3-MPA treatment group under different lighting conditions. In the gradient light (GL) group treated with 25 µM of 3-MPA, a longer hypocotyl was observed compared to the control and the 50 µM 3-MPA group. Hypocotyl length did not change significantly under the other two light conditions (TL, GL) in either the control or 3-

MPA treatment groups. Changes were also noted in the total dark (TD) group, which showed an increase in hypocotyl length when treated with 3-MPA at both concentrations. However, hypocotyl growth did not respond to shoot dark (SD) and shoot dark with light blocker (SDB) conditions at the 3-MPA concentrations tested.

Arabidopsis roots express all 12 photoreceptors (Mo et al., 2015) and can detect light and evaluate its spectrum and intensity using various photoreceptors, allowing for the integration of growth between aboveground and underground organs. In laboratory settings, the roots of Arabidopsis seedlings should be kept in darkened Petri plates. Exposing roots to light affects not only the roots themselves but also the overall morphology and physiology of the seedlings. Recent discoveries that plant GABA can regulate ion channels (ALMTs) have spurred GABA research, though many aspects of GABA's regulation of plant physiology and development remain unclear. Further research has shown that plant hormones (such as ethylene and ABA) and ROS production can influence GABA metabolism in plants, and experimental evidence indicates that high GABA concentrations inhibit root growth. Our research has provided additional insights into this topic and our findings suggest that GABA levels modulate plant growth responses under different environmental conditions. However, future experiments treating young seedlings with exogenous GABA will further elucidate GABA's role in plant growth.

4.3. Chapter 3

"Investigation of *Arabidopsis* root skototropism with different distance settings"

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Yan, X., Liang, Y., **Yamashita, F**., & Baluška, F. Investigation of *Arabidopsis* root skototropism with different distance settings. *Plant Signaling & Behavior*, 19, 1 (2024).
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"Investigation of *Arabidopsis* root skototropism with different distance settings" was published in 2024 as an open access article in Plant Signaling & Behavior. The original publication is attached in the appendix of this dissertation. The abstract below is intended to highlight my personal contribution to this document. As an abstract focusing only on the main important points to provide clarity and brevity, the appropriate references to some statements have been omitted, which can be found in the full article attached (Appendix 3).

This publication, in which I am the third author, investigates root skototropism and its connection to the distance between root and light. Approximately 70% of the experiments were carried out by Xingyu Yan and Yongshun Liang, and the other 30% by me. The data generated was discussed with all the co-authors before a manuscript was prepared. I wrote the first manuscript version and Xingyu Yan and Yongshun Liang finalized it. All the co-authors helped to revise its content. All the co-authors approved the final manuscript version before I sent it to the corresponding journal.

Continuing in the same research direction as the previous paper (Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions), we analyzed the relationship between light and root and hypocotyl growth of *A. thaliana*. Light is vital for plant processes like photosynthesis, photomorphogenesis, and phototropism, where plants grow towards light due to asymmetric auxin distribution. Conversely, in roots, the growth direction is the opposite of the light source, in other words, towards darkness. This light escape tropism of roots could be defined as the combination of negative root phototropism and increased root growth, also known as skototropism.

Skototropism is active plant tropism that represents the directional search behavior performed by the shoot apex to detect a potential host tree for support, or the root apex to

navigate towards darkness. In *A. thaliana*, two transporter families play a role in auxin transportation: the long PINs (PIN 1-4) and PIN7. Additionally, the phototropin (PHOT) blue light receptors are important as they interact with PIN2 in light-induced root responses.

Therefore, in this paper, we use *AtPIN2* and *AtGLR3.7* Arabidopsis mutant lines to examine skototropic root behavior along with the mutants. The *AtGLR3.7* mutant lines are plants with more glutamate-like channels 3.7. Glutamate is a neurotransmitter that is present in humans and is approximately 25 years has also been found in plants. In humans, glutamate receptors are responsible for cell-cell signaling and communication, acting as calcium channels. In plants, glutamate channels also act as calcium channels in cell-cell communication and long-distance signaling. In Arabidopsis, GLRs contribute to stress responses, reproduction, and growth regulation. Despite extensive research, the precise biological functions of GLRs are still being elucidated.

We used four different types of Petri dishes, always leaving half of the dish covered. This half was either inside black boxes or with a black cover. On the illuminated side of the plate, the seedlings were placed at distances of 0, 10, 20, 30, and 40 mm from the edge of the darkness for 96 hours, after which the root bending angle was measured. The four groups were therefore: (A) Small round dishes (92 × 16 mm) with three columns of seedlings were inserted into black boxes, resulting in light intensity on the darkness side of 39.74 μmol s^{-1} m^{-2}; (B) Small round dishes with three columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 15.34 μmol s^{-1} m^{-2}; (C) Large round dishes (150 × 20 mm) with five columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 19.10 μmol s^{-1} m^{-2}; (D) Square dishes (120 × 120 × 17 mm) with five columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 20.17 μmol s^{-1} m^{-2}. The light source was at the growth chamber ceiling.

Seedlings and their roots cannot detect darkness as distances grow considerably, leading to the absence of root escape tropism. Our experiments also showed that as the distance reached 40 mm, the relative difference between positive and negative root skototropism decreased. Even though the majority of plant roots grow underground in dark conditions, all photoreceptors are present at the root tips. While weak light does not stress the roots, they attempt to avoid strong light. Recent research indicates that Arabidopsis roots exhibited accelerated growth in a light gradient environment, growing towards darkness. Both the root skototropism (growth towards darkness) and the skototropic root growth (acceleration of root growth within light-dark gradient) suggest that there may be some form of vision at the root tips. The concept that plants might possess a type of plant-specific vision was initially proposed by Gottlieb Haberlandt in

1905 and based on "Plant Ocelli." He contended that the leaf epidermis could function as a convex or plano-convex lens.

In line with the concept of plant ocelli, the way *phot1* is distributed in the transition zone of the root apex indicates that this area may be involved in sensing blue light, whereas the root cap is specifically adapted for sensing red light. Recent studies have shown that both red light and blue light can increase the transcription levels of various genes that encode GLR proteins. Under red light conditions, the upregulation of AtGLRs at the transcriptional level is mainly controlled through processes mediated by pigments. Our findings indicate that the *atglr3.7* knockout displayed a higher degree of positive skototropism in comparison to the wild-type (Col-0) line, implying that *AtGLR3.7* may have a role in regulating the skototropic response in the roots of Arabidopsis. Conversely, the overexpression of *AtGLR3.7* led to a reduction in positive skototropism and an increase in negative skototropism, suggesting that overexpressing *AtGLR3.7* could disturb the typical skototropic response. Furthermore, the *pin2* knockout mutants exhibited a different response to darkness compared to the wild-type line (Col-0). The root curvature did not significantly change in response to the distance from darkness, indicating that the PIN2 protein, responsible for auxin transport, likely has a key role in mediating the seedling's response to darkness by skototropic Arabidopsis roots. Interestingly, even in the absence of functional PIN2 protein, about 50% of the *pin2* mutant seedling roots displayed bending towards darkness. This indicates that there may be other auxin transporters besides PIN2 involved in root skototropism. One potential candidate for this role could be the ABCB auxin transporter, which has been demonstrated to have a significant impact on root phototropism.

In essence, this research offers a valuable understanding of the skototropic tendencies of Arabidopsis roots and the potential participation of *AtGLR3.7* and *AtPIN2* in regulating this reaction. Additional investigations are required to better clarify the underlying molecular mechanisms and signaling pathways responsible for root skototropism. Grasping these mechanisms could have implications for enhancing plant growth and development in various environmental circumstances

4.4. Chapter 4

"Potential plant–plant communication induced by infochemical methyl jasmonate in sorghum (*Sorghum bicolor*)"

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This publication, in which I am the first author, analyzes plant communication through chemical stimuli. The experiments were carried out by me in Brazil during my master's degree. The data was analyzed and discussed with all co-authors prior to the preparation of the manuscript during my PhD in Germany. I wrote the first manuscript version and all the co-authors helped to revise its content. All the co-authors approved the final manuscript version before I sent it to the corresponding journal.

In this paper, we performed an experiment to investigate root behavior. Besides, this research also explores plant memory and plant communication through root exudates. Plants communicate with neighboring plants, herbivores, and predators by releasing various chemical compounds from their shoots and roots. These chemicals influence the behavior of neighboring plants and can also lead to physical and physiological changes in them. To examine communication between emitters and receivers of *Sorghum bicolor* plants, we utilized methyl-jasmonate (MeJa), a natural plant infochemical. We divided the roots of two plants and placed

them in three different pots, with one pot containing the roots of both plants and the other pot containing the roots of one plant each. We used a randomized block design with four groups, distinguished as follows: (1) Mock (M): Roots came in contact with a mock solution (without MeJa), and were split into two parts, with one half in pot 1 and the other half in pot 2. (2) Mock neighbor (MN): Roots did not come in contact with any solution, split into two parts, with one half in pot 2, in direct contact with the roots of the mock group, and the other half in pot 3. (3) Treated (T): Roots came in contact with the MeJa solution, split into two parts, with one half in pot 1 and the other half in pot 2. (4) Treated neighbor (TN): Roots did not come in contact with the MeJa solution, split into two parts, with one half in pot 2, in direct contact with the roots of the treated group, and the other half in pot 3. We applied 2 mL of MeJa solution to the first fully expanded leaf of the plants in group T. The application was performed twice, with an interval of 10 days between them, to analyze the plants' memory effect.

The results showed that in the first exposure to MeJa, we observed a smaller CO_2 net assimilation rate (A) in the treated (T) plants in comparison to the mock (M) plants. In the T group, the A was smaller than that of the M group by 23.5% at 5 h and 20.4% at 7 h after application (HAA). Five days after the first contact, we applied MeJa for a second time and observed that A did not differ between the T and M groups. In contrast, by just comparing the A of the plants that received the infochemical (T) between the first and second contact, we observed that the A was greater during the second contact than in the first contact by 44.31% at 5 and 31.67% at 7 HAA. Similarly to A, stomatal conductance (g_S) decreased after MeJa contact. Just 3 HAA of MeJa, we observed smaller g_S in the plants of the T group compared to those of the M group. This pattern continued until at 7 HAA, only equaling out at 9 HAA. We observed a 68% higher stomatal conductance of the plants in the T group during the second contact when we compared it to the first contact at 5 HAA.

The signaling led to changes in the physiological patterns of the stages in the photochemical phase of photosynthesis. During the second contact, we observed that the plants of the treated neighbor (TN) group had a higher maximum fluorescence adapted to light (F_M) compared to the other groups. This difference was found in neighboring plants (mock neighbor (MN) × TN) at 5, 7, and 9 HAA during the second contact with MeJa. We also recorded the same difference in patterns at the same hours between the T and TN groups, being that the maximum fluorescence of TN was higher by 75.4% at 5 HAA, 57.3% at 7 HAA, and 39.9% at 9 HAA.

The morphological analysis of the *S. bicolor* adventitious roots after going through two rounds of contact with MeJa showed variations regarding the intercellular space in the cortex and the area occupied by the stele. Roots with smaller intercellular spaces were observed in the

plants of the T and TN groups. The roots of the M group had a cortical intercellular space area that was 45.9% greater than that of the T group. The plants in the MN group had a cortical intercellular space that was 25.2% greater compared to that of the plants in the TN group. In contrast, the plants of the T and TN groups had larger steles. The plant roots in the T group showed twice the area occupied by the stele (101.6%) concerning those of the M group. The plants of the TN group showed roots with an area occupied by the stele that was 41.17% greater than those of the MN group.

Upon initial contact with MeJa, the plants in the treated (T) group displayed changes in their physiological parameters. However, their responses during the subsequent contact did not show any variance from those of the mock (M) group, suggesting that sorghum plants developed reduced sensitivity to MeJa after the initial treatment. It was also observed that plants from the T group potentially conveyed sensory information through their roots to neighboring plants (TN group). However, our findings do not exclude the potential role of shoot volatiles in this form of plant-plant communication, as previous studies have shown their impact on gene expression and stomatal opening. In conclusion, MeJa may have facilitated plant-plant communication and affected the physiological and morphological characteristics of neighboring plants. In the future, it will be crucial to examine plant-plant communication concerning key physiological parameters of plant responses to environmental challenges, to anticipate responses and enhance the likelihood of overcoming future stress events.

4.5. Chapter 5

"*Boquila trifoliolata* mimics leaves of an artificial plastic host plant"

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"*Boquila trifoliolata* mimics leaves of an artificial plastic host plant" was published in 2022 as an open access article in the Plant Signaling & Behavior. The original publication is attached in the appendix of this dissertation. The abstract below is intended to highlight my personal contribution to this document. As an abstract focusing only on the main important points to provide clarity and brevity, the appropriate references to some statements have been omitted, which can be found in the full article attached (Appendix 5).

This publication, in which I am the second author, analyzes the intriguing mimicking ability of *Boquila trifoliolata*. The experiments were carried out by Jacob White and all data was evaluated and analyzed by me. Data was discussed with the first author prior to the preparation of the manuscript. Jacob White and I wrote the manuscript version. Both authors approved the final manuscript version before I sent it to the corresponding journal.

In our study, we examined a different type of plant motion by observing the *B. trifoliolata*, a distinctive vine plant from South America that adjusts the shape of its leaves based on the surrounding plants. Despite confirming the Boquila's capacity for adaptable leaf mimicry, the mechanism underlying this ability remains unknown. We provide evidence suggesting that plant vision, potentially through plant-specific ocelli, is a possible hypothesis.

Boquila belongs to the Lardizabalaceae family, consisting of 8 genera and 45 species. Six of these genera are found in East Asia (from the Himalayas to Japan) and only two genera are found in South America, in Chile. The two species found in Chile are: *Lardizabala biternata* and *B. trifoliolata*. Boquila is endemic to the temperate rainforests of southern South America, mainly in south-central Chile, and also in some regions of Argentina.

In 2014, a research group from Chile revealed that the leaves of the Boquila mimic the leaves of the host trees in terms of size, color, orientation, shape, and venation pattern. Surprisingly, a single Boquila individual plant extended itself under three host plants of three different species and mimicked the leaves of all three host plants. This ability to mimic multiple species had never been observed in science before. The researchers did not conclude how Boquila mimics the leaves of host trees. In their paper, they proposed two hypotheses. The first suggests that Boquila alters the shape of its leaves in response to volatile compounds received from the host plant, as leaf mimicry was observed even when Boquila was not in direct contact with the host plant. The second hypothesis proposes that a vector, such as microorganisms present in the air around the host plant's leaf, might transport genetic material from one plant to another.

After five years of home experiments performed by Jacob White and my analysis of all these data, we published our paper with a third hypothesis. We did not find the Chilean researcher's hypothesis convincing, so we chose to investigate an alternative. This alternative explanation needed to contradict the previous two theories, meaning there should be no evidence of volatile chemical compounds or genetic material from the host plant for any microorganism to transmit between individuals. As a result, we opted to use a synthetic plant, a plant constructed from plastic material. Consequently, it would be impossible for Boquila to absorb any volatile chemical compounds triggering leaf modifications, and it would also be unable to obtain genetic material, as the plastic plant lacks genetic material altogether.

Four Boquila plants were placed in a row and their opaque shelves above them. As they are lianas, which means climbing plants, a wooden support was placed so that the plants could have a support to grow on. Plastic plants were placed above the first shelf so that new Boquila leaves would come into contact with the artificial plastic leaves. After a while, the Boquila leaves that grew next to the plastic leaves stopped showing a three-lobed shape and grew into a more elongated shape, similar to the plastic plants.

Boquila leaves change shape as the vine grows towards the artificial plant. The plants attempt to mimic the closest false leaves of model plants, although some leaves still keep a single lobe. However, all leaves exhibit more elongated shapes. We utilized a leaf recognition algorithm to assess the mimicry of Boquila leaves, focusing on quantifying leaf forms. We noticed significant variations in leaf widths. By establishing a correlation between leaf area, perimeter, length, and width, we derived various parameters. It was observed that the non-mimic leaves displayed greater rectangularity, taking on a uniform, rectangle-like form.

The aspect ratio and form factor show us that mimic leaves are generally longer rather than wider, indicating that they are more similar to the elongated plastic leaves that were placed next to the Boquila plants, as a model of the host plant. The non-mimic leaves showed similar values for lengths, having their form factor values close to 1 (similar width and length values result in the form factor values close to 1), the more similar the leaves are in length and width. Corroborating these data, we obtained rectangularity, showing us that the non-mimics are more roundish in shape, in comparison to the slender mimic leaves.

The mimicry began just below the artificial vine (between shelves 1 and 2) and when more leaves were directed toward the model leaves, it seemed to affect the detail of mimicry. This indicates that the lower leaves copy the details of the adjacent leaves and transfer that information to the next set of developing leaves. Fresh leaves take form in the mimic shape and young leaves grow larger in that same shape. This implies that the lower leaves have some involvement in leaf mimicking.

An interesting finding was made regarding the venation pattern when examining the leaves under binocular microscopy. It was noted that non-mimic leaves exhibited a higher prevalence of free-ending veinlets, which are tiny veinlets with their ends extending freely into the leaf mesophyll. The presence of more free-ending veinlets was observed in non-mimic leaves across all stages of leaf development - from young to middle-aged to old leaves. It is well known that vein development and patterning progress in a direction from the leaf apex to the base, therefore, the leaf apex tends to have a more advanced venation network compared to the base. In contrast to non-mimic standard leaves, mimic leaves show a reduced number of free-ending veinlets. This characteristic suggests elevated auxin concentrations at the leaf margins, indicating potential alterations in auxin biosynthesis and polar auxin transport in these leaves. These findings could be indicative of an effort to modify the leaf shape, possibly mimicking the characteristics of plastic leaves.

Observations have shown that all shoots growing near the artificial model (host) plant have attempted leaf mimicry. Some of the mimicking leaves do not perfectly, similar to their attempts at serrated leaves in nature. This imperfection could be attributed to the uneven edges of the artificial plant. As a result, all leaves in contact with the artificial vine have a notably different shape compared to the non-mimic leaves located below the shelf. Our research demonstrated that leaves of *B. trifoliolata* mimic artificial leaves by altering their shape to a longer, lobeless form. This goes in the opposite direction of the two hypotheses proposed by Gianoli & Carrasco-Urra 2014, which proposed that Boquila's leaves could adopt airborne chemicals

released by other trees or acquire genes from its host through a parasite or microbe. Our current analysis supports the theory of plant vision based on Haberland's plant-specific leaf ocelli.

4.6. Chapter 6

"Algal Ocelloids and Plant Ocelli"

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"Algal Ocelloids and Plant Ocelli" was published in 2023 as an open access article in the Plants Journal. The original publication is attached in the appendix of this dissertation. The following summary aims to highlight my personal contribution. As an abstract that only focuses on the major points of importance to provide conciseness and clarity, appropriate references to some statements have been left out, but they can be found in the full article attached (Appendix 6).

This publication, of which I am the first author, examines different types of vision between kingdoms, considering the appearance of eyes in cyanobacteria and algae, and how the vision could plausibly occur in plants. With this mini review, we suggest that such an efficient characteristic across kingdoms has been passed on through evolution, so the plant kingdom probably also has this characteristic, it just has not been discovered yet. I carried out all of the literature research with the support of František Baluška.

The range of vision in animals is remarkably varied and has developed separately multiple times. Although there are many different types of visual organs, an eye can be described as having a cornea and/or lens that concentrates light onto a sensory area, like the eye retina or other light-sensitive structures and tissues, where photo-responsive proteins change the light signal into electrical and chemical signals.

In algae, more specifically in the green alga *Chlamydomonas reinhardtii*, the eye apparatus can be called an eyespot. The subcellular apparatus of eyespot is anchored at the cell periphery, with photoreceptors (Channelrhodopsin) in the algae membrane, just below with the presence of two layers of carotenoid globules positioned between the thylakoid and chloroplast membranes. In addition, is the rhizoplast, which is a contractile centrin-based structure connecting the basal bodies of the flagella with the nuclear surface. An important detail is the electrical currents in the eyespot induced by light, which activates and controls the flagella motions through a bioelectric process similar to the action potential.

In addition to *C. reinhardtii*, another type of algae possessing a photosensitive apparatus is *Euglena gracilis*. This organism, which is well-known for unicellular vision, exhibits two kinds of photo movement, namely phototopic and phototactic behavior. The movement of this algae in response to light stimuli is also reliant on carotenoids, and its plastids do not transform into chloroplasts because of the absence of chlorophyll. Recent studies have indicated that Euglena lacking carotenoids have lost their capacity to react to light, indicating the essential nature of carotenoids for light detection.

In 1967, David Francis described an eyespot in *Nematodinium armatum*, describing lenses capable of focusing light rays and concentrating them into a structure called a pigment cup. This structure is supposed to be a light-sensitive retinoid and may have a role in image formation. In 2015, surprising discovery of eye-like ocelloids in warnowiid dinoflagellates. These single-celled organisms that float in the water have specialized organelles that function as eye-like structures called ocelloids. The outer layer, which is based on mitochondria, forms a cornea-like surface covering a lens structure, while the retinal body of the ocelloids is created from a membrane network derived from plastids. To confirm these observations using a microscopic approach, the researchers analyzed the DNA of a warnowiid retinal body. They found that it contained a much larger proportion of DNA originating from plastids compared to other samples from the entire cell. Warnowiid dinoflagellates are the only type of single-celled microorganisms that possess eye-like structures similar to cameras for vision-like capabilities.

In bacteria, vision in cyanobacterium *Synechocystis sp*. PCC 6803 is accomplished by the whole cell acting as a lens, focusing light on a small patch of the plasma membrane. The entire cell acting as a lens is a principle found also in eukaryotic volvocine algae, suggesting that plant cells may also use this feature through their ocelli. Evolution makes use of successful structures and processes that enhance survival chances. Even complex vision organs like animal and human eyes are part of this evolutionary continuum. In multicellular volvocine algae, cells' light-focusing roles affect adjacent cells, influencing morphological symmetries and colony behavior. In Synechocystis, light perception at the photosensitive patch of the plasma membrane electrically controls a motility apparatus based on type IV pili. The pili near the light focal spot are inactivated, whereas those facing the light source are active, enabling movement toward the light. This ancient prokaryotic vision based on the type IV pili complex in cyanobacteria evolved over three billion years ago and has proven to be a very successful solution to environmental challenges.

In plants, Gottlieb Haberlandt proposed the plant ocelli concept for leaf epidermis in which the upper epidermal cells resemble convex or planoconvex lenses, converging light rays on the

light-sensitive subepidermal cells. The concept was revisited years later when it was observed that young seedlings of the tropical vine *Monstera gigantea* demonstrated the ability to grow in the direction of darkness, a behavior known as skototropism. This behavior allowed the Monstera seedlings to locate and effectively attach themselves to host trees for support. Skototropism refers to the directional movement of a plant organ toward darkness. Another example is the Boquila, which, based on the article described in Chapter 5, may have some kind of structure that makes it possible to see the plastic leads around it. So, they modify their leaves, accordingly, mimicking the more elongated shape of the false leaves and avoiding the traditional three-lobed shape of the species.

Nonetheless, human eyes and specialized insect eyes both likely originated from a common predecessor, possibly from eyespots similar to those found in cyanobacteria. Biological evolution does not always follow a linear path, but many useful traits have emerged and persisted across all kingdoms over millions of years. While there is no proof that plants possess ocelli, the absence of evidence does not rule out the possibility. Importantly, several technical papers published by the group of Thomas Vogelmann in the 90-ties confirmed that leaf epidermal cells have lense-like Properties. It is not implausible for plants to have a primitive vision structure. The sophisticated vision organs found in humans and animals are also a significant aspect of the biological evolutionary process.

4.7. Chapter 7

"Root apex cognition: from neuronal molecules to root-fungal networks"

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Baluška, F., **Yamashita, F.**, and Mancuso, S. Root apex cognition: from neuronal molecules to root-fungal networks. In: Baluška F, Mukherjee S. Rhizobiology: Molecular Physiology of Plant Roots, Signaling and Communication in Plants. Cham: Springer International Publishing: 1-24 (2021).
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"Root apex cognition: from neuronal molecules to root-fungal networks" was published in 2021 as a chapter in the book "Rhizobiology: Molecular Physiology of Plant Roots", which is part of the book series "Signaling and Communication in Plants" by Springer Nature. The original publication is attached in the appendix of this dissertation. The following summary aims to highlight my personal contribution. As an abstract that only focuses on the major points of importance to provide conciseness and clarity, appropriate references to some statements have been left out, but they can be found in the full article attached (Appendix 7).

This publication, of which I am the second author, explores the sophisticated and dynamic behaviors exhibited by plant roots, particularly focusing on the root apex as a cognitive entity. František Baluška, Stefano Mancuso and I carried out all of the literature research.

In their book "The Power of Movement in Plants" published in 1880, Charles and Francis Darwin proposed that the root apex functions as a brain-like organ. They described the tip of the radicle as being equipped with the power to control the movements of the adjacent parts, likening it to the brain of lower animals situated at the anterior end of the body. It receives input from the sense organs and coordinates various movements. In essence, Darwin suggested that the root apex processes information from its environment and influences root development.

The root transition zone, which functions similarly to a Darwinian root brain, processes various environmental signals to direct the root's growth and behavior. This region integrates information from the environment, such as light, gravity, water, and nutrient availability, allowing the plant to make crucial adaptive decisions for survival. Also found in this region are neuronal molecules (glutamate, GABA) that play a role in root development and response mechanisms such as signaling and communication between cells. Other neuronal molecules,

including peptides and neurotransmitter-like substances, also contribute to the root's ability to perceive and respond to the environment, demonstrating functional parallels with the neuronal systems of animals.

Not only substances from the plant itself can help communication and signaling between individuals. There is a symbiotic relationship between plant roots and mycorrhizal fungi. This symbiosis forms an extensive underground network, often called the " wood-wide web", which facilitates communication and the exchange of resources between plants. Mycorrhizal fungi increase nutrient absorption, improve resistance to pathogens, and contribute to the overall health of plants and the stability of the ecosystem. The coevolution of roots and mycorrhizal fungi has driven the development of sophisticated sensory and signaling mechanisms, allowing roots to effectively navigate and exploit the soil environment.

Roots exhibit a form of swarm intelligence, in which the collective behavior of individual root apices results in efficient soil exploration and resource acquisition. This behavior is similar to the swarm intelligence observed in social insects such as ants and bees, which coordinate their activities to accomplish complex tasks. Root systems show coordinated growth patterns, resource allocation strategies, and adaptive responses to environmental changes, reflecting a high degree of organizational complexity.

Understanding root apex cognition has profound implications for agriculture, ecology, and plant biotechnology. Insights into root behavior and communication can inform the development of crops with greater growth efficiency, stress resistance, and nutrient use efficiency. Furthermore, this knowledge challenges traditional views of plants as passive organisms, highlighting their dynamic and responsive nature. Recognizing that plants are capable of complex interactions with their environment can lead to innovative agricultural practices and ecosystem management strategies.

In conclusion, the root apex, equipped with molecules similar to those found in neurons and involved in symbiotic relationships with fungi, acts as a central hub for processing environmental information and orchestrating adaptive behaviors. This research highlights the sophistication of plant sensory systems and their crucial role in plant survival and adaptation. Exploring the parallels between plant and animal cognition highlights the complexity of plant behavior and its importance for agricultural and ecological research. Understanding these mechanisms can lead to the development of more resilient and efficient crops, better ecosystem management practices, and a deeper appreciation of the intricate ways in which plants interact with their environment.

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6. Eidesstattliche Erklärung

Hiermit versichere ich, dass diese Dissertation von mir selbst und ohne unerlaubte Hilfe angefertigt wurde. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Ferner erkläre ich, dass die vorliegende Arbeit an keiner anderen Universität als Dissertation eingereicht wurde.

Felipe Yamashita

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8. Appendix 1

Chapter 1

"Root and hypocotyl growth of Arabidopsis seedlings grown under different light conditions and influence of TOR kinase inhibitor AZD"

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Root and hypocotyl growth of *Arabidopsis* seedlings grown under different light conditions and influence of TOR kinase inhibitor AZD

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We set up six light conditions to investigate the changes in the development of *Arabidopsis thaliana* hypocotyls and roots. Seedlings grown for 96 h under darkness were scored with shorter roots and longer hypocotyls. In shoot-shaded conditions, seedlings were unable to carry out photosynthesis, resulting in insufficient stored nutrients for root development. In the three groups of different light intensities applied to the roots, total light caused stress in the entire seedlings and the length of roots and hypocotyls were shorter than in conditions when roots were growing within light-dark gradients. Importantly, root lengths were higher within light-dark gradients than in total light. Different light treatments did significantly affect root growth and hypocotyl growth. The addition of ATP-competitive mTOR kinase inhibitor (AZD), drastically reduced root, however, this did not occur with hypocotyl length.

Key words: Total light, total dark, gradient light, shoot dark with light blocker, light blocker, shoot dark.

INTRODUCTION

To adapt to a changing environment, all living organisms have to respond appropriately to circumstances. Unlike animals, plants are unable to move away from extremes in their surrounding environment or move towards a nutrition source. However, plants have a flexible pattern of development that allows them to adjust their organ number and size (architecture) to the changing environment. The fundamental body plan of the mature plant is generated during the early stages of embryogenesis (Jürgens et al., 1991). This process involves the production of shoot and root meristem, cotyledons, radicle and hypocotyls.

In animals, most organs are already present by the time the embryo is fully formed. On the contrary, most organs in plants are formed after embryogenesis is finished. Once dormancy is broken, the seeds begin to

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germinate with the formation of primary plant organs: roots, shoots, and leaves (Kadereit et al., 2014). Roots emerge from root meristems located at the tip of the root, while the aboveground shoot system generates from shoot meristems (Brand et al., 2001). Roots are the underground part of the plant body that is required for anchorage in the substrate, uptake of water and ion, and synthesis of phytohormones (Kadereit et al., 2014). The root apex also has an oscillatory zone (Baluška and Mancuso, 2013). The root apex is subdivided into four zones: Meristematic (Kadereit et al., 2014), transition (Verbelen et al., 2006, Baluška et al., 2010), elongation, and differentiation zones. The root cap is the structure that detects the pull of gravity and thus controls the downward growth of roots (Petricka et al., 2012). Statocytes are specialized root-cap cells that contain amyloplasts, which will precipitate if the root is reoriented (Kadereit et al., 2014). Cells in the elongation zone elongate and allow root growth (Crang et al., 2018). Root growth is regulated and fine-tuned by several phytohormones. The first phytohormone to be discovered was auxin, which is crucial for cell elongation and lateral root growth (Petricka et al., 2012).

Light is one of the most important environmental factors for plant development. Light cues of varied intensity and quality cause plants to change their morphological traits (Yadav et al., 2020). Besides that, light and temperature also modulate phytochrome growth via phytochrome (Ibrahim and František, 2022). Light perceived by photosensory systems in above-ground tissues can affect the roots via long-distance signal transduction pathways (Qu et al., 2017). Sunlight, on the other hand, can reach root tissues that are several centimeters underground (Qu et al., 2017). Plants use dedicated photoreceptors to receive light signals of various wavelengths. Activated photoreceptors trigger a signal transduction cascade, which results in a wide range of gene expression modifications that affect physiological and developmental responses (Su et al., 2017). Most known plant photoreceptors, including phytochromes, cryptochromes, phototropins, and ultraviolet receptors (UVR), are expressed in the root tissues (Qu et al., 2017). Phytochromes are a class of red (R)/far-red (FR) light photoreceptors in plants that mediate the expression of various genes and are involved in root development and structure (Briggs et al., 2001).

When seedlings receive light, the elongation of the hypocotyls is carefully regulated to match the intensity and quality of light around them, and the phenomenon of de-etiolation occur, in which the development of leaves and chloroplasts inhibits stem elongation and promotes root growth and lateral root development, a process known as photomorphogenesis. The initial response of plants to light is photomorphogenesis, in which the shoot and root meristems are activated, and a series of changes, such as cell division and expansion, lead to changes in the differentiation structure and function of plant cells, and eventually the formation of tissues and organs (McNellis and Deng, 1995).

Light, as we have seen, has a rapid and dramatic impact on root development and physiology. Light is shown to have a great impact on adventitious roots and hypocotyles (Zeng et al., 2022). That aspect should be taken into account while doing experiments with plants, particularly those focusing on roots. Seeds are usually planted in transparent agar medium in hyaline Petri dish plates in laboratory settings, exposing roots and shoots to light in a similar way. Sucrose is also added to the growth medium of the classic agar plate culture technique (TPG, traditional plant-growing). However, because this is not a natural environment for roots, certain artifacts may occur (Xu et al., 2013). Improved approaches are available to make root experiments more efficient. An improved agar-plate method (IPG, improved plant-growing) is one example, in which shoots are lighted while roots are grown in a media without sucrose under dark circumstances. When comparing IPG to TPG, the root and lateral root lengths are both shorter, and the root hair density is lower. The primary root, on the other hand, was much longer. As a result, IPG provides a better and more natural environment for investigating *A. thaliana* root development and responses (Xu et al., 2013).

The target of rapamycin (TOR) is a large and highly conserved protein belonging to the family of phosphatidylinositol 3-kinase-related kinases (PIKKs). In response to environmental changes such as nutrients, energy status, and growth factors, TOR serves as a key sensor of cell growth and metabolism. Recent research has discovered that the conserved TOR pathway is vital in coordinating plant development at the whole plant level (Barrada et al., 2015). Furthermore, the Arabidopsis genome seems to have a single critical TOR gene, which down-regulation results in reduced plant growth, stress resistance (Menand et al., 2002), and increased life span (Ren et al., 2013). Moreover, *Arabidopsis* plants silenced for TOR expression display significantly reduced polysome abundance (Deprost et al., 2007), indicating that TOR plays a function in plant translational regulation. TOR inhibitors restrict the meristematic cell proliferation capability by reducing the number of cells in the MZ, mostly via encouraging differentiation (Montané and Menand, 2013). One of the most effective TOR inhibitors is AZD. As a second-generation mTOR inhibitor (known as ATP-competitive mTOR kinase inhibitor), it is developed not only to suppress mammalian TOR for cancer therapy but also to inhibit TOR in plants. AZD can bind to the TOR kinase domain within the ATP-binding pocket and inactivates the TOR complex (Montané and Menand, 2013). The aim of this study was to show the effect of AZD on Arabidopsis seedlings grown for 96 h under different light conditions. We could show that when Arabidopsis is treated with AZD and grown for 96 h under different light conditions, the root and hypocotyl are significantly modulated.

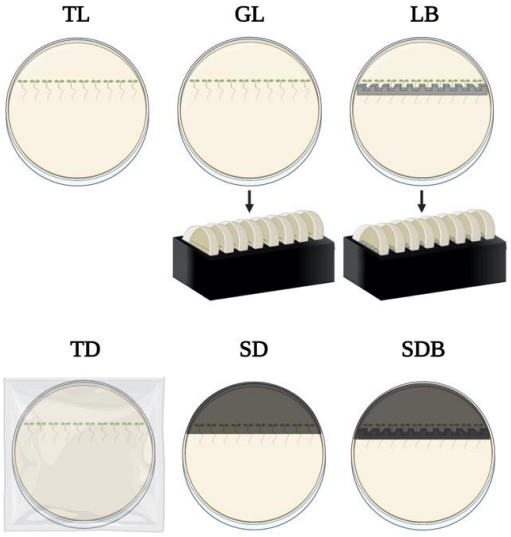

Plate 1. Experimental setup performed in this study: Different light conditions for *A. thaliana* seedlings growth. First line from left to right: total light (TL), gradient light (GL), light blocker (LB). Second line from left to right: total dark (TD), shoot dark (SD), shoot dark with blocker (SDB).
Source: Authors

MATERIALS AND METHODS

Arabidopsis thaliana seedlings grown under different light conditions

Growth media preparation

The growth medium was prepared by mixing the MS medium salt (with vitamins), saccharose and dH_2O. After adding each to a 1 L container, the pH was adjusted to 5.8 using KOH or HCl. After that, 4 g of phytagel was added to 1 L. The medium was mixed and autoclaved at 120°C. The medium was placed in Petri dishes of different sizes and prepared under a sterile bench, for further usage. For the stupor experiment with *A. thaliana* seedlings, medium was added on round Petri dishes with AZD at 5 µM concentration.

Seeds preparation

A. thaliana seeds were sterilized in a plastic tube for 3 min with 1 mL of 70% ethanol. This was followed by a 5 min treatment with 1 mL sodium hypochlorite solution. The plastic tube was inverted many times in each phase. The seeds were washed five times in distilled water. Sterilized seeds were sown on square Petri dishes with ½ MS medium under the sterile bench. The square Petri dishes with sterilized seeds were stored in the fridge for stratification for 48 h at 4°C and transferred to the growth chamber for 96 h for seed germination.

Experimental preparation with AZD

To investigate the influence of AZD on *A. thaliana* root and hypocotyl growth, *A. thaliana* seedlings were transferred to round Petri dishes with phyto agar and AZD (5 µM). The 48 h stratified seedlings were placed side by side with straightened roots in a horizontal position. Control plates were treated in the same way but, only containing phyto agar. After placing the seedlings, all dishes were sealed with parafilm and then transferred to different light conditions (Plate 1). Each treatment was repeated in triplicate. The six light conditions used are as follows: (1) Total light (TL): Round Petri dishes with *A. thaliana* seedlings were placed under the light of the growth chamber with the intensity of 100 µmol s^{-1} m^{-2}. (2) Total dark (TD): Plants were kept in total darkness (covered with aluminum foil) for 96 h. Moreover, shaded seedling roots create two forms of light; gradient light and light blocker. Shoot dark and shoot dark with a light blocker were two types of light conditions created by shading seedling shoots. (3) Gradient light (GL): Plants in the Petri dish were introduced in a black box, where the roots were inside the box and the hypocotyl outside, resulting in a slight gradient with a value of 39.74 µmol s^{-1} m^{-2}. (4) Light blocker (LB): A light blocker strip was placed inside the medium, perpendicular to the Petri dish, preventing light from reaching below the blocker. Subsequently, they were introduced into a black box, resulting in light intensity of 7.27 µmol s^{-1} m^{-2}. (5) Shoot dark (SD): The hypocotyls of *A. thaliana* were covered resulting in light intensity of 7.91 µmol s^{-1} m^{-2}. (6) Shoot dark with light blocker (SDB): The light blocker strip (same as LB) was placed on medium in a round Petri dish and then the hypocotyls of the seedlings were covered, resulting in light intensity of 2.03 µmol s^{-1} m^{-2}.

Root and hypocotyl lengths measurements

After 24, 48, 72 and 96 h the round Petri dishes were scanned. Based on the digital images, the root length and hypocotyl length were measured via Fiji software.

Skototropism experimental preparation

To investigate the influence of distance to darkness on *A. thaliana* roots for the skototropism experiment, *A. thaliana* seedlings were transferred to round Petri dishes with phyto agar. The seedlings were placed in a vertical position, one below the other with straightened roots. After placing the seedlings, all dishes were sealed with parafilm and then placed in construction that held one-half of the Petri dish in darkness. Dishes were aligned in the construction and set with different distance patterns (0, 10 and 20 mm) from the *A. thaliana* seedlings to the darkness and then placed under artificial light (100 µmol s^{-1} m^{-2}) for 96 h in the growth chamber.

Measurements and evaluation

After 96 h, the round Petri dishes were scanned. Based on the digital images, the root bending angle was measured. Values for root bending were sorted into 3 groups: (1) Positive values, showing a bending towards darkness; (2) Negative values, indicating a bending away from darkness; (3) A group zero, exhibiting no visible behavior towards or away from light. Collected data for the experiment were evaluated with Fiji ImageJ software. The length standard for these measurements was set with the help of a ruler which was calibrated with the respective samples. Statistical analysis was performed using Graphpad Prism (version 9.1.1)

RESULTS

Treatment with AZD (5 µM) under shaded light conditions

We found that the growth of the AZD treatment groups

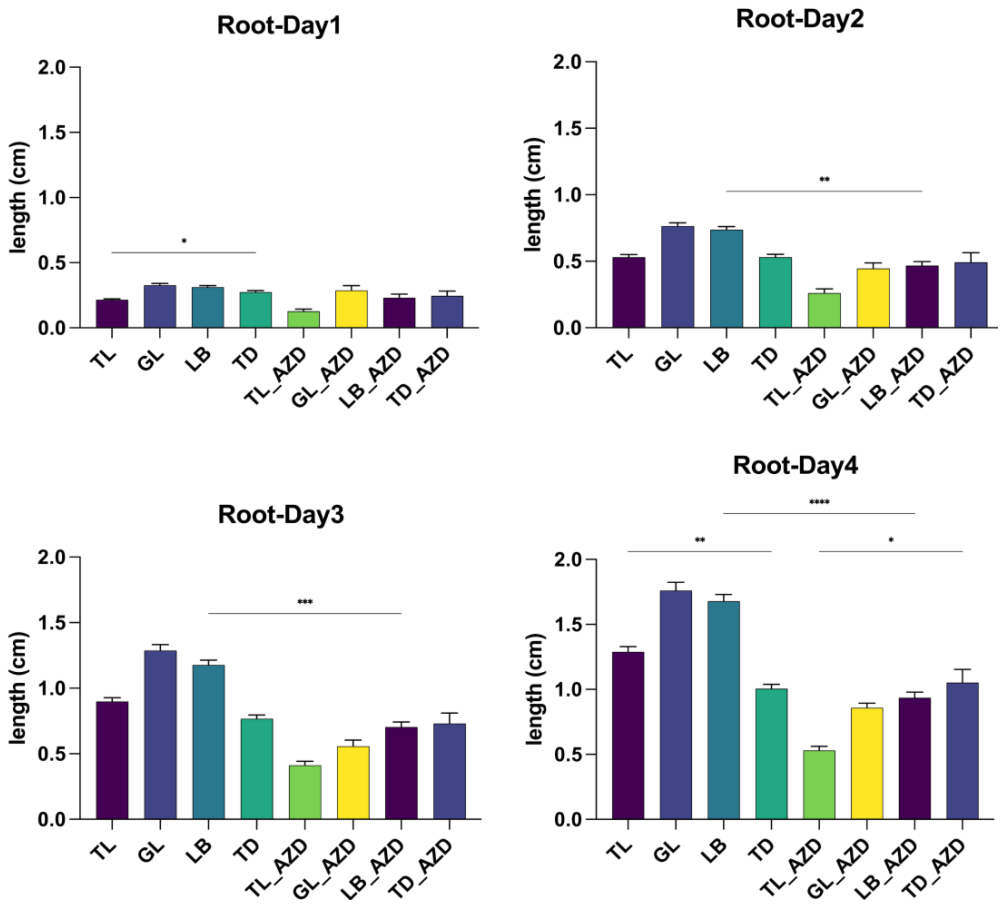

Figure 1. The root length of *A. thaliana* seedlings was measured for 24, 48, 72 and 96 h. The x-axis shows the seedlings were grown for Control as well as AZD [5μM] treatments under different light conditions. The y-axis represents the average root length in cm. Bars in different colors represent different light conditions.
Source: Authors

was substantially slower than the control groups, but no significant difference was seen in the total dark condition (TD). For control groups, the root length in the total dark condition was always the shortest compared with that in other lighting conditions, and in the gradient light (GL) and light blocker (LB) settings. Importantly, root lengths were longer in the gradient light (GL) and light blocker (LB) conditions than in the total light condition (TL). This shows that root growth speeds up if it grows within light-dark gradients and also there is a clear inhibitory role of AZD in root growth. Figure 1 shows a clear difference after four days of growth.

Root growth under different light conditions

When comparing the hypocotyl length in different illumination conditions under the same treatment, it was noted that the length of hypocotyls under total dark conditions (TD) was much larger than that under other illumination conditions and that the length of hypocotyls under complete illumination was always the shortest. Furthermore, there was no significant difference in hypocotyl development between the control and AZD treatment groups (Figure 2). This result shows that AZD has no significant influence on hypocotyl growth under our studied condition.

Hypocotyl growth under different light conditions

Shoot-shaded light conditions

Seedling root and hypocotyl growth of the control group and AZD treatment group was compared under shoot-shaded light conditions (Figures 3 and 4). The root growth of the AZD treatment group was significantly slower than that of the control group. Moreover, root

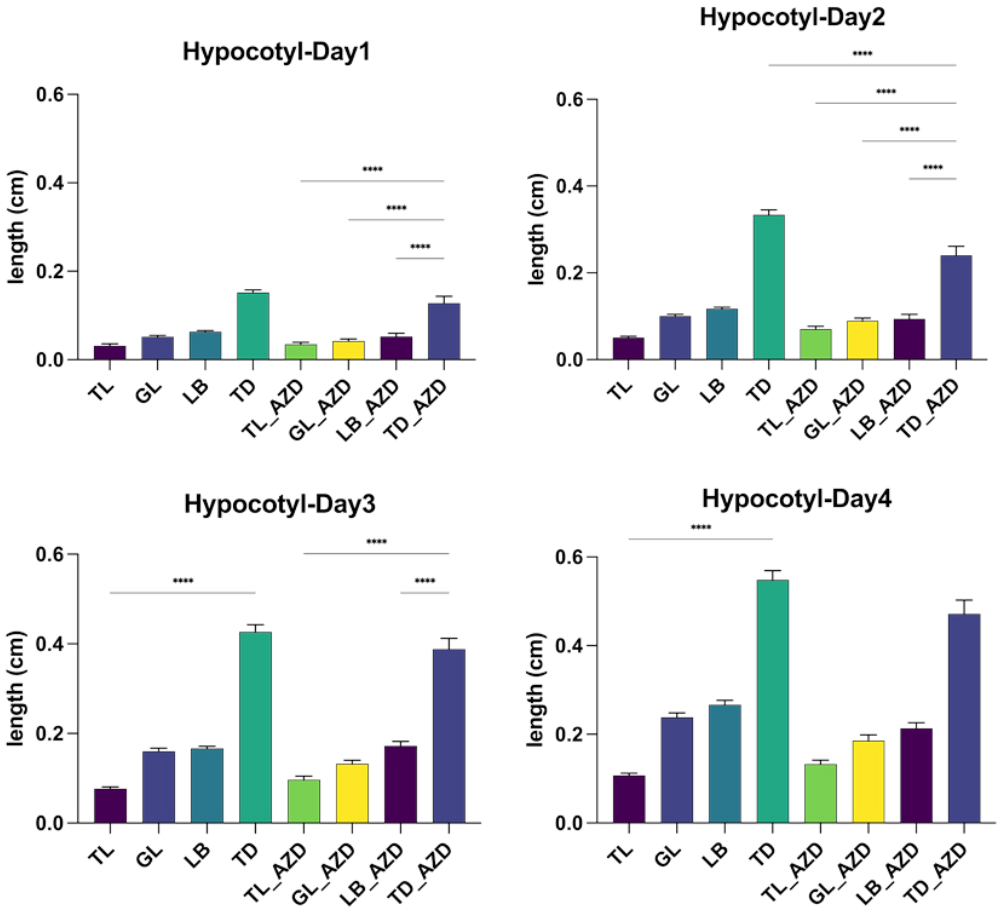

Figure 2. Hypocotyl length of *A. thaliana* seedlings was measured for 24, 48, 72 and 96 h. The x-axis shows the seedlings were grown for Control as well as AZD (5 μM) treatments under different light conditions. The y-axis represents the average root length in cm. Bars in different colors represent different light conditions.
Source: Authors

development of shoot dark (SD) condition was faster than shoot dark with light blocker (SDB) condition. Importantly, after 96 h of development, the root growth length in the AZD treatment group was less than 0.2 cm under different light conditions, far less than the control group (Figure 3).

Root growth under different light conditions

In total light conditions (TL), hypocotyl development was slower than in the other two light conditions (SD, SDB). For the control treatment, the hypocotyl length increased more as the hypocotyl light intensity declined, as determined by comparing hypocotyl development under shoot dark (SD) and shoot dark with light blocker (SDB) conditions. For AZD treatments, it is worth mentioning that hypocotyl growth in the SD condition is faster than that in the SDB condition (Figure 4).

DISCUSSION

Arabidopsis was selected as a model plant about decades ago because of the unique traits that made it ideal for laboratory research. *Arabidopsis* has been cultivated on Petri dishes since then, and the vast majority of root biology research has been done with the root system exposed to light. Light appears to have a direct influence on root development and responses, according to recent research (Yokawa et al., 2014; Meng, 2015). Our results have revealed a role for light in both root growth and hypocotyl growth. Six different light conditions (total light, gradient light, light blocker, total dark, shoot dark, shoot dark with blocker) were set up since the influence of light on seedling growth and differentiation can be divided into direct and indirect aspects. The light conditions (shoot dark and shoot dark with blocker) are the indirect ways to explore the influence of light on root growth via changing the light

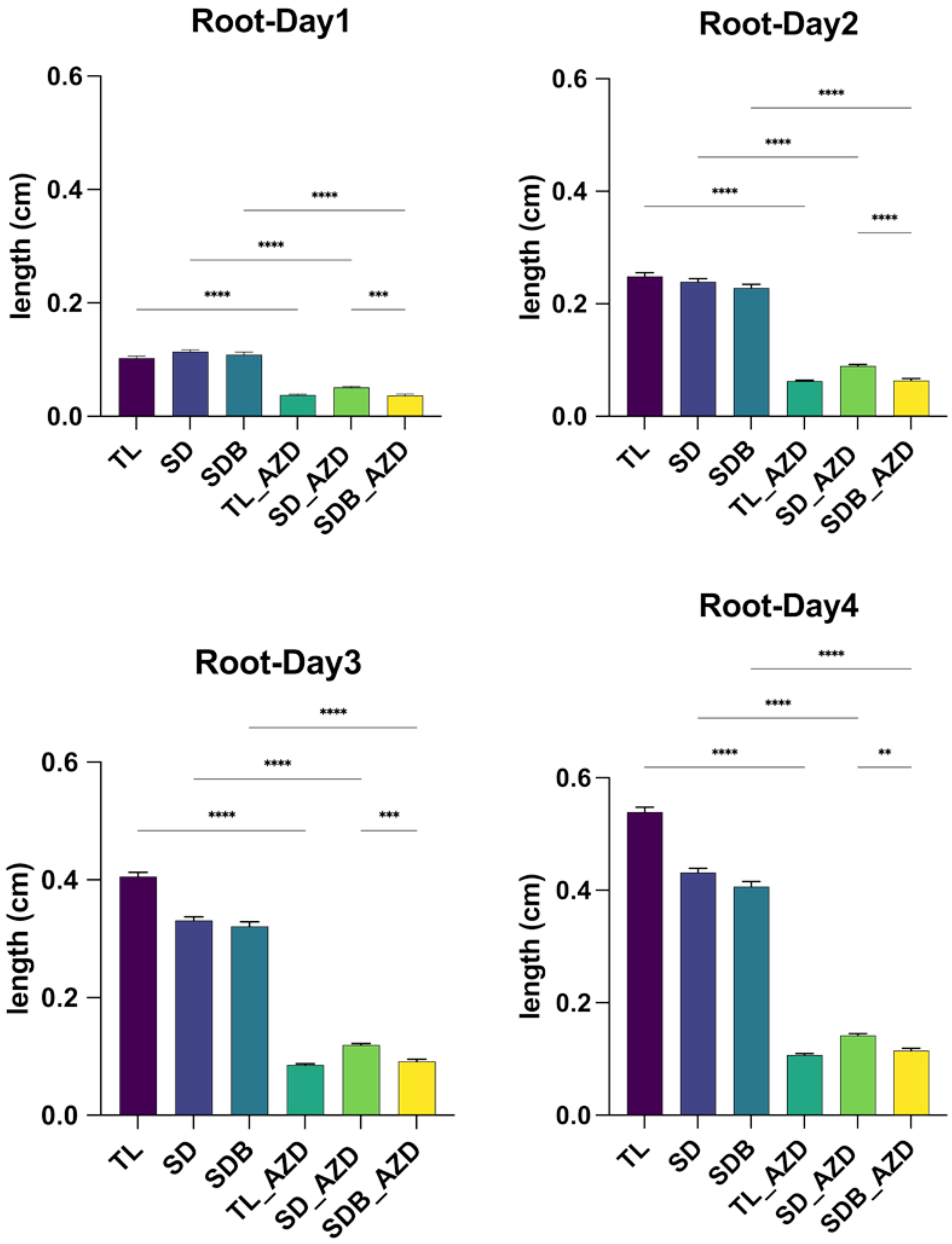

Figure 3. The root length of *A. thaliana* seedlings was measured for 24, 48, 72 and 96 h. The x-axis shows the seedlings were grown for Control as well as AZD (5 μM) treatments under different light conditions. The y-axis represents the average root length in cm. Bars in different colors represent different light conditions.
Source: Authors

intensity on the shoot. The investigations have found that the total root length under shoot dark (light intensity 7.91 μmol s^{-1} m^{-2}) and shoot dark with blocker (light intensity 2.03 μmol s^{-1} m^{-2}) circumstances was substantially less than under total light conditions. Within 24 and 48 h, there was no significant difference in root length across the three lighting conditions (SD, SDB and TL), but from 72 to 96 h, the total light condition had a considerably longer root length than the other two illumination conditions. This situation can be explained by the fact that photosynthesis mainly occurs in plant shoots, and plants are unable to produce enough organic matter (sucrose) to fulfill their growth requirements after a period of limited light. Yokawa et al. (2011) reported on the root-

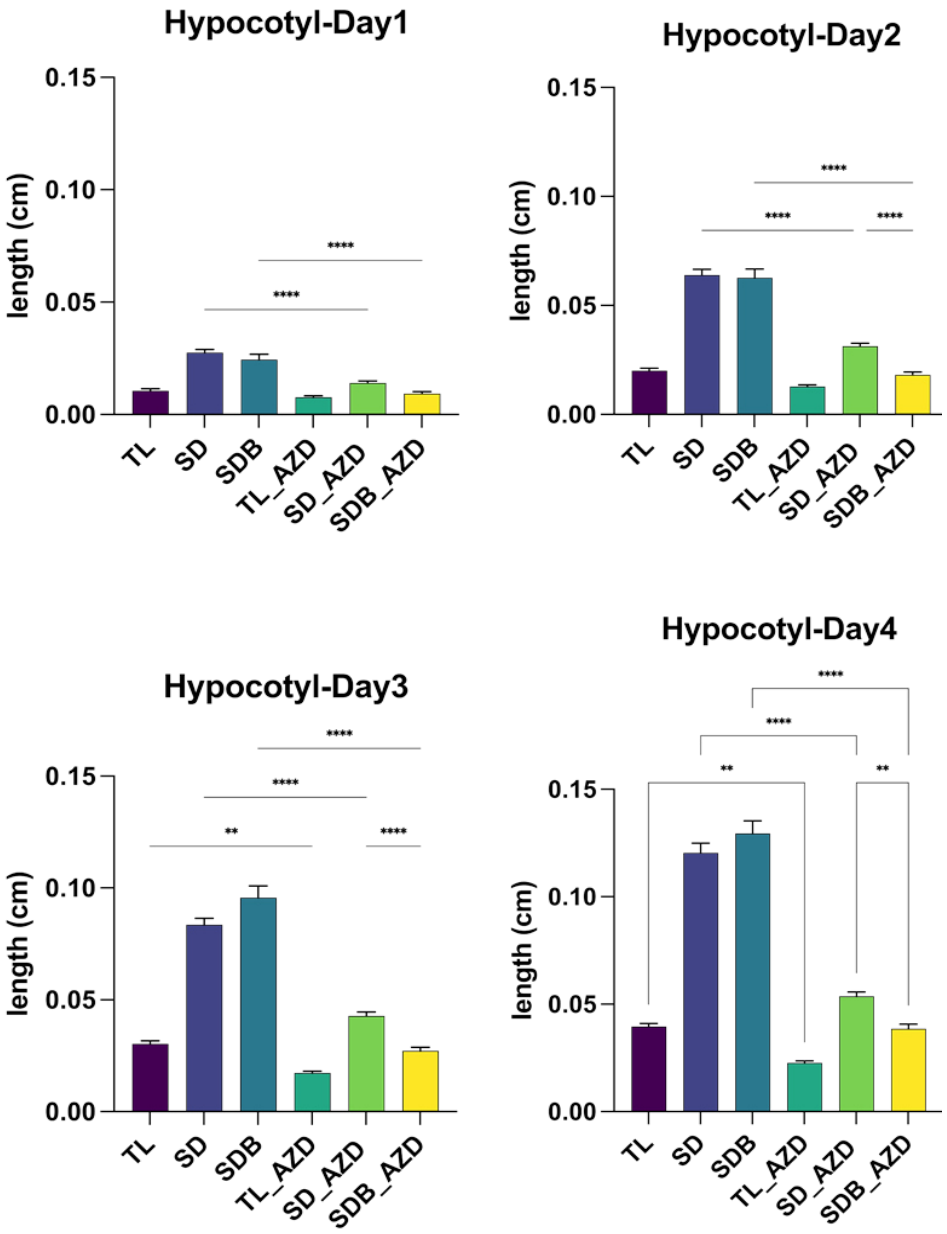

Figure 4. Hypocotyl length of *A. thaliana* seedlings was measured for 24, 48, 72 and 96 h. The x-axis shows the seedlings were grown for Control as well as AZD (5 µM) treatments under different light conditions. The y-axis represents the average hypocotyl length in cm. Bars in different colors represent different light conditions.
Source: Authors

shoot ratio of *Arabidopsis* seedlings growing in the soil (whose roots are almost completely dark) is 1:1. The exposure of roots to light causes stress in the entire plant, and roots normally respond by increasing their growth. This indicates that illumination of the roots disturbs the balance of the root-shoot ratio, which is approximately 1:1 in a normal physiological situation. The analysis of our experimental data also supported this conclusion. The gradient light condition had a higher light intensity than the simulated natural condition (light blocker), and the gradient light condition had a longer root length than the light blocker condition. Meanwhile, in the gradient light condition, the hypocotyl length was less than in the light blocker condition. Most *Arabidopsis*

studies are conducted out in transparent Petri dishes, ignoring the extra effects that the additional light may have on the roots. Results reported the root length under total light conditions was significantly shorter than that under gradient light and simulated natural conditions (light blocker), confirming the shortcomings of the traditional plant-growing (TPG) technique. Silva-Navas et al. (2015) demonstrated root illumination shortens root length and increases the early development of lateral roots, promoting root system expansion. Hypocotyl is a highly plastic organ whose length is controlled by a network of interacting elements including light and plant hormones (Vandenbussche et al., 2005). In continuous darkness, the process of hypocotyl elongation differs significantly from that in uniform light. TOR is critical for plant translational control (Méndez-Gómez et al., 2022). Translation re-initiation at upstream ORFs (uORFs) in genes that play crucial roles in stem cell control and organogenesis in plants is significantly reliant on TOR (Schepetilnikov et al., 2017). Many important proteins are encoded by uORF-mRNAs, including transcription factors, protein kinases, cytokines, and growth factors. The results from the study indicated that seedlings of *A. thaliana* treated with AZD (an ATP-competitive inhibitor of TOR) efficiently inhibited root and hypocotyl growth when compared to plants grown under control conditions. Our findings are consistent with previous research, which indicated that at the whole plant level, AZD treatment of *A. thaliana* delayed cotyledon and leaf development while also shortening root length (Montané and Menand, 2013). The situation is assumed to occur because AZD limits meristem activity in plants and may diminish the size of differentiated cells. Meanwhile, AZD may induce AtTOR haplo-insufficiency which results in reduced plant growth and stress resistance (Montané and Menand, 2013).

The control of root-to-shoot is a complex physiological process in plant. ROS-regulating factor was shown to play a key role in Arabidopsis (Jin et al., 2022). TOR signaling activity, which promotes growth and cell division, may be suppressed in QC. The presence of TOR in both the apical and basal meristems of the root shows that TOR is involved in both root proliferation and cell expansion (Montané and Menand, 2013). Furthermore, Barrada et al. (2015) stated that the TOR kinase is emerging as a key regulator of plant environmental and hormonal responses. TOR increases BR signaling, most likely via a signaling relay mediated by BIN2 substrates (Wu et al., 2019). Interestingly, by comparing the experimental data, it was discovered that hypocotyl length in the control group under shoot dark with blocker condition was larger than that under shoot dark condition; however, it was the opposite after AZD therapy. The outcome can be explained by the following reason: The use of AZD suppresses the activity of TOR, which indirectly affects the BR signaling, leading to a significant influence on the hypocotyl being almost completely dark condition (shoot dark with blocker condition).

Conclusions

A number of conclusions were drawn from this study: (1) That root growth speeds up if it grows within light-dark gradients and AZD shows a clear inhibitory role in root growth. (2) The length of hypocotyls under total dark conditions was much larger than that under other illumination conditions and that the length of hypocotyls under complete illumination was always the shortest. AZD has no significant influence on hypocotyl growth under the studied condition. (3) Root development of shoot dark (SD) condition was faster than shoot dark with light blocker (SDB) condition. Also, after 96 h of development, the root growth length in the AZD treatment group was less than 0.2 cm under different light conditions, far less than the control group (4). For AZD treatments, the hypocotyl growth in the SD condition is faster than that in the SDB condition. This study shows that AZD, a TOR inhibitor, drastically reduced root and hypocotyl length. The findings are consistent with previous research, which indicated that at the whole plant level, AZD treatments of *A. thaliana* delayed cotyledon and leaf development while also shortening root length (Montané and Menand, 2013).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

RECOMMENDATION

Further experiments are needed to pool-down other kinases that may be involved in this pathway.

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9. Appendix 2

Chapter 2

"Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions"

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Effect of GABA-Transaminase Inhibitor 3-MPA on *Arabidopsis thaliana* Grown Under Different Light Conditions

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Abstract: Plants adapt to stress by sensing their surroundings and generating signals that trigger changes in growth and defense. Light is one of the key environmental factors that modulate the physiology of both plants and animals via the diverse photoreceptors found in them. Both plants and animals have various signal molecules, like γ-aminobutyric acid (GABA), a non-protein amino acid, that regulates plant growth and accumulates in response to stress. Light and neurotransmitters like GABA play a key role in this process. A recent discovery of the first bona fide GABA target proteins in plants, the aluminum-activated malate transporters (ALMTs) suggests that GABA indeed could be one of the signaling molecules in plants. All this research did not address in detail the relationship between light and GABA. To better understand the role of GABA concerning light we set up six light conditions to investigate the changes in the hypocotyl and root growth in *Arabidopsis thaliana* under different light conditions, including total light, total dark, light blocker, gradient light, shoot dark, shoot dark with blocker. We treated the seedlings with 3-mercaptopropionic acid (3-MPA), a GABA inhibitor, using different concentrations grown under different light conditions between 1 to 4 days. Our results show that both the root and hypocotyl are modulated by GABA when grown under different light conditions. These results suggest a link in the signaling pathway of GABA with photoreceptor signaling pathways.

Keywords: hypocotyl growth; neurotransmitter; root growth; skototropism; γ-aminobutyric acid

1. Introduction

To adapt to a changing environment, all living organisms have to respond appropriately to circumstances. Unlike animals, which are mobile, plants grow in the soil and are comparatively fixed. However, plants have a flexible pattern of development that allows them to adjust their organ architecture to the changing environment. The fundamental body plan of the mature plant is generated during the early stages of embryogenesis (Jurgens et al., 1991). This process involves the production of shoot and root meristem, cotyledons, radicle, and hypocotyl. Embryogenesis of Arabidopsis begins with the already heavily polarized zygote, which divides

into the upper apical and the lower basal cell respectively. The basal daughter cell further differentiates into the suspensor and the hypophysis. The suspensor is needed to stabilize the embryo in the seed and later enter apoptosis. The hypophysis divides asymmetrically and then develops into the quiescent center of the root apical meristem and the columella the radicle (Kadereit et al., 2014). In animals, most organs are already present by the time the embryo is fully formed. On the contrary, most organs in plants are formed after embryogenesis is finished. GABA is a four-carbon non-protein amino acid found in all life domains. It was discovered in plants some years ago (Steward et al., 1949). In plants, it was shown to play a critical role in pollen-tube guidance in the process of reproduction (Palanivelu et al. 2003). They reported that a gradient of GABA is formed from the stigmatic surface toward the ovary, which is essential for successful guidance of the pollen tube and fertilization (Palanivelu et al. 2003). One of the signaling roles of GABA was reported to be stomatal regulation under water deficiency (Mekonnen et al., 2016). The modulation of reactive oxygen species was also reported as one of the key functions of GABA (Bouché et al., 2003), recently various developmental effects of GABA modulation both exogenous application (Du et al., 2020) and genetic engineering (Xie et al., 2020) was reported. GABA was also suggested to function in plant communication with bacterial (Lang et al., 2016) interactions. Furthermore, GABA-binding sites have been detected on plant cell membranes (Yu et al., 2006, Wudick et al., 2018). These discoveries further suggest that GABA functions as a signaling molecule in plants.

It is well established that in acidic soils TaALMT1 confers Al^{3+} tolerance in wheat by exuding malate from the root tips and chelating toxic Al^{3+} (Delhaize and Ryan, 1995; Ma et al., 2001; Sasaki et al., 2004). Exogenous application of GABA or muscimol to the roots of wheat seedlings with high TaALMT1 expression inhibited malate efflux and impaired root growth in the presence of Al^{3+}, which looks phenotypically like a near-isogenic line with less expression of TaALMT1 and less Al^{3+} tolerance (Ramesh et al., 2015). Interestingly, in these conditions, it was observed that when root efflux of malate was high, endogenous GABA concentrations in the cells were low and vice versa (Ramesh et al., 2015). This reciprocal relationship remained unexplained and may indicate either TaALMT1 activation caused changes in higher GABA or maybe that a higher concentration of GABA is altered in some way that regulates TaALMT1. Much data about a decade ago revealed that GABA negatively regulates the aluminum (Al^{3+})-activated Malate Transporters (ALMTs) in plants. Polygenic proteins, ALMTs, are frequently occurring in plants, and they can be expressed not only in various organs but also on different membranes (Dreyer et al., 2012). Extracellular GABA interacts with ALMTs on the plasma membrane, modifying the membrane potential and causing membrane hyperpolarization and desensitization (Žárský, 2015). GABA-related biosynthesis might be selectively disrupted in vivo and in vitro to explore the potential action sites and impacts of GABA in plants under different conditions. Compared to other previously known GAT inhibitors such as all glycine 3-Mercaptopropionic acid (3-MPA) is a relatively specific inhibitor that reduces the enzymatic activity of GAD and thereby suppresses the production of GABA (Horton and Meldrum, 1973).

In addition, GABA has been found in all organs in plants, including the embryo, cotyledon, roots, shoot, flowers, fruit, nodule, xylem, and phloem (Kinnersley and Turano, 2000; Hijaz and Killiny, 2020). Its concentrations vary significantly in different organs, tissues, and compartments (Ramesh et al., 2017), e.g., 100 –150 μM in the xylem of soybean (Wallace et al., 1984) and up to 20 mM in tomato fruit (Yin et al., 2010). This broad range in GABA

concentrations, from low micro to millimolar, may indicate its function as a signaling molecule and primary metabolite, respectively. However, none of these studies provided solid proof of the occurrence of a GABA signaling system in plants. GABA also plays a role as a primary metabolite related to the balance between nitrogen and carbon metabolism (Fait et al., 2011). Combined transcriptomics and metabolomics of Arabidopsis seedlings exposed to exogenous GABA suggest its role in plants is pre-dominantly metabolic (Batushansky et al., 2014). Light cues of varied intensity and quality cause plants to change their morphological traits (Yadav et al., 2020). Besides that, light also directs plant growth in a specific direction, shoots, for example, bend towards the light, which is called phototropism. Our studies show GABA's effect on plants' growth under different light conditions.

2. Results

It was observed differences under control, 25 µM, and 50 µM groups. Seedlings under gradient light (GL) and light blocker (LB) with 3-MPA treatment show longer roots than control (Figure 1 A, B, and C). It seems that treatment with 3-MPA did not influence the size of roots that were fully exposed to light (TL). On the other hand, the roots that were gradually covered (GL) and covered from light exposure (LB) showed enhanced root size when they were treated with 25 µM, and 50 µM of 3-MPA, compared to the control (Figure 1 A, B, and C). When the whole plant was without light (TD), its roots were larger only at the 25 µM concentration of 3-MPA (Figure 1B). No difference in root size was observed between the SD and SDB groups with or without 3-MPA treatment (Figure 1 A, B, C, and D).

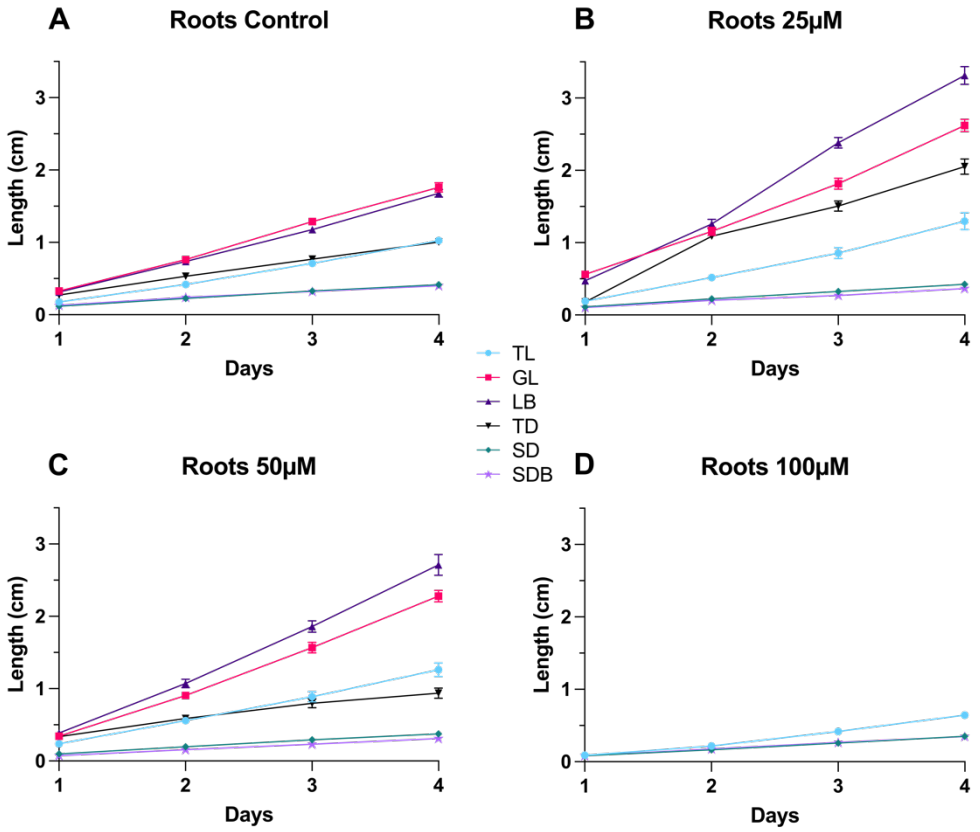

Figure 1 - The effect of 3-MPA on *Arabidopsis thaliana* roots at three different concentrations. The x-axis shows the days under treatment, and the y-axis shows the root length. Total light (TL), gradient light (GL), light blocker (LB), total dark (TD), shoot dark (SD), and shoot dark blocker (SDB). The data refer to means ($n \geq 20$); error lines indicate the standard error, $P < 0.001$.

After four days, there was a significant difference in root development between both concentrations of 3-MPA [25 µM] and [50 µM] and control (Figure 1). It has been discovered that when light intensity decreases, the rate of hypocotyl development increases (Figure 2).
The length of the hypocotyl changes in the 3-MPA treatment group under varied lighting settings, as seen in Figure 2. In the gradient light (GL) group with 25 µM of 3-MPA was observed a bigger hypocotyl (Figure 2B) if compared with the control and 50 µM 3-MPA (Figure 2 A & C). Hypocotyl length in the other two light conditions (TL, GL)did not alter remarkably in the control group or 3-MPA treatment. There were changes also in the total dark (TD) group, which showed a bigger hypocotyl when treated with 3-MPA (both concentrations) (Figure 2 B & C).
The growth of hypocotyl, on the other hand, did not respond to shoot dark (SD) and shoot dark with light blocker (SDB) conditions with the 3-MPA concentrations (Figure 2).

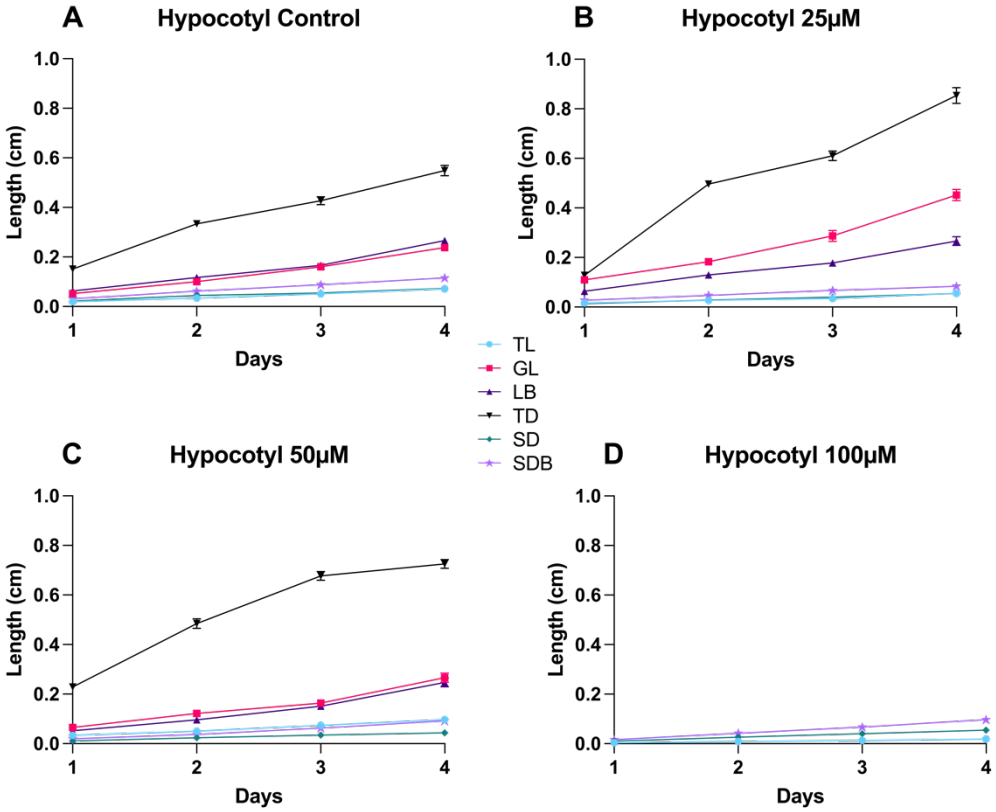

Figure 2 - The effect of 3-MPA on *Arabidopsis thaliana* hypocotyl at three different concentrations. The x-axis shows the days under treatment, and the y-axis shows the hypocotyl length. Total light (TL), gradient light (GL), light blocker (LB), total dark (TD), shoot dark (SD), and shoot dark blocker (SDB). The data refer to means ($n \geq 20$); error lines indicate the standard error, $P < 0.001$.

3. Discussion

Arabidopsis is commonly used as a model plant to study plant physiology due to its unique traits that make it ideal for laboratory research. Arabidopsis has been cultivated on Petri dishes since then, and the vast majority of root biology research has been done with the root system exposed to light. Some of these fail to point out the direct influence of light on root development and responses. Recently these influences became more visible according to recent research (Yokawa et al., 2014; Meng, 2015).

Our results have revealed a role for light in both root growth and hypocotyl growth. We set up six different light conditions (total light, gradient light, light blocker, total dark, shoot dark, and shoot dark with blocker) since the influence of light on seedling growth and differentiation can be divided into direct and indirect aspects. The light conditions (shoot dark and shoot dark with blocker) are the indirect ways to explore the influence of light on root growth via changing the light intensity on the shoot. Our investigations have found that the total root length under shoot dark and shoot dark with blocker circumstances were substantially less than under total light conditions. Within 1 and 2 days, there was no significant difference in root length across the three lighting conditions, but from 3 to 4 days, the total light condition had a considerably longer root length than the other two illumination conditions.

Yokawa et al. (2011) discovered that the root-shoot ratio of Arabidopsis seedlings growing in the soil (whose roots are almost completely dark) is 1:1. The exposure of roots to light causes stress in the entire plant, and roots normally respond by increasing their growth. This indicates that illumination of the roots disturbs the balance of the root-shoot ratio, which is approximately 1:1 in a normal physiological situation. The analysis of our experimental data also supported this work. The gradient light condition had a higher light intensity than the simulated natural condition (light blocker), and the gradient light condition had a somewhat longer root length than the light blocker condition. Meanwhile, in the gradient light condition, the hypocotyl length was less than in the light blocker condition. Most Arabidopsis studies are conducted out in transparent Petri dishes, ignoring the extra effects that the additional light may have on the roots, as explained in the introduction. Our results reported the root length under total light conditions was significantly shorter than that under gradient light and simulated natural conditions (light blocker), confirming the shortcomings of the traditional plant-growing (TPG) technique. Silva-Navas et al. (2015) demonstrated root illumination shortens root length and increases the early development of lateral roots, promoting root system expansion. They discovered that roots grown under full light produce shorter roots and more emerged lateral roots than roots produced in nearly full darkness (simulated natural condition). This is also in exact agreement with the results of our experiments. This situation can be explained by the potential reasons: (1) light diminishes the accumulation of potassium, sodium, and molybdenum in roots while dramatically increasing the absorption of iron in roots and shoots (Silva-Navas et al., 2015). Because light photocatalysis reactive oxygen species (ROS) formation in roots (Yokawa et al., 2011) and iron solubilization happens via redox processes, iron buildup in roots under light might be due to ROS activation. All living organisms require iron, and it may be a growth-limiting resource for plants since it is a fundamental component of redox reactions in photosynthesis and respiration (Silva-Navas et al., 2015). (2) As root illumination induces the burst of ROS (Yokawa et al., 2011). Higher levels of ROS in roots may break the equilibrium between root development and lateral root emergence (Tsukagoshi et al., 2010; Passaia et al., 2014). Furthermore, ROS is a strong oxidant that may react with a wide range of biomolecules, causing significant damage to plant tissues (Petrov and Van Breusegem, 2012).

The hypocotyl is a highly plastic organ whose length is controlled by a network of interacting elements including light and plant hormones. In continuous darkness, the process of hypocotyl elongation differs significantly from that in uniform light. GA, BR, and auxin may induce hypocotyl elongation in etiolated plants via downstream actuators (Figure 2).

It is well known that photoreceptors in light-grown plants suppress the biosynthesis of the hormones GA, BR, and auxin. Cytokinins stimulate ethylene synthesis, and ethylene influences actuators via regulation of the auxin or GA signal or directly on downstream actuators. Our results demonstrated that the length of the hypocotyl under total dark conditions was considerably longer than the hypocotyl under light conditions (total light, light blocker, and gradient light) (Figure 2), and the root length of the total dark condition was shorter than other light conditions (total light, gradient light, and light blocker), especially under gradient light and light blocker conditions (Figure 1). Plants that do not have access to light will develop a skotomorphogenesis pattern, which leads to etiolation (Yokawa et al., 2011).

As described in the introduction, modulation of ALMT activity by GABA leads to altered root development and tolerance to alkaline pH, acid pH, and aluminum ions. AtALMT1, the first recognized *Arabidopsis thaliana* homolog of ALMTs, was likewise shown to be involved in Al-resistance (Hoekenga et al., 2006). Aluminum ions in acid soils are toxic to plants and excessive quantities of soluble aluminum in soil solutions result in poor plant growth (Matsumoto, 2000). Plants resistant to higher soluble aluminum concentrations in soil release malate anions from their root cells, which chelate the toxic Al3+ cations in the apoplast (Delhaize, Ryan, and Randall, 1993). Thus, the efflux modulation of organic acids from plant roots, such as malate, plays a significant role in the aluminum resistance controlled by the ALMT1 gene (Ryan, Delhaize, and Jones, 2001). According to Ramesh et al. (2017), the application of 3-MPA may interact with the predicted GABA-binding region in ALMTs, elucidating the molecular identity and basis of GABA control of plant ion fluxes. We set up treatment groups with different concentrations of 3-MPA (25 μM, 50 μM, and 100 μM). Our experimental results show that when the 3-MPA concentration is 25 μM or 50 μM compared to the control group, the development of Arabidopsis roots and hypocotyl under six distinct light conditions is increased. Comparing the groups with the highest (50 μM) and lowest (25 μM) dosages of 3-MPA, as the 3-MPA concentration increased to 50 μM, there was no significant increase in root length. We can see that root growth is relatively higher with 25 μM, which indicates that above this concentration, 3-MPA may start to be toxic to the roots (Ryan, Delhaize, and Jones, 2001). This situation may be explained as follows: 3-MPA inhibits the biosynthesis of GAD which catalyzes the formation of GABA, resulting in a negative regulating impact on ALMTs on the membrane. Low concentrations of GABA increase inward currents, which in root and hypocotyl growth used could have been malate efflux or cation influx. Interestingly, the promoting tendency of root and hypocotyl development became less noticeable as 3-MPA concentration increased. The higher the 3-MPA concentration, the less GABA is produced and the more malate effluxes. This excessive carbon loss pathway is damaging to plant growth and stress resistance, this was also reported by Ramesh et al. (2017).

4. Materials and Methods

Arabidopsis (*Arabidopsis thaliana* L.) Columbia ecotype seeds were soaked in the sterilizing solution containing 10% sodium hypochlorite and 0.1% Triton X-100 for 15 min and washed several times with sterilized distilled water. Sterilized seeds were planted on the phytagel-fixed half-strength of the Murashige-Skoog medium as further described in Njimona et al. (2022).
Petri dishes with sterilized seeds were stored in the fridge for stratification for 2 days at 4°C and transferred to the growth chamber at 23 ℃ for 4 days for seed germination. To investigate the influence of 3-MPA on *A. thaliana* root growth and hypocotyl growth. *A. thaliana* seedlings were transferred to new Petri dishes with MS medium having different concentrations of 3-MPA (25 μM, 50 μM and 100 μM). Control dishes for this experiment were treated in the same way but, only containing MS medium. The treatment of 3-MPA [100 μM] was only carried out under total light and two kinds of shoot-shaded light conditions (SD and SDB). The Petri dishes (control or 3-MPA treatment) with seedlings were placed vertically in the growth chamber (temperature: 23 °C; light intensity: 100 mmol m^{-2} s^{-1}; photoperiod: 16 h light/8 h dark; humidity: 70%) with continuous illumination from an LED light source on top. A detailed experimental setup was described in our previous publication (Yan et al., 2022). The six light

conditions (Figure 3) are as follows: Total light (TL): Petri dishes with *A. thaliana* seedlings were placed under the light of the growth chamber with the intensity of 100 µmol s^{-1} m^{-2}. Total dark (TD): the plants were kept in total darkness. Moreover, shaded seedling roots create two forms of light: gradient light and light blocker. Shoot dark and shoot dark with a light blocker were two types of light conditions created by shading seedling shoots. Gradient light (GL): plants in the Petri dish were introduced in a black box resulting in a light gradient with a value of 39.74 µmol s^{-1} m^{-2}. Light blocker (LB): a light blocker is placed on the plants in the Petri dish and then they were introduced into a black box, resulting in light intensity of 7.27 µmol s^{-1} m^{-2}. Shoot dark (SD): The hypocotyls of *A. thaliana* were covered resulting in a light intensity of 7.91 µmol s^{-1} m^{-2}. Shoot dark with light blocker (SDB): a light blocker was placed on the seedlings in a round Petri dish and then the hypocotyls of the seedlings were covered, resulting in a light intensity of 2.03 µmol s^{-1} m^{-2}.

After 24 h, 48 h, 72 h, and 96 h the round Petri dishes were scanned with a photo scanner. When moving the Petri dishes (SD, SDB, and TD conditions) from the growth chamber to the photo scanner, they were kept shaded to prevent the etiolated seedlings from staying exposed to external light. Root and hypocotyl length was determined as described previously by Lucas et al. (2011) and Yokawa et al. (2011). Collected data for the experiment were evaluated with Fiji ImageJ software. Data were analyzed using the statistical software GraphPad Prisma (9.1.1, GraphPad Software, San Diego, CA, USA). All data were obtained from at least 20 biological repetitions in four independent experiments. One-way analysis of variance (ANOVA) was used to identify significant differences between groups. The mean values were compared by the Tukey test (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represents standard error.

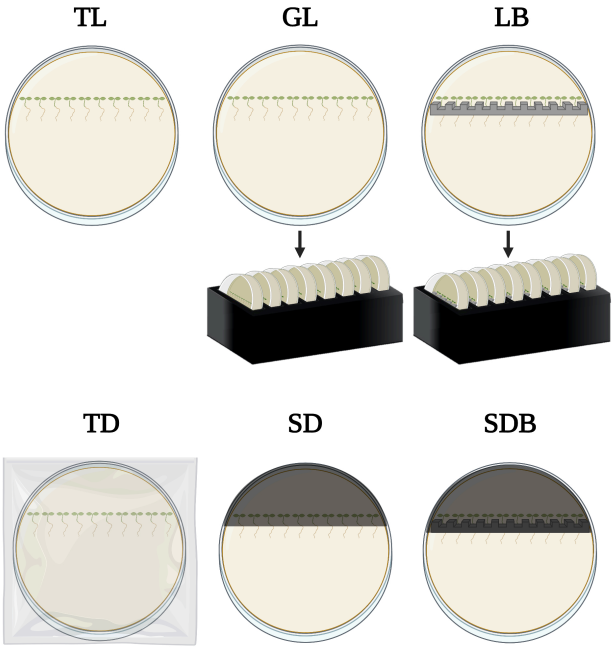

Figure 3 - Experimental setup performed in this study: Different light conditions for *A. thaliana* seedlings growth. First line from left to right: total light (TL), gradient light (GL), light blocker (LB). Second line from left to right: total dark (TD), shoot dark (SD), shoot dark with blocker (SDB).

5. Conclusions

Plant roots can sense light and assess spectrum and light intensity using various photoreceptors, thus integrating the growth of aboveground and underground organs. The roots of laboratory-grown Arabidopsis seedlings should be kept in darkened Petri plates. The illumination of roots influences not only the roots but also the morphology and physiology of the whole seedlings. Additionally, the recent discovery that plant GABA can regulate ion channels (ALMTs) has promoted GABA research, although there are still many gaps in the regulation of GABA on plant physiology and development. Meanwhile, further research has revealed that plant hormones (ethylene, ABA) and ROS production can alter GABA metabolism in plants, and some experimental evidence has shown that high GABA concentrations inhibit root growth. In our work, we have provided further clarity on this topic. However future experiments in which young seedlings shall be treated with exogenous GABA will further provide more evidence of the role of GABA in plant growth.

6. Summary

We set up six light conditions (TL, TD, GL, LB, SD, SDB) to investigate the changes in *Arabidopsis thaliana* hypocotyl and root development. *A. thaliana* seedlings developed under absolute darkness (TD) with shorter roots and longer hypocotyls. Shoots were shaded in SD and SDB conditions, and seedlings were unable to carry out photosynthesis, resulting in insufficient stored nutrients for root development. In the three groups of different light intensities on the root (TL, GL, LB), light causes stress in the entire plant under total light, the length of the root and hypocotyl in TL condition was shorter than GL and LB conditions. The stimulated natural condition, LB, had a bigger root and longer hypocotyl than the GL condition. Different light treatments did significantly affect root growth and hypocotyl growth. We developed three treatment groups 3-MPA [25 μM], 3-MPA [50 μM], and 3-MPA [100 μM]. Root and hypocotyl growth was promoted at the concentration of 3-MPA [25 μM], and the development of root and hypocotyl was suppressed gradually as the 3-MPA concentration increased.

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Conflicts of Interest: The authors declare no conflicts of interest.

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10. Appendix 3

Chapter 3

"Investigation of *Arabidopsis* root skototropism with different distance settings"

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Investigation of *Arabidopsis* root skototropism with different distance settings

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ABSTRACT

Plants can activate protective and defense mechanisms under biotic and abiotic stresses. Their roots naturally grow in the soil, but when they encounter sunlight in the top-soil layers, they may move away from the light source to seek darkness. Here we investigate the skototropic behavior of roots, which promotes their fitness and survival. Glutamate-like receptors (GLRs) of plants play roles in sensing and responding to signals, but their role in root skototropism is not yet understood. Light-induced tropisms are known to be affected by auxin distribution, mainly determined by auxin efflux proteins (PIN proteins) at the root tip. However, the role of PIN proteins in root skototropism has not been investigated yet. To better understand root skototropism and its connection to the distance between roots and light, we established five distance settings between seedlings and darkness to investigate the variations in root bending tendencies. We compared differences in root skototropic behavior across different expression lines of *Arabidopsis thaliana* seedlings (*atglr3.7 ko, AtGLR3.7 OE*, and *pin2 knockout*) to comprehend their functions. Our research shows that as the distance between roots and darkness increases, the root's positive skototropism noticeably weakens. Our findings highlight the involvement of GLR3.7 and PIN2 in root skototropism.

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1. Introduction

To adapt to various environments (e.g., freezing conditions, dry conditions, light environments, etc.), each living organism has to respond properly to its surroundings. Plants cannot move away from extremes in their environment, but they do have their own adaptive adjustment mechanisms, modifying their developmental architecture or behavioral characteristics to cope with environmental stresses. In a general plant life cycle, there are six typical stages involved, including seed germination, vegetative development, inflorescence development, inflorescence, fertilization, and ripening.[1] After seed germination, one of the first organs that start to develop is the roots.

Roots are the underground part of the plant body and are required for anchorage in the substrate, water and ions uptake, phytohormones synthesis, nutrient storage, vegetative growth, etc. The root apex is subdivided into four zones: meristematic, transition, elongation, and differentiation zones[2,3]). Also at the root apex, can be found the root cap, responsible for sensing the gravity pull, protects the root apical meristem (RAM) from physical damage (such as stones), and controls the root's downward growth.[4] This downward root growth is carried out by specialized cells of the root cap, called statocytes. Statocytes are cells that contain amyloplasts, plastids filled with starches, which sediment in the lower part of the cells, hence allowing the root to be reoriented. The cells in the elongation zone elongate, allowing root growth.[5] One of the main growth-related phytohormones is auxin, which is crucial for cell elongation and lateral root growth[4] and light plays a key role in auxin production and transport.[6,7]

One of the most important environmental factors for plant growth and development throughout their life cycle is light. For example, light controls seed germination, plant development, flowering, and metabolism.[8] Light also provides energy for both photosynthesis and photomorphogenesis, two mechanisms that determine plant growth. Light sensing is an essential factor for plants and changes in intensity and quality cause plants to change their morphological traits.[9] Besides that, light also directs the movement of plant organs in a specific direction, which is defined as phototropism, as early described by Charles Darwin.[10] This dynamic plant growth and morphogenesis is mainly under control of the plant hormone auxin.[11]

The asymmetric distribution of auxin causes the cells on the plant's darker side to elongate, leading the plant to bend toward the light source. Under normal physiological conditions, a significant proportion of apoplastic auxin exists in its protonated form, indole-3-acetic acid (IAAH), which can freely permeate cell membranes. This process is facilitated by members of the AUXIN/LIKE AUX1 (AUX/LAX) family of auxin importers. Upon entering the cell, where the intracellular pH is neutral, the weak acid form of auxin, indole-3-acetate (IAA^-), becomes trapped and necessitates the activity of efflux carriers for extrusion, allowing for intercellular transport.[12] Two families of transporters are involved in this process. The

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long-PINs, including PIN1–4 and PIN7 in *Arabidopsis thaliana*, serve as efflux carriers responsible for the directional movement of auxin between cells. Additionally, the ATP-binding cassette B (ABCB) class, comprising several multidrug resistance transporters, also participates in auxin efflux and facilitates intercellular transport of auxin.[13] Moreover, the phototropin (PHOT) blue light receptors are important,[14] interacting with the PIN2 in light-induced root responses.[15]

In *Arabidopsis* and other flowering plants, there are two PHOTs present, namely phot1 and phot2. Phot1 primarily acts as the photoreceptor for root phototropism and hypocotyl phototropism across a wide range of blue light intensities. In contrast, the involvement of phot2 in hypocotyl phototropism is limited to high light intensities. This restriction is mainly attributed to the increase in protein abundance of phot2 mediated by light exposure.[16] Interestingly, this phototropic response is observed across a wide range of light intensities, spanning from very low levels of light to the intensity of blue light experienced on a sunny day.[17]

In contrast to phototropism, skototropism is the term given to growth or movement of plant organs toward the darkness,[18] emphasizing the movement of roots seeking darkness. Roots normally grow downwards, following the gravity vector.[19] However, under natural conditions, roots can encounter sunlight in the soil's upper layers. Once this happens, they bend or stretch away from the light source to search for darkness, which allows them to avoid exposure to potentially unfavorable light conditions. Unfortunately, roots of seedlings grown in the transparent Petri dishes are exposed to strong light causing seedling stress and altered seedling morphogenesis and root-shoot ratio.[20,21]

According to Gottlieb Haberlandt's[22] hypothesis of plant ocelli, the upper epidermal cells of leaves are shaped like convex or Plano convex lenses.[23] By gathering light rays together, these "lenses" allow light-sensitive epidermal cells to recognize the size and shape of other plants in their surroundings. In addition to the leaves, the root apex may also have ocelli, since the roots can adapt to lower levels of light in the soil. The major factors that influence the negative phototropic response in plant roots are primarily the blue light signal and the activity of the PHOT blue light receptors.[24,25] In roots, the presence of blue light triggers a signaling cascade inducing root growth away from the light source (negative phototropism), a response mediated by the PHOT1 receptor.[15,26]

Calcium (Ca^{2+}), a key second messenger in plant cells, plays an important role in signaling responses to environmental changes. To produce free cytosolic Ca^{2+} transients, Ca^{2+} permeable channels, such as GLRs,[27] must be opened to control the influx of cytosolic Ca^{2+}.[28] In animals, one important channel responsible for cytosolic Ca^{2+} is the ionotropic glutamate receptor channels (iGluRs). The opening of iGluR allows glutamate entrance into the postsynaptic neuron and allows calcium (Ca^{2+}) transport.[29] Glutamate is involved in signal transmission between neurons, particularly at synapses. It has been extensively studied and recognized as a fundamental signaling molecule in animals for more than five decades.[30] It is essential for cognitive functions, learning, memory, and various other important biological processes. Moreover, glutamate, which is synthesized by the enzyme glutamate synthase using the substrates glutamine and 2-oxoglutarate, plays a crucial role in the metabolism of amino acids in plants.[31]

Since the discovery of the 20 genes in *Arabidopsis* as homologs of iGluRs, it has led to extensive research on these genes in plants.[32] Plant glutamate-receptor-like receptors (GLRs) exhibit significant similarity to their animal counterparts in terms of their nucleotide and amino acid sequences.[33] While iGluRs mediate neurotransmission in mammals, GLRs in plants serve crucial roles in various plant-specific physiological processes such as stress response and adaptation, sexual reproduction, pollen tube growth, stomata aperture regulation, innate immune and wound responses.[27–36] One of their key functions is the regulation of Ca^{2+} signaling. In the presence of specific amino acids, GLRs can facilitate the movement of various cations, including Ca^{2+}, across the cell membrane and into the cytoplasm.[37,38] This influx of Ca^{2+} acts as a major signaling player within the cell, having a vital role in intracellular signaling pathways in plants. GLRs in *Arabidopsis*, known as AtGLRs, serve as both sensors and mediators for a wide range of external and internal signals in plants.

Despite enormous advancements in the comprehension of the function of GLR in plants, the understanding of the biological function of these receptors is still in a stage of development. Therefore, the aim of this study was to demonstrate the skototropic root behavior of *Arabidopsis* seedlings positioned at different distances from darkness (0, 10, 20, 30, and 40 mm), including wild-type (Col-0), AtGLR3.7 knockout line *(atglr3.7 ko)*, AtGLR3.7 over-expression line *(AtGLR3.7 OE)*, and AtPIN2 deletion mutants *(pin2 knockout)*.

2. Material and methods

2.1. Growth media preparation

The growth medium was prepared by mixing the Murashige and Skoog (MS) media salt (with vitamins), saccharose, and dH_2O. After adding each to a 1 L container, the pH was adjusted to 5.8 using KOH or HCl. After that, 4 g of phytagel was added to the prepared mixed solution of 1 L. The medium was mixed and autoclaved at 120°C. The medium was placed in Petri dishes of different sizes and prepared under a sterile bench for further usage.

2.2. Seeds preparation

All plant genotypes used in this study had the background of *Arabidopsis thaliana* Col-0. The AtGLR3.7 knockout line (*atglr3.7 ko*) was kindly provided by Prof. Lai-Hua Liu (China Agricultural University, Beijing, China). The AtGLR3.7 over-expression line (*AtGLR3.7 OE*) was provided by Dr. Matthias Weiland, a former student at our laboratory (Institute of Cellular & Molecular Botany, University of Bonn, Bonn, Germany). The AtPIN2 deletion line (*pin2 knockout*) and *Arabidopsis* wild-type (Col-0) seeds were ordered from the European Arabidopsis Stock Centre (Nottingham, United Kingdom). Sterile growth conditions were maintained by surface sterilization of *Arabidopsis* seeds. Rough sterilization was done in 70% ethanol for 3 min, followed by sodium hypochlorite solution for 5 min.

Seeds were washed five times in distilled water. Sterilized seeds were sown on square Petri dishes with ½ MS medium under the sterile bench. Petri dishes with sterilized seeds were stored in the fridge for stratification for 48 h at 4°C and transferred to the growth chamber for 36 h for seed germination. The conditions of the growth chamber were as follows: the temperature was 17–24°C, and the light intensity was 121.43 μmol s^{-1} m^{-2}.

2.3. Skototropism experimental preparation

To investigate the influence of distance to darkness on *A. Thaliana* roots for the skototropism experiment, seedlings were transferred to various-sized Petri dishes. After 36 h for seed germination, seedlings were transferred to new Petri dishes, according to the treatment, and placed in a vertical position, one below the other with straightened roots. Depending on the different sizes of the Petri dishes, the *Arabidopsis* seedlings were put in three or five columns, resulting in settings with various distance patterns (0, 10, 20, 30, and 40 mm) from the seedlings to the darkness (Figure 1). After placing the seedlings, all dishes were sealed with parafilm and then placed in construction that held one-half of the Petri dish in darkness or shaded with a black cover (Figure 1). Four groups of Petri dishes with shades were arranged as below: (A) Small round dishes (92 × 16 mm) with three columns of seedlings were inserted into black boxes, resulting in light intensity on the darkness side of 39.74 μmol s^{-1} m^{-2}; (B) Small round dishes with three columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 15.34 μmol s^{-1} m^{-2}; (C) Large round dishes (150 × 20 mm) with five columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 19.10 μmol s^{-1} m^{-2}; (D) Square dishes (120 × 120 × 17 mm) with five columns of seedlings were placed into black covers, resulting in light intensity on the darkness side of 20.17 μmol s^{-1} m^{-2}. Light source was at the growth chamber ceiling (Figure 1).

2.4. Measurements and evaluation

After 96 hours, the Petri dishes were scanned. The root bending angle was measured via Fiji ImageJ software based on digital images. The values for root bending were sorted into 3 groups: (1) Positive values, showing a bending toward darkness; (2) Negative values, indicating a bending away from darkness; (3) A group zero (180° ± 1°), exhibiting no visible behavior toward or away from light darkness. Statistical analysis, graphing, and data visualization were performed using GraphPad Prism (version 9.5.1) software.

3. Results

3.1. Treatment of small round Petri dishes (92 × 16 mm) within black boxes

We found that for the Col-0 seedlings positioned on the borderline between light and darkness, 90.74% of them were bent to the darkness, implying a positive bending angle, after a 96-hour growing period. When the seedlings were placed on the light-biased side, 10 mm from the dividing line, 83.89% of them bent toward the darkness, and only 2.68% were grown toward the light. Further expanding the distance between Col-0 seedlings and the light-dark borderline to 20 mm, 74.64% of them were positively bent, 12.68% of them were bent to light, and 12.68% of them were grown without any preferred direction, growing downwards. Figure 2a shows a clear difference in root bending directions as the distance between the Col-0 seedlings and the light-dark borderline increases after 96 hours of growth.

For the seedlings of the *atglr3.7 ko*, there were 92.54% of them placed on the borderline between light and darkness (0 mm) bending to darkness. When the distance toward darkness increased to 10 mm, 81.13% of them bent toward

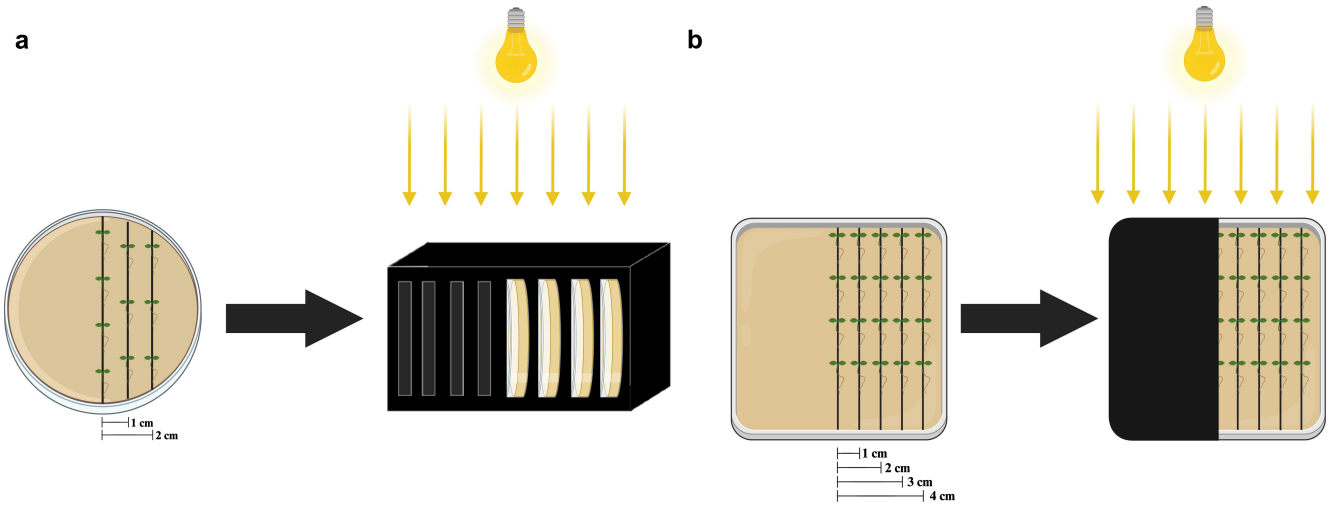

Figure 1. Experimental setup in this study. Examples of different shade approaches we adopted: (a) treatment of small round Petri dishes (92 × 16 mm) within black boxes with a light source at the growth chamber ceiling. (b) Treatment of large square Petri dishes darkened with black covers with a light source at the growth chamber ceiling. Based on the sizes of the petri dishes, three rows of *Arabidopsis* seedlings were positioned in a and five rows in B, respectively. Each column was spaced 1 cm (10 mm) apart from each other as the label. To ensure consistent positioning, the inner row of seedlings was aligned with the border of the covers.

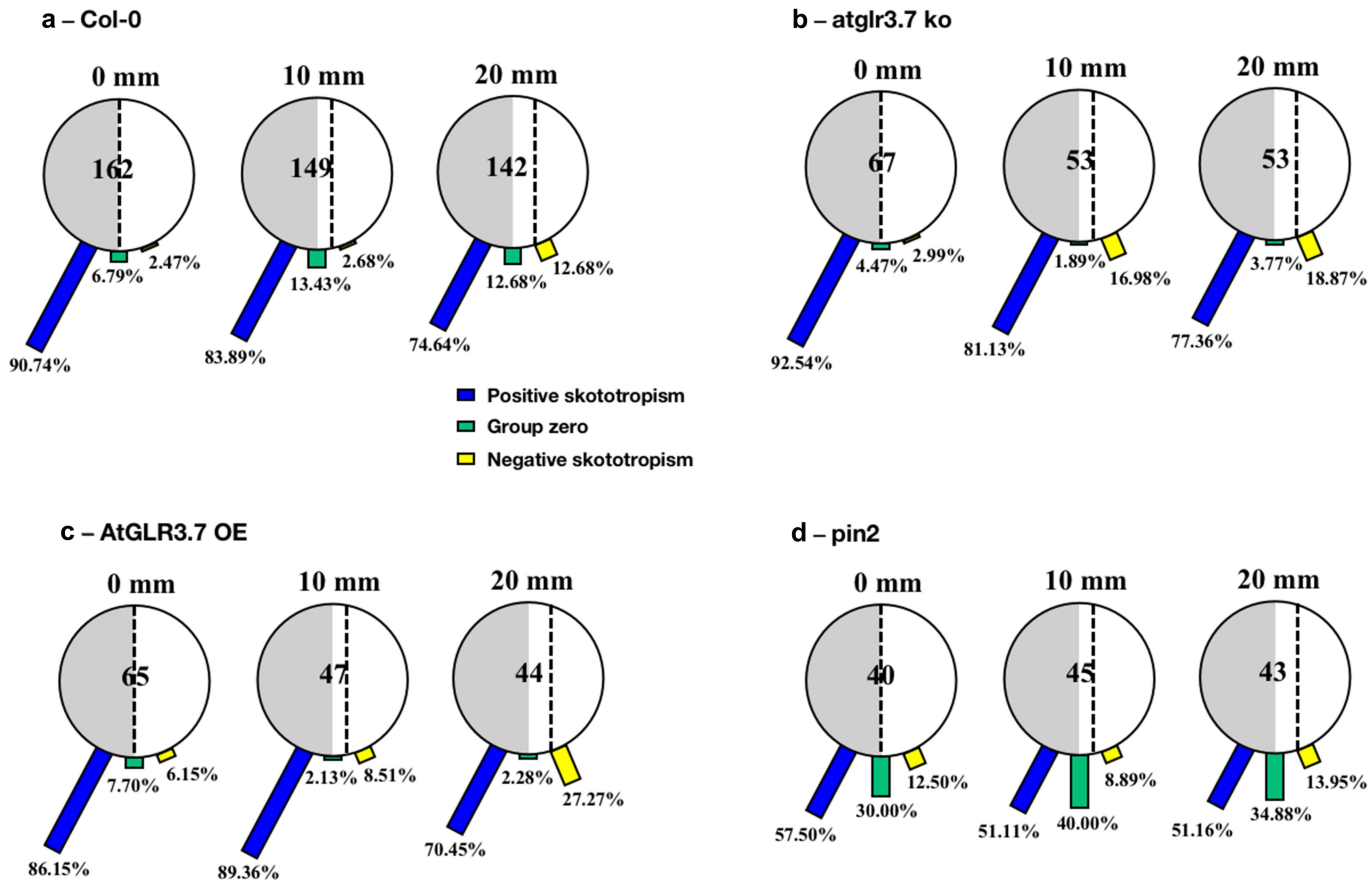

Figure 2. Skototropic response of *Arabidopsis* roots after 96 h growth within small round dishes inserted into the black box. Four lines of *Arabidopsis* seedlings were adopted: (a) *Arabidopsis thaliana* (col-0), (b) AtGLR3.7 knockout line (*atglr3.7 ko*), (c) AtGLR3.7 over-expression line (*AtGLR3.7 OE*), and (d) AtPIN2 deletion line (*pin2 knockout*). The circle contains the total number of *Arabidopsis* seedlings used in the experiment at the following distance settings: 0, 10, and 20 mm. The blue bars, green bars, and yellow bars, respectively, show the percentages of seedlings positively bending toward darkness, seedlings with no discernible bending trend, and seedlings bending away from darkness.

darkness, and the other 16.98% bent away from the light. When it came to the distance of 20 mm to the darkness in the *atglr3.7 ko* line, there was a more noticeable increase in positive skototropism compared to the Col-0 line, reaching 77.36% (Figure 2b).

For the seedlings of the *AtGLR3.7 OE*, out of the 65 grown in the black box treatment for 96 hours at the border of darkness (0 mm), 86.15% bent toward the darkness, while 6.15% bent away from the darkness. The percentages of mutants bending toward darkness changed to 89.36% and 70.45% (Figure 2c) as the distances between the mutants and the darkness increased to 10 mm and 20 mm, whereas the proportions of those bending toward light rose to 8.51% and 27.27%, respectively.

Moreover, seedlings of AtPIN2 deletion lines (*pin2 knockout*) with a distance of 0 mm, 10 mm, and 20 mm from the light-dark borderline had almost the same proportion of bending to darkness, with bending to darkness proportions ranging from 50% to 60%. *Pin2* mutants that were 0 mm away from the borderline showed a proportion of bending to the darkness of 57.50%, which was slightly higher than seedlings in the other two circumstances (10 mm and 20 mm) (Figure 2d).

3.2. Treatment of small round Petri dishes (92 × 16 mm) darkened with black covers

The experimental results of Col-0 seedlings grown in the small round Petri dishes with black covers are shown in Figure 3a. Most of them (81.54%) were positioned at the border of darkness (0 mm), showing positive root skototropism with bending angles to darkness, while 12.31% of them bent away from darkness. Furthermore, Col-0 seedlings that were 10 mm and 20 mm away from the darkness-light borderline showed the proportions of bending to the darkness of 80.00% and 72.73%.

The *atglr3.7 ko* had larger percentages of root-positive skototropism than the Col-0 line at distances of 0, 10, and 20 mm from the light-dark borderline, exhibiting correspondingly 91.30%, 80.00%, and 82.14% (Figure 3b).

When the distances between the darkness-light borderline and seedlings were increased to 10 mm and 20 mm for the *AtGLR3.7 OE*, there was a significant decrease in the proportion of positive root skototropism compared to the other two lines, and the proportion of bending away from darkness increased sharply, reaching about 38% (Figure 3c).

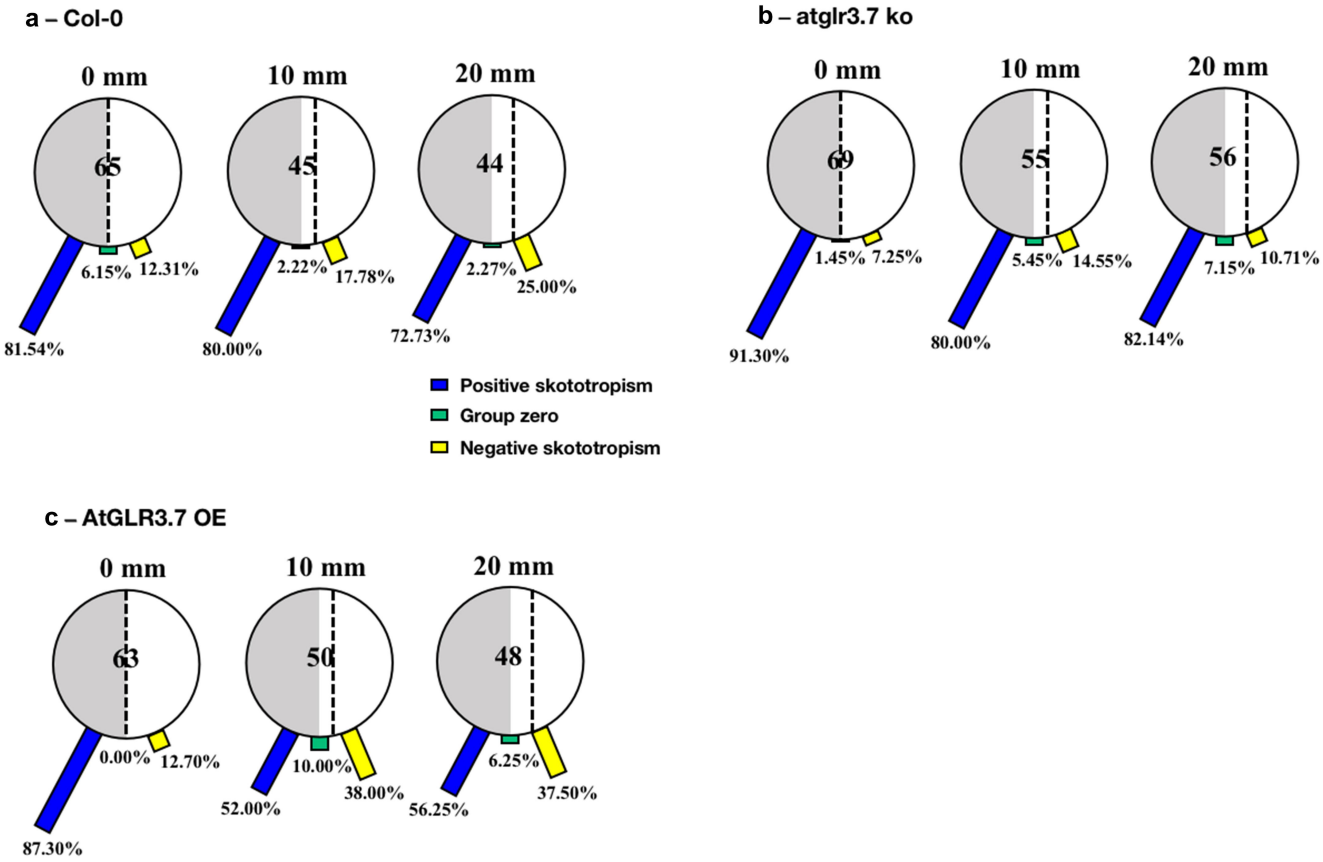

Figure 3. Skototropic response of *Arabidopsis* roots after 96 h growth with small round dishes darkened with black covers. Three lines of *Arabidopsis* seedlings were adopted: (a) *Arabidopsis thaliana* (col-0), (b) AtGLR3.7 knockout line (*atglr3.7 ko*), and (c) AtGLR3.7 over-expression line (*AtGLR3.7 OE*). The circle contains the total number of *Arabidopsis* seedlings used in the experiment at the following distance settings: 0, 10, and 20 mm. The blue bars, green bars, and yellow bars, respectively, show the percentages of seedlings positively bending toward darkness, seedlings with no discernible bending trend, and seedlings bending away from darkness.

3.3. Treatment of large round Petri dishes (150 × 20 mm) darkened with black covers

After the growth period of 96 h, with the increase of distances between Col-0 seedlings and the light-dark borderline from 0, 10, 20, 30, and 40 mm, the proportion of positive skototropism of Col-0 seedlings grown on black-covered large round Petri dishes showed a significant downward trend, with specific values of 86.42%, 81.93%, 72.09%, 73.91%, and 66.67%, and the portions of seedlings bending away from the light also increased in accordance (Figure 4a). The seedlings of the *atglr3.7 ko* and the *AtGLR3.7 OE* also showed root skototropic behavior consistent with the Col-0 line (Figures 4b,c).

3.4. Treatment of large square Petri dishes (120 × 120 × 17 mm) darkened with black covers

When *Arabidopsis* seedlings were grown in square Petri dishes with black covers, the tendency of wild-type (Col-0), *atglr3.7 ko*, and *AtGLR3.7 OE* seedlings to bend to darkness reduced with the increase in distance between seedlings and darkness (Figure 5a–c). Importantly, for seedlings of the *atglr3.7 ko*, when the distance from the darkness reached 30 mm and 40 mm, the proportion of seedlings bent toward darkness (positive skototropism), and the proportion of seedlings bent away from darkness (negative skototropism) were nearly identical, approximately 40% (Figure 5b).

4. Discussion

Even though plant roots develop in soil that is almost completely dark in nature, they are highly sensitive to light. Light stress conditions stimulate root growth as the roots try to escape light by increasing their growth rate, a strategy known as "root escape tropism".[21] The combination of light-induced root development and negative phototropism can be regarded as a physiologically relevant reaction since it induces light-exposed roots to return to the dark soil in nature.[21] The analysis of our experimental data supports Yokawa's study in that the general trend of decreasing skototropism with increasing distance to darkness remained consistent across the different cover treatments. This skototropic behavior is believed to be an adaptive mechanism that allows roots to avoid potentially unfavorable light conditions like in the upper layers of the soil. Furthermore, as shown in Figure 6, when the seedling with a diameter of 100 μm is 20 mm (2 cm) away from darkness, this corresponds to a person with a diameter of 0.7 m (70 cm) being 140 m away from the darkness (Figure 6). When the seedling is 10 mm (1 cm) away from the darkness, it is equivalent to 70 m away from the darkness. Plants are unable to sense dark

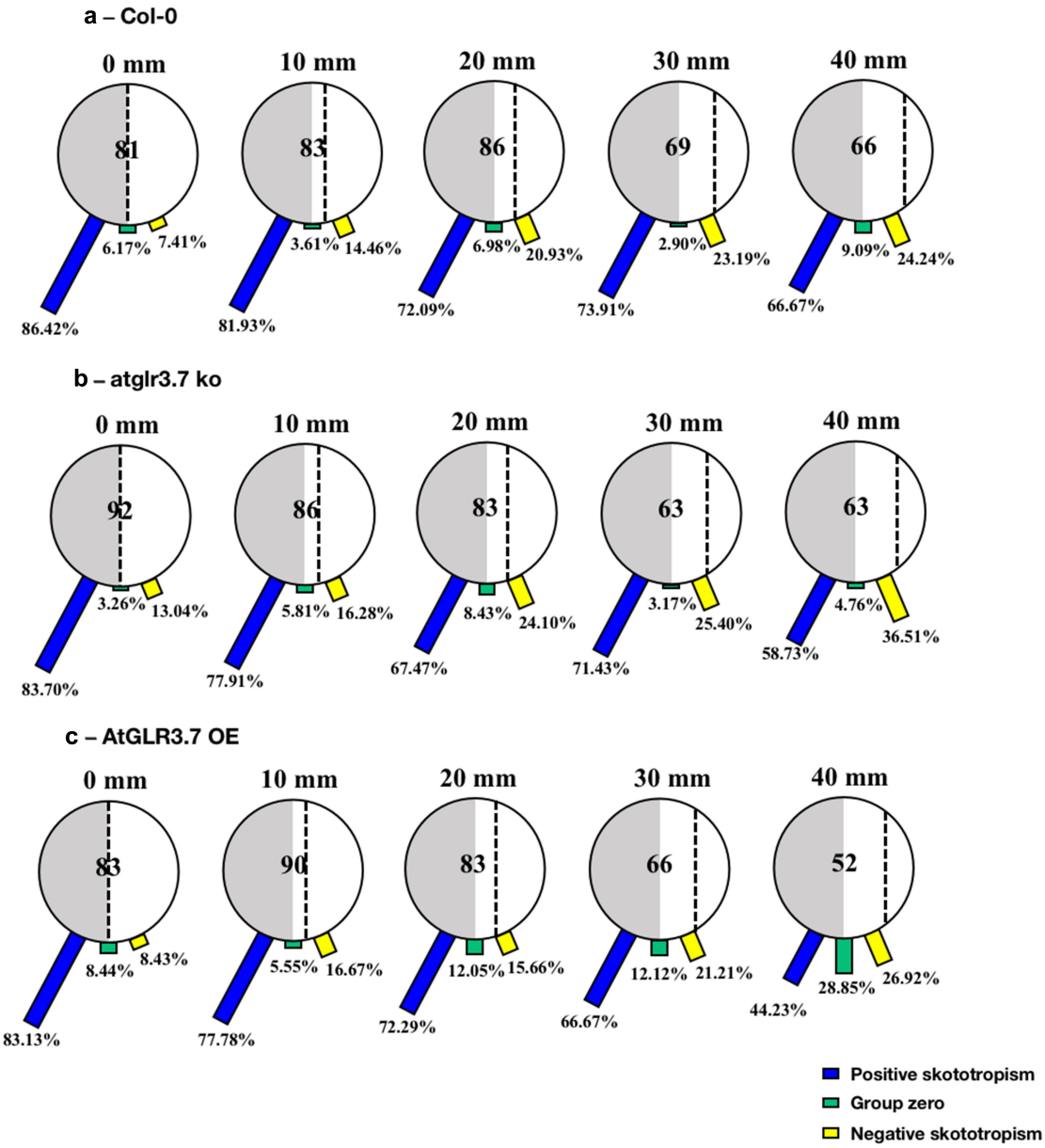

Figure 4. Skototropic response of *Arabidopsis* roots after 96 h growth with large round dishes partially covered with black covers. Three lines of *Arabidopsis* seedlings were adopted: (a) *Arabidopsis thaliana* (col-0), (b) AtGLR3.7 knockout line (*atglr3.7 ko*), and (c) AtGLR3.7 over-expression line (*AtGLR3.7 OE*). The circle contains the total number of *Arabidopsis* seedlings used in the experiment at the following distance settings: 0, 10, 20, 30, and 40 mm. The blue bars, green bars, and yellow bars, respectively, show the percentages of seedlings positively bending toward darkness, seedlings with no discernible bending trend, and seedlings bending away from darkness.

surroundings as distances expand significantly, resulting in no escape tropism or behavior. Our experimental results also revealed that when the distance increased to 40 mm, the proportionate gap between positive and negative root skototropism decreased.

Despite the fact that almost all plant roots growing in nature are underground, in darkness, all photoreceptors are expressed at the root apices.[39] Although a weak light is not stressful for the roots, they try to avoid strong lights. Recent studies have shown that *Arabidopsis* roots grew faster when grown in a light gradient environment, growing toward darkness. Based on this growth, one can imply some kind of vision through the root apex.[39–41]

The hypothesis that plants can have some sort of vision was first proposed by Gottlieb Haberlandt, in 1905 and called "Plant Ocelli". He argued that the leaf epidermis can resemble a convex or Plano convex lens.[22] Haberlandt's theory was tested experimentally[42] as well as supported by studies of a mimicking plant *Boquila trifoliolata*.[23,43,44] This plant has the intriguing ability to change the shape of its leaves according to the host plant. When plastic leaves were presented to *Boquila trifoliolata*, it changed the shapes of leaves from three-lobed leaves to longitudinal leaves, mimicking the plastic leaves too.[45]

Parallel to the hypothesis of plant ocelli, the distribution pattern of phot1 in the transition zone of the root apex suggests a role for this region in blue light sensing, while the root cap is

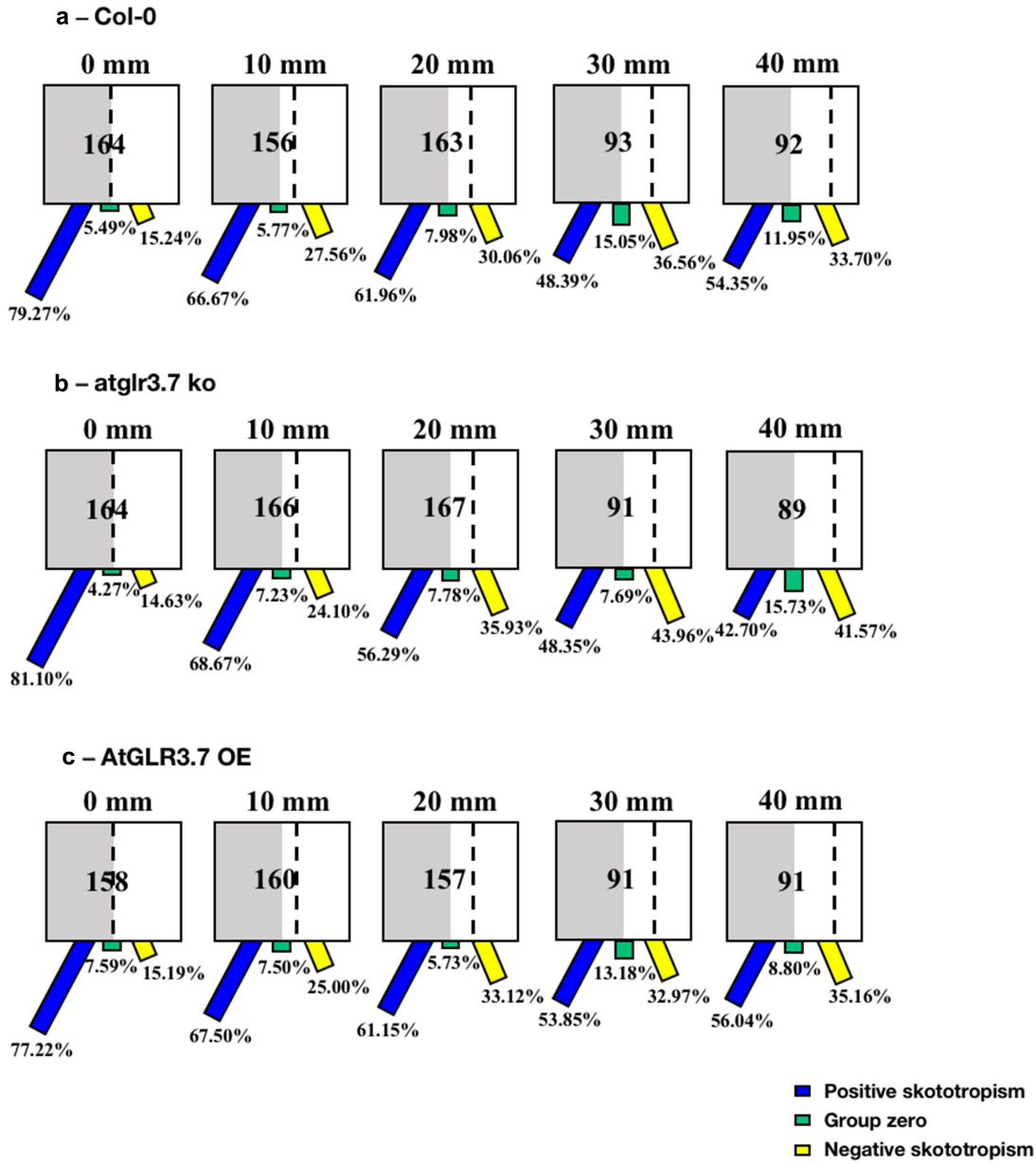

Figure 5. Skototropic response of *Arabidopsis* roots after 96 h growth with square dishes partially covered with black covers. Three lines of *Arabidopsis* seedlings were adopted: (a) *Arabidopsis thaliana* (col-0), (b) AtGLR3.7 knockout line (*atglr3.7 ko*), and (c) AtGLR3.7 over-expression line (*AtGLR3.7 OE*). The circle contains the total number of *Arabidopsis* seedlings used in the experiment at the following distance settings: 0, 10, 20, 30, and 40 mm. The blue bars, green bars, and yellow bars, respectively, show the percentages of seedlings positively bending toward darkness, seedlings with no discernible bending trend, and seedlings bending away from darkness.

specialized for red light sensing.[39] Recent research findings have demonstrated that red light and blue light can upregulate the transcription levels of several genes encoding GLR proteins. The transcriptional upregulation of AtGLRs under red light conditions is primarily regulated through pigment-mediated processes. The involvement of cryptochromes in this process is less evident, as some mutants show a significant reduction in red light induced AtGLRs transcriptional upregulation, while the high-level blue light upregulation by cryptochromes remains unaffected. These findings not only highlight the complex regulation of AtGLR upregulation but also suggest the possibility of AtGLR playing an important role in skototropism.[46] According to our results, the *atglr3.7 ko* showed a higher proportion of positive skototropism compared to the wild-type (Col-0) line, which suggests that AtGLR3.7 may play a role in modulating the skototropic response in *Arabidopsis* roots. On the other hand, the *AtGLR3.7 OE* showed a decrease in positive skototropism and an increase in negative skototropism, indicating that overexpression of AtGLR3.7 may disrupt the normal skototropic response.

Moreover, the AtPIN2 deletion mutants (*pin2 knockout*) showed different skototropic behavior to the wild-type line (Col-0), the curvature of the root hardly changes according to

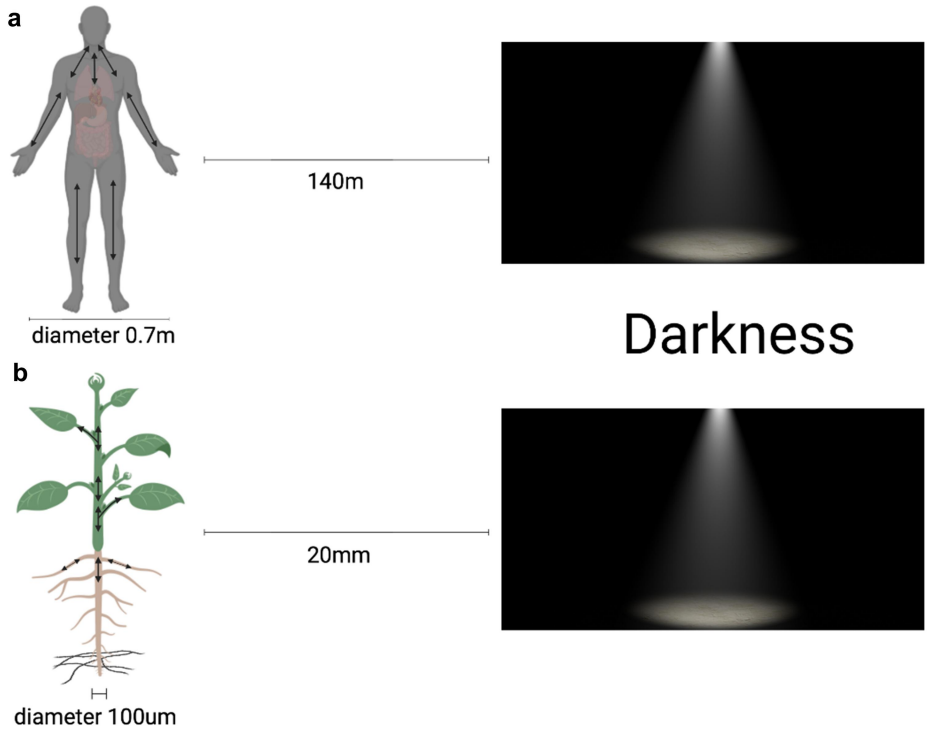

Figure 6. Comparative perception of darkness in *Arabidopsis* and human being. The *Arabidopsis thaliana* seedling (about 100 μm in diameter) positioned at 20 mm from darkness is equivalent to a person with a diameter of 0.7 m being situated 140 m away from darkness.

the distance from darkness, implying that the PIN2 protein, which is involved in auxin transport, may play a major role in mediating the skototropic response in *Arabidopsis* roots. As mentioned, the localization of PIN proteins, responsible for polar auxin transport in the root apex, undergoes constant recycling between the plasma membrane and endosomal compartments.[47] PIN2 protein has been identified to be involved in root negative phototropism.[48] In dark-grown roots, PIN2 is not polarly localized at the plasma membrane but accumulates within endosomes/vacuoles.[48,49] However, despite the absence of functional PIN2 protein, approximately 50% of the *pin2* mutant seedling roots still exhibited bending toward darkness. This suggests the involvement of other auxin transporters besides PIN2 in root skototropism. One potential candidate could be the ABCB auxin transporter, which has been shown to play an important role in root phototropism.[15]

Different shade approaches, resulting in reduced light intensity on the dark side of the Petri dish, have different effects on the skototropic response of *Arabidopsis* roots. The use of a black cover creates a complete blockage of light on one side of the Petri dish, providing a clear and distinct contrast between the light and dark conditions. This setup ensures that the roots experience a sharp transition from light to darkness, allowing for a strong skototropic response. The black cover effectively prevents any light leakage and provides a well-defined boundary for the roots. However, the black box induces a strong gradient of light intensity within the Petri dish.[50] Although the reduced light intensity can influence the strength of the light stimulus perceived by the roots, it may result in a less pronounced skototropic response compared to the black cover setup. Additionally, the presence of some residual light in the dish due to partial blocking may introduce a more gradual transition between light and darkness, potentially affecting the roots' perception and response. While the distribution pattern of bending angles may differ between the treatments, with the small round Petri dishes with black cover treatment showing a more dispersed pattern than the black box treatment, the overall trend of decreasing skototropism with increasing distance to darkness remains consistent. Moreover, Petri dish shapes may affect factors such as air circulation and humidity within the Petri dish, which can indirectly impact root growth and behavior. Our data show that the round Petri dishes display a more prominent skototropic response compared to the square ones.

Several conclusions were drawn from this study: (1) Plants show root skototropic behavior when they are under light stress conditions. As the distance between seedlings and darkness increases, it becomes more challenging for them to perceive the darkness and exhibit this "escape tropism." (2) In contrast to the wild-type (Col-0) line, the *atglr3.7 ko* demonstrated a larger percentage of positive root skototropism (bending toward darkness), whereas the *AtGLR3.7 OE* exhibited reverse bending trends, suggesting the AtGLR3.7 may play an important role in root skototropism. (3) The root-positive skototropism of *pin2 knockout* mutants was significantly lower than that of Col-0 seedlings, and there was no noticeable change in root skototropism of *pin2 knockout* mutants under different distance-pattern settings.

Summarily, this study provides valuable insights into the skototropic behavior of *Arabidopsis* roots and the potential involvement of AtGLR3.7 and AtPIN2 in mediating this

response. Further studies are needed to elucidate better the underlying molecular mechanisms and signaling pathways involved in root skototropism. Understanding these mechanisms could have implications for improving plant growth and development in various environmental conditions.

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Additional information

Supplementary information. For, pre data supporting this study see Supplementary Data 1.

Author contributions

F.B. and F.Y. conceived and designed research studies; X.Y. and Y. L. performed the experiments, analyzed the data, and wrote the manuscript; F.Y. contributed to methodology and reviewed the manuscript; F. B. supervised the study and reviewed the manuscript.

Data availability statement

The authors declare that all relevant data supporting the findings of this study are available within the paper and its supplementary files. All data for the main figures are provided in Supplementary Data 1. All other data will be available from corresponding authors upon reasonable request.

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11. Appendix 4

Chapter 4

"Potential plant–plant communication induced by infochemical methyl jasmonate in sorghum (*Sorghum bicolor*)"

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Potential Plant–Plant Communication Induced by Infochemical Methyl Jasmonate in Sorghum (*Sorghum bicolor)*

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Abstract: Despite the fact that they are sessile organisms, plants actively move their organs and also use these movements to manipulate the surrounding biotic and abiotic environments. Plants maintain communication with neighboring plants, herbivores, and predators through the emission of diverse chemical compounds by their shoots and roots. These infochemicals modify the environment occupied by plants. Moreover, some infochemicals may induce morphophysiological changes of neighboring plants. We have used methyl-jasmonate (MeJa), a plant natural infochemical, to trigger communication between emitters and receivers *Sorghum bicolor* plants. The split roots of two plants were allocated to three different pots, with the middle pot containing the roots of both plants. We scored low stomatal conductance (g_S) and low CO_2 net assimilation (A) using the plants that had contact with the infochemical for the first time. During the second contact, these parameters showed no significant differences, indicating a memory effect. We also observed that the plants that had direct leaf contact with MeJa transmitted sensory information through their roots to neighboring plants. This resulted in higher maximum fluorescence (F_M) and structural changes in root anatomy. In conclusion, MeJa emerges as possible trigger for communication between neighboring sorghum plants, in response to the environmental challenges.

Keywords: carbon assimilation; infochemical; plant signaling; photosynthesis; physiological memory; root anatomy; stomatal conductance

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1. Introduction

The main cognitive functions of the nervous system, such as speech, memory, learning ability, and cognition, are strictly attributed to humans and some animals. Any attempt to compare such cognitive attributes to plants has been, and still is, labeled anthropomorphism, an attempt to humanize what is not human [1]. Obviously, plants have no neurons or a brain, so their sensory perceptions and the coordination of their organs must differ from those found in animals [2,3]. Nevertheless, plants are not senseless automatons and their adaptation and survival are based on plant-specific sensory systems continuously monitoring their environment [2–8].

Although plants are sessile organisms, they are able to actively move their organs (e.g., leaves and roots) and also to use these movements to interact with and manipulate the surrounding biotic and abiotic environments [4–8]. Plants generate electrical signals through membrane polarization and depolarization [9], as well as volatile chemical substances [6]. It is known that plants generate numerous different volatile substances, both from their shoots and roots [10], as well as root exudates [11]. These compounds help plants to communicate with herbivores, predators, and the parasites of their herbivores, and even with neighboring plants, may helping their defense strategy. However, for

plant–plant communication to be accomplished, two factors are necessary. The first factor is that emitter plants exists. Second, it is necessary that receiver plants can capture, translate, and respond to these emitted signals [7]. The signal emitter can be a plant that, after an attack from a herbivore, activates mechanisms of response, triggering a cascade of internal signaling and long distance communication from the shoot to the root or vice versa. After this process of internal signaling, plant–plant signaling via volatiles can occur [12].

A relevant infochemical in plant signaling is methyl jasmonate (MeJa) [13]. This chemical compound is a phytohormone that acts as a natural plant regulator and plays a key role in a physiological pattern, plant growth, and development [13–15]. MeJa modulates root and shoot growth, leaf growth and senescence, pollen maturation, and formation of secondary metabolites [16,17]. This infochemical can also induce stomatal closure, consequently modifying water loss and CO_2 absorption by the leaf, leading to a direct impact on photosynthetic machinery due to limited CO_2 availability [17,18]. In addition, the exogenous application of MeJa can activate a signaling cascade for jasmonate production [19], inducing the accumulation of reactive oxygen species (ROS), inhibiting synthesis, and promoting the degradation of chlorophyll and rubisco, thereby causing a reduction in photochemical efficiency [20,21]. Consequently, plant growth and development can be modified.

Otherwise known as a stress hormone, MeJa and jasmonates plays a crucially role in response to biotic and abiotic stresses. In response to environmental stimuli, such as herbivory, plants typically release MeJa [13,15,22], and neighboring plants can capture this infochemical and begin a process of preparation and regulation of its defense mechanism against this biotic attack [14,23]. Recent research has shown that a slight touch of the aerial part from one plant to another can trigger responses in neighboring untouched plants through underground communication [24]. Still, in the same study, it was proven that roots have the ability to detect the altered physiological state of neighboring plants through chemical signals released as a root exudates. However, signaling to the environment through the roots [23,25,26] due to the contact of shoots with MeJa has not been examined thus far.

It is already known that after an initial stressful event, plants can modify their development patterns. In subsequent stressful events, plants can then adapt to environmental changes through a plant-specific learning process [27–29]. This learning process is based on developing anticipatory behavior without the need to learn from scratch during every environmental disturbance situation. Walter et al. (2013) called this learning process "stress memory" [30]. At the very beginning of a stressful event, the plant captures information (alarm phase) and throughout this period changes its physiological processes, which may promote the memory effect [30]. Therefore, these physiological adjustments can generate a stress "impression" that can enhance adaptive responses to subsequent stress events [28–32]. This process of memory in plants hardly resembles the neuronal networks and brains found in animals, but neurons may not be the only essential way of learning [33].

In this context, considering that plant communication can occur also through root–root signaling, the hypothesis of this study was that MeJa induces root communication between neighboring plants. The chemical signaling received by neighboring plants can cause morphophysiological alterations in receiver plants, which would favor tolerance to recurrent stress events. To test these hypotheses, this study aimed to evaluate: (i) The occurrence of changes in gas exchange and photosynthesis after first contact with MeJa; (ii) during the second contact, the plants become less sensitive to MeJa; (iii) neighboring plants can capture information about stressful events and alter their morphophysiological patterns accordingly. We designed a hydroponic experiment in which leaves of just one sorghum plant were exposed to MeJa, while their roots came into physical contact with the roots of neighboring plants using an experimental split root system [34].

2. Results

2.1. Assimilation Rate

We analyzed the effects of MeJa on the physiology of sorghum seedlings, applying the infochemical two times on the leaf surface of future emitter seedlings.

In the first exposure to MeJa, we observed a smaller CO_2 net assimilation rate (*A*) in the treated (T) plants in comparison to the mock (M) plants. In the T group, the *A* was smaller than that of the M group by 23.5% at 5 h and 20.4% at 7 h after application (HAA) (Figure 1A). Five days after the first contact, we applied MeJa for a second time and observed that *A* did not differ between the T and M groups (Figure 1B). In contrast, by just comparing the *A* of the plants that received the infochemical (T) between the first and second contact, we observed that the *A* was greater during the second contact than in the first contact by 44.31% at 5 and 31.67% at 7 HAA.

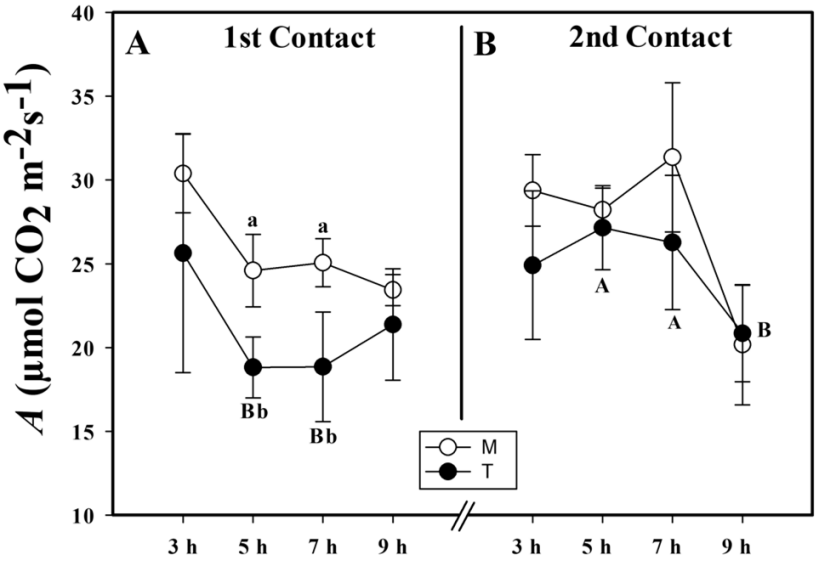

Figure 1. CO_2 net assimilation (*A*, µmol CO_2 $m^{-2}s^{-1}$) of the first (**A**) and second (**B**) contact with the methyl jasmonate (MeJa) (treated (T)) and mock (M) groups. MeJa was applied to the first fully expanded leaf of the plants in the T group at 6:00 a.m., 0 h after application (HAA), and evaluations were made at 3, 5, 7, and 9 HAA. The results are from a one-way ANOVA repeated measures, followed by Tukey's test with a significance level of 5% ($p < 0.05$). The data refer to means ($n = 4$), error lines indicate standard deviation. Lowercase letters indicate differences between the T and M groups in the respective contact, while uppercase letters indicate differences between the first and second contact in the T group ($p < 0.002$).

2.2. Stomatal Conductance

Similarly to *A*, stomatal conductance (g_S) decreased after MeJa contact. Just 3 HAA of MeJa, we observed smaller g_S in the plants of the T group compared to those of the M group. This pattern continued until at 7 HAA, only equaling out at 9 HAA (Figure 2A). We observed a 68% higher stomatal conductance of the plants in the T group during the second contact, when we compared it to the first contact at 5 HAA.

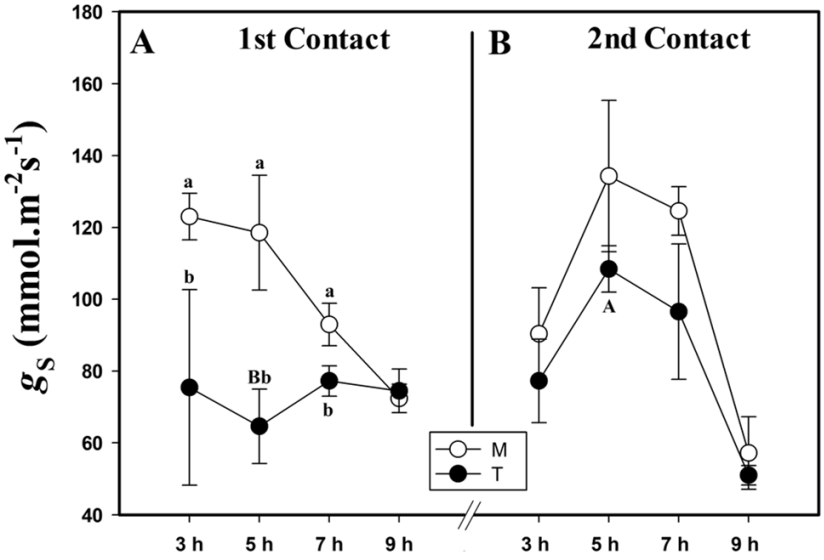

Figure 2. Stomatal conductance (g_S, mmol.m^{-2}s^{-1}) of the first (**A**) and second (**B**) contact with MeJa (treated (T)) and mock (M) groups. MeJa was applied to the first fully expanded leaf of the plants of the T group at 6:00 am, 0 h after application (HAA), and evaluations were made at 3, 5, 7, and 9 HAA. The results are from a one-way ANOVA repeated measures, followed by Tukey's test with a significance level of 5% ($p < 0.05$). The data refer to means ($n = 4$), error lines indicate standard deviation. Lowercase letters indicate differences between the T and M groups in the respective contact, while uppercase letters indicate differences between the first and second contact in the T group ($p < 0.01$).

2.3. Maximum Fluorescence

The signaling led to changes in the physiological patterns of the stages in the photochemical phase of photosynthesis. During the second contact, we observed that the plants of the treated neighbor (TN) group had a higher maximum fluorescence adapted to light (F_M) compared to the other groups. This difference was found in neighboring plants (mock neighbor (MN) × TN) at 5, 7, and 9 HAA during the second contact with MeJa. We also recorded the same difference in patterns at the same hours between the T and TN groups, being that the maximum fluorescence of TN was higher by 75.4% at 5 HAA, 57.3% at 7 HAA, and 39.9% at 9 HAA (Figure 3).

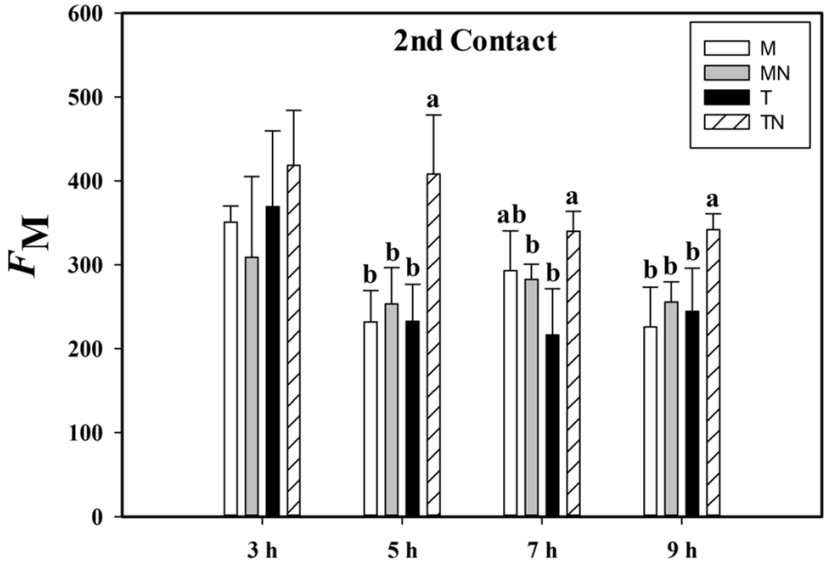

Figure 3. Maximum fluorescence (F_M) of the second contact with the MeJa (treated (T)), mock (M), treated neighbor (TN), and mock neighbor (MN) groups. MeJa was applied to the first fully expanded leaf of the plants in the T group at 6:00 a.m., 0 h after application (HAA), and evaluations were made at 3, 5, 7, and 9 HAA. The results are from a one-way ANOVA repeated measures, followed by Tukey's test with a significance level of 5% ($p < 0.05$). The data refer to means ($n = 4$), error lines indicate standard deviation. Lowercase letters indicate differences between the groups in the respective evaluations ($p < 0.001$).

2.4. Anatomical Analyses of Adventitious Roots

The morphological analysis of the *Sorghum bicolor* adventitious roots after going through two rounds of contact with MeJa showed variations regarding the intercellular space in the cortex and the area occupied by the stele. Roots with smaller intercellular spaces were observed in the plants of the T and TN groups. The roots of the M group had a cortical intercellular space area that was 45.9% greater than that of the T group. The plants in the MN group had a cortical intercellular space that was 25.2% greater compared to that of the plants in the TN group. In contrast, the plants of the T and TN groups had larger steles.

The plant roots in the T group showed twice the area occupied by the stele (101.6%) in relation to those of the M group. The plants of the TN group showed roots with an area occupied by the stele that was 41.17% greater in relation to those of the MN group (Figure 4).

Discussed data that are not presented were collected, but no significant differences were observed. This data is in the supplementary tables S1 and S2.

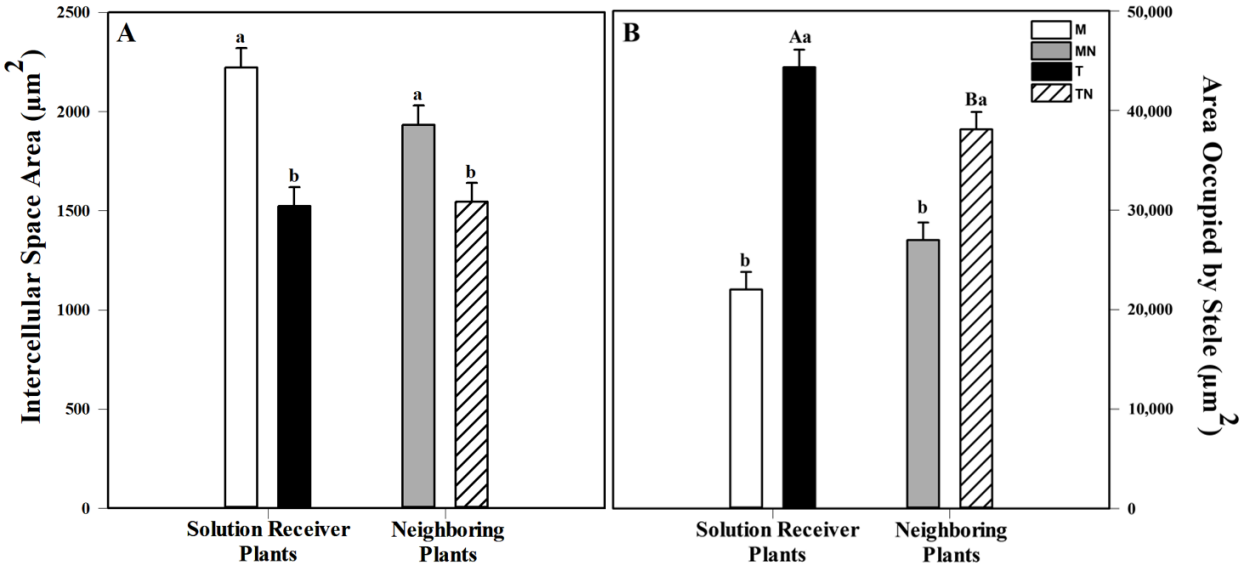

Figure 4. Anatomical aspects of *S. bicolor* on the 18th day of the experiment of all four groups: Mock (M), mock neighbor (MN), treated (T) and treated neighbor (TN). (**A**) Intercellular space area in the cortex (µm²). (**B**) Area occupied by the stele (µm²). The results are from a one-way ANOVA repeated measures, followed by Tukey's test with a significance level of 5% ($p < 0.05$). The data refer to means ($n = 4$), error lines indicate standard deviation. Uppercase letters indicate differences between the plants that received the mock or MeJa solution and the neighboring plants (M × MN and T × TN), while lowercase letters indicate differences between the plants that received the solution and the neighboring plants (M × T and MN × TN) ($p < 0.001$).

3. Discussion

Contact of *S. bicolor* leaves with MeJa simulates stressful stimuli resembling herbivorous injuries and/or disease [22]. Only a few hours after contact with MeJa, we observed low stomatal conductance and carbon assimilation of the treated group (T) in relation to naive plants of mock group (M), especially the 5 h after application (HAA), the time of day when C4 plants are showing their optimal photosynthetic performance [35]. This might indicate that contact of the leaf with MeJa stimulated stomatal closure, reducing its conductance [21]. The strategy of stomatal closure triggered a series of physiological changes, one of them being the reduction of CO_2 incorporation in the Calvin–Benson cycle and this lower carbon uptake reduced the net assimilation rate (*A*). In cases of photosynthesis reduction, this causes less sugar availability for the plant, consequently leading to the leaves consuming all of the sugar that they have already photosynthesized before complete stomatal closure. Depending on the severity and the stress prolongation, this can lead to senescence and eventual leaf fall, among other morphological responses [36].

During the second contact with MeJa, the plants of the T group had no variation regarding the mock group in the physiological parameters of either their stomatal conductance or their carbon assimilation, equalizing these parameters to those of the mock group. The MeJa-exposed plants may have demonstrated the ability to store information of the first contact and to react more quickly and efficiently in response to the second contact [37]. Initial exposure to stress can activate an epigenetic marker in a set of genes, facilitating faster and more efficient responses to future stresses [38]. This result may lead to the existence of physiological memory. This type of memory was named by Walter et al. (2011) as "stress imprint" [39], related to the phenotypic plasticity of a species. Regarding plant phenotypic plasticity, it is important to point out the plant specialization in a given environment; in other words, the greater the species plasticity, the greater is the acclimatization in contrasting environments [40].

The challenges induced by biotic and abiotic stress factors are interpreted by the plant after an internal signaling cascade has been accomplished [41], thus allowing the response of the whole plant. However, this signaling is not only restricted to the individual plant,

but it can also be shared with the plants or organisms around them. This information can be shared by the roots, which are the main organ responsible for detecting the altered physiological state of their neighboring plants [24]. We tried to identify which chemical substance was responsible for the shared information between roots. For this, we analyzed the nutritive solutions of pots 1, 2, and 3 through HPLC–MS/MS. However, we did not find any differences in the solutions of the different pots, nor we did identify MeJa in the solutions (data not shown).

Although there were no differences in the nutritive solutions, we could observe the altered state in the maximum fluorescence (F_M) patterns, indicating changes in photosystem II (PSII). This protein complex is among the first structures affected by exposure to stress [42]. Therefore, it is essential to re-organize the photosynthetic apparatus to dissipate the excess light energy absorbed in a metabolism weakened by a stressor. This regulation is observed with the chlorophyll *a* fluorescence parameter through photochemical and non-photochemical dissipation [43,44]. Our fluorescence data indicate that there may have been an indirect communication between plants, because chlorophyll fluorescence provides information about the PS II state [45] and damage to PSII reaction centers has been used to estimate the quantum efficiency of PSII [46]. Therefore, stressed plants with damaged photosynthetic tissues increase their nonphotochemical quenching processes, consequently decreasing F_M [47]. Nevertheless, our data show a higher F_M in the TN group. Even without any contact with MeJa, the plants of the TN group showed higher F_M in relation to the T and MN groups, increasing their fluorescence rates hours after the stimulus.

Physiological memory is indicated via the maximum observed fluorescence, when F_M could be an indicator of communication between adjacent plants, as well as the perception of stressors. In a previous study, similar effects were observed after sulfur dioxide exposure in an urban landscape [48]. This implies that damage promotes the rebuilding of photochemical apparatus and the optimization of physiological responses, bringing about better photochemical performance in recurrent stress via the stimulation of MeJa biosynthesis and signaling. Roots are well known to activate both jasmonate synthesis and signaling in response to shoot stress [49,50]. Intriguingly, even very weak mechanical stimuli induced by water droplets mimicking rain show this phenomenon [51].

Parallel to the physiological changes caused by the disturbance imposed on the plants, we observed structural changes in the roots of the plants in contact with MeJa and their neighbors. The roots from both groups of plants (T and TN) exhibited reduced area occupied by the intercellular spaces in the cortex and larger steles. Thus, concerning these anatomical parameters, it is remarkable that the naive neighbor plants responded to the MeJa treatment similarly to the treated plants, showing the structural plasticity of the tissues. In *S. bicolor*, the arrangement of the cortical cells is categorized as Panicoid-type and is characterized by the cuboidal packing of the inner cortical cells [52–54] with little extensive aerenchyma formation. The even smaller area occupied by the cortical intercellular spaces, as observed here in the roots of the plants of the T and TN groups, can be explained by a likely increased number of such cortical cells, an increased radial dimension of the parenchyma cells, or both. As it was observed in the roots of different plants under mechanical stress conditions [54–57], or can still be indirectly related to the higher stele size in these plants. The enlarged steles in the sorghum plants treated with MeJa (T) and in its neighbors (TN) could be associated with the overexpression of the genes related to stress, as reported for rice roots [58]. The stele size and the area occupied by the intercellular spaces in the cortex influence the rate of water and solute uptake by roots and their distribution between roots and shoots, involving coordinated activity of transport systems [59]. The structural parameters analyzed here could reflect important alterations in functioning of roots. Considering that the regulation of root water uptake is crucial to overcome stress injury [60], an increased volume of the stele may play an essential role in plant performance under these conditions.

4. Materials and Methods

4.1. Plants and Growth Conditions

This study took place in a greenhouse under natural light conditions located in the Department of Biostatistics, Plant Biology, Parasitology and Zoology of the Institute of Biosciences of São Paulo State University (UNESP), Botucatu, Brazil. Gas exchange, chlorophyll *a* fluorescence, and morphostructural changes were evaluated.

Sorghum bicolor seeds of the BRS 332 variety were used. The seeds were provided by the Brazilian Agricultural Research Corporation (Embrapa Maize and Sorghum, Sete Lagoas, MG, Brazil) and were sown in styrofoam trays with vermiculite substrate and irrigated once a day for germination and rooting.

At 20 days after sowing, the moment defined in this study as the juvenile growth phase, when the average height of seedlings was 25 cm, the seedlings were transplanted into a hydroponic system with Hoagland and Arnon (1950) nutrient solution nº 2, with 50% ionic strength, electrical conductivity of 1.2 mS, and pH 6.0 [61].

Plants were acclimatized for 10 days in different pots with a 500 mL capacity, filled with nutritive solution. This 10-day period was established after continuous physiological monitoring according to previous experiments of the research group [62] for better adaptation to the new culture medium and for the root growth needed to use specific techniques for this experiment.

During the whole experimental period, the condition of the greenhouse was monitored, with an average temperature of 27 °C, light intensity of 800 μmol m^{-2} s^{-1}, and average relative humidity of 70%.

4.2. Plant–plant Communication Experimental Design

After the acclimatization period, the roots were split in two portions to assess the possibility of root communication induced by external stimuli. A randomized block design with four groups was used. The groups were as described below and shown in Figure 5:

(1) Mock (M): Contact with the mock solution (without the addition of MeJa), with its root separated into two parts, where half remained in pot 1 and the second half was allocated to pot 2.

(2) Mock neighbor (MN): Without contact with any solution, with its root also separated into two parts, where the first half was in pot 2, allowing direct contact with the roots of the mock group, while the second half was in pot 3.

(3) Treated (T): Contact with the MeJa solution, with its root separated into two parts, where half remained in pot 1 and the second half was allocated to pot 2.

(4) Treated neighbor (TN): Without contact with the MeJa solution, with its root also separated into two parts, where half was in pot 2, allowing direct contact with the roots of the treated group, while the second half was in pot 3.

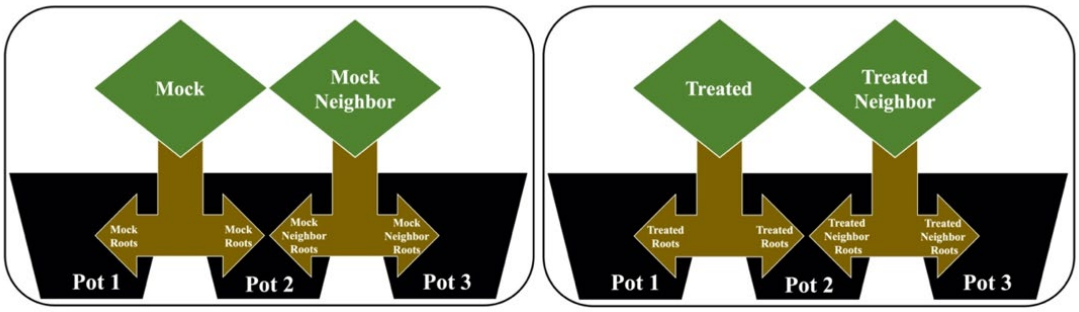

Figure 5. Experimental model for root communication following the model in [34,62]. The mock group had their roots divided and allocated to pots 1 and 2; the mock neighbor group had their roots divided and allocated to pots 2 and 3; the treated group had their roots divided and allocated to pots 1 and 2; the treated neighbor group had their roots divided and allocated to pots 2 and 3.

In the proposed experimental model, designed according to Figure 5, pots with a capacity of 500 mL of nutritive solution were used (model adapted from [34,62]). The roots were separated in two parts, allowing physical contact between the roots of two different plants in the same pot (pot 2) and to verify the difference in exudates from the three different pots. Thirty days after germination (20 days in Styrofoam tray + 10 days for acclimatization in hydroponic system), treatment with MeJa (Sigma-Aldrich, Munich, Germany) was started to simulate a possible signal received by this chemical compound.

At 6:00 a.m. on days 1 and 10, we brushed 2 mL of the MeJa solution (in a Becker, 0.75 mM MeJa was diluted in 5% ethanol and 0.5% surfactant %*v*/*v*.) on the first fully expanded leaf until total exhaustion of the solution in the plants of the T group. The excess solution was gently removed with soft paper. For the M group, we used the same methodology, but with the mock solution (deionized water, ethanol, and surfactant). The solutions were not applied to the plants of the MN and TN groups. The surfactant agrex'oil (Microquimica Tradecorp, Campinas, SP, Brazil) was used to reduce the volatility and to enhance the adherence of the MeJa to the leaf surface, since the infochemical, despite being in the liquid phase, has volatile characteristics. For 4 days (96 h), the plants shared root exudates in pot 2, where there was contact between their roots. On day 5, all roots were carefully washed with deionized water for total removal of root exudates and the nutritive solutions of pots 1, 2, and 3 were replaced, starting the recovery period. This period lasted 5 days (days 5–10), until the physiological patterns returned to the initial state (same pattern as the day before the experiment started). On day 10, a new washing process took place, where a new nutritive solution was added to all of the pots and a new application of the MeJa and mock solutions occurred in their respective groups. On day 14, the nutritive solutions of all of the pots were replaced again, characterizing the end of the second cycle of the experiment. The end of the experiment occurred on day 18, with the collection of biological root material for anatomical analysis.

Eight gas exchange analyses were performed to monitor the plants' physiological state. Four analyses were performed on day 1 (first contact with MeJa) and the other four analyses were performed on day 10 (second contact with MeJa). Data collection of the gas exchange was performed in 4 repetitions per group and occurred at 3, 5, 7, and 9 h after application (HAA) of the MeJa or mock solution on the leaves, as shown in Figure 6.

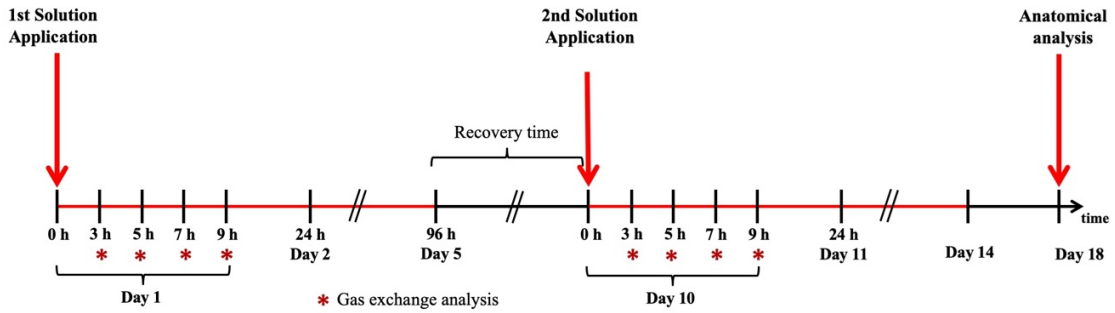

Figure 6. Time line of the experimental design for the methyl jasmonate (MeJa) application, gas exchange and anatomical analysis that were performed. The first and second red arrows show MeJa application at days 1 and 10 at 0 h. The third red arrow shows root collection for anatomical analysis. Red stars indicate gas exchange measurements.

The complete set of collected data is shown in the Supplementary Material.

4.3. Physiological Analysis

Gas exchange measurements were carried out using equipment with an open photosynthesis system with CO_2 and a water vapor analyzer by infrared radiation (Infra-Red Gas Analyzer (IRGA) and Fluorescence System, GFS 3000, Walz, Effeltrich, Germany). Analyses were carried out with four replicates per treatment at approximately 3, 5, 7, and 9 HAA (9:00 a.m., 11:00 a.m., 1:00 p.m., and 3:00 p.m., respectively) during the two

application cycles, totaling 8 measurements. The evaluated parameters were the CO_2 net assimilation (A, μmol CO_2 m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}). Measurements were standardized using the IRGA: 400 ppm for CO_2 concentration, 20,000 ppm for H_2O concentration, and 30 °C for leaf temperature.

The maximum fluorescence adapted to light (F_M) was evaluated using a fluorometer (luminous intensity of 1500 μmol m^{-2} s^{-1} for photon flux density) coupled to the IRGA, so the times and number of evaluations were the same as those of the gas exchanges. Experiments were performed in the greenhouse with a constant average e temperature, air humidity, and vapor pressure deficit (VPD) (22.06 °C, 79.71%, and 0.54 kPa, respectively).

4.4. Morphological Analysis

Samples were taken from 0.5 cm above the tips of adventitious roots. The samples were fixed in FAA 50 (formaldehyde, acetic acid, 50% ethyl alcohol) [63] for 48 h and then stored in 70% alcohol. Afterward, they were dehydrated in ethanol series and embedded in methacrylate resin (Leica HistoResin, Leica, Wetzlar, Germany) [64]. The samples were sectioned on a semi-automatic rotary microtome and cross-sections (4-μm-thick) were stained with 0.05% Toluidine Blue pH 4.7 [65]. The slides were analyzed on a Leica DMR photomicroscope with DFC 425 camera (Leica, Wetzlar, Germany) attached. Quantitative analyses were performed using LAS software (V3.8 Leica, Wetzlar, Germany).

4.5. Data Analysis

Data were analyzed using the statistical software SigmaPlot (12.0, Systat Software Inc. San Jose, CA, USA) All data were obtained from four biological repetitions and, after being submitted to the Shapiro–Wilk normality test ($p < 0.05$), were statistically analyzed by one way analysis of variance repeated measures (ANOVA). The mean values were compared by Tukey's test ($p < 0.05$).

5. Conclusions

In *Sorghum bicolor*, during the first contact with MeJa, the plants of the treated (T) group showed changes in their physiological parameters. However, during the second contact, their responses did not differ from those of the mock (M) group, indicating that sorghum plants became less sensitive to MeJa after the first treatment. We also observed that the plants from the T group may have signaled their sensory information through their roots to their neighboring plants (i.e., the TN group). Nevertheless, our data do not exclude the contribution of shoot volatiles [66,67] in this plant–plant communication, since some studies have already demonstrated that it has an impact on gene expression and stomatal opening [68]. Altogether, MeJa may have led to plant–plant communication and altered the physiological and morphological patterns of the neighboring plants. In future, it will be important to study plant-plant communication from the perspective of critical physiological parameters of plant responses to environmental challenges, anticipating responses and increasing the chances of tolerating a possible future stress event. Intriguingly, in this respect, anesthesia induced with diethyl ether prevents both sensitivity to and accumulation of jasmonic acid in *Venus flytrap* plants [69]. Future studies should focus on the illumination of those mechanisms that interlink plant communication, behavior, and memory with jasmonate signaling related to the sensitivity of plants to anesthetics.

Supplementary Materials: The following are available online at www.mdpi.com/2223-7747/10/3/485/s1, Table S1: Physiological analysis, Table S2: Anatomical analysis.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

MeJa	Methyl jasmonate
ROS	Reactive oxygen species
A	CO_2 net assimilation
T	Treated group
M	Mock group
HAA	Hours after application
g_S	Stomatal conductance
TN	Treated neighbor group
MN	Mock neighbor group
F_M	Maximum fluorescence adapted to light
VPD	Vapor pressure deficit
FAA 50	Formaldehyde, acetic acid, 50% ethyl alcohol
HPLC–MS/MS	High-performance liquid chromatography-mass spectrometry

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12. Appendix 5

Chapter 5

"*Boquila trifoliolata* mimics leaves of an artificial plastic host plant"

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Boquila trifoliolata mimics leaves of an artificial plastic host plant

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ABSTRACT

Upon discovery that the *Boquila trifoliolata* is capable of flexible leaf mimicry, the question of the mechanism behind this ability has been unanswered. Here, we demonstrate that *plant vision* possibly via plant-specific ocelli is a plausible hypothesis. A simple experiment by placing an artificial vine model above the living plants has shown that these will attempt to mimic the artificial leaves. The experiment has been carried out with multiple plants, and each plant has shown attempts at mimicry. It was observed that mimic leaves showed altered leaf areas, perimeters, lengths, and widths compared to non-mimic leaves. We have calculated four morphometrical features and observed that mimic leaves showed higher aspect ratio and lower rectangularity and form factor compared to non-mimic leaves. In addition, we have observed differences in the leaf venation patterns, with the mimic leaves having less dense vascular networks, thinner vascular strands, and lower numbers of free-ending veinlets.

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Introduction

Seven years ago, Gianoli and Carrasco-Urra reported on their discovery of *Boquila trifoliolata* (Lardizabalaceae), a woody vine from temperate rainforests of southern Chile, capable of complex leaf mimicry, when leaves of up to three different host plants were mimicked by leaves of one *B. trifoliolata* plant.[1] However, according to a side-by-side published commentary, the absence of any plausible hypothesis for such a phenomenon makes this report unexplainable and mysterious.[2]

Gianoli and Carrasco-Urra preferred some chemical volatile signals released from the host plants, which would allow the *B. trifoliolata* to mimic leaves of host plants.[1,3] As an alternative proposal, they also speculated that horizontal gene transfer between host plant and Boquila vine, mediated perhaps via airborne microbes, might allow this leaf mimicry. They proposed this scenario because *B. trifoliolata* leaves mimic the nearest foliage, irrespective if these leaves are from the host plants or some other neighboring plants.[1,3] The complexity of this mimicry, when *B. trifoliolata* leaves were shown to mimic shapes, colors, leaf orientations, petiole lengths, and vein conspicuousness and patterns may have a third hypothesis, totally different from the volatile signals from host plants or gene transfer via airborne microbes. This third hypothesis would support the possibility that *plant vision* based on plant ocelli[4,5] is behind this unique form of plant behavior.[6,7]

The plant ocelli concept was elaborated by Gottlieb Haberlandt in 1905 and two years later supported by Francis Darwin[8] which consists of the upper epidermis cells have a planoconvex or convex shape acting as lenses, allowing the convergence of light radiation into light-sensitive subepidermal cells.[5] With the discovery that the *B. trifoliolata* is able to mimic the leaves of the nearest plant,[1,3] we have been given a rare opportunity to test *plant vision* in more detail. The simplest way to test the vision hypothesis with the *B. trifoliolata* would be to see if it would mimic a non-living leaf shape from an artificial plant. In this study, *B. trifoliolata* was exposed to the artificial plastic plant with a characteristic leaf shapes. The results of this study show that this is indeed the case as leaves of *B. trifoliolata* mimicked leaves of the artificial plant. Hopefully, this report will stimulate more experiments in future to improve our understanding of the plant sensory abilities.

Results and discussion

Boquila trifoliolata grows in very wet conditions in the Valdivian temperate rainforest. The standard leaves of the *B. trifoliolata* plants show a variation of leaf shapes and the number of lobes. The majority of leaves have three lobes with blunted tips (Figure 1a). Variation of the number of lobes can be seen with some leaves having multiple lobes and others having less than three. Some leaves showed similar pattern to the fake leaves with respect to lobe variation (Figure 1b). In this research, lower leaves were used as control (non-mimic) leaves due to being below the line of the opaque shelf 1, therefore without direct visual contact with the false leaves (Figure 2).

As the vine grows toward the artificial plant, the leaves of *B. trifoliolata* take a much different shape. The plants show obvious mimicry attempts to the closest false leaves of model plants, though some leaves still maintain a single lobe (Figure 3). The artificial plant, due to the imperfections in manufacture, has differently shaped leaves. However, all leaves showed more longitudinal shapes (Figure 4). To evaluate the mimicry attempts of Boquila leaves, we have classified them, regarding their age, into three basic groups of young, middle-age and old age leaves.

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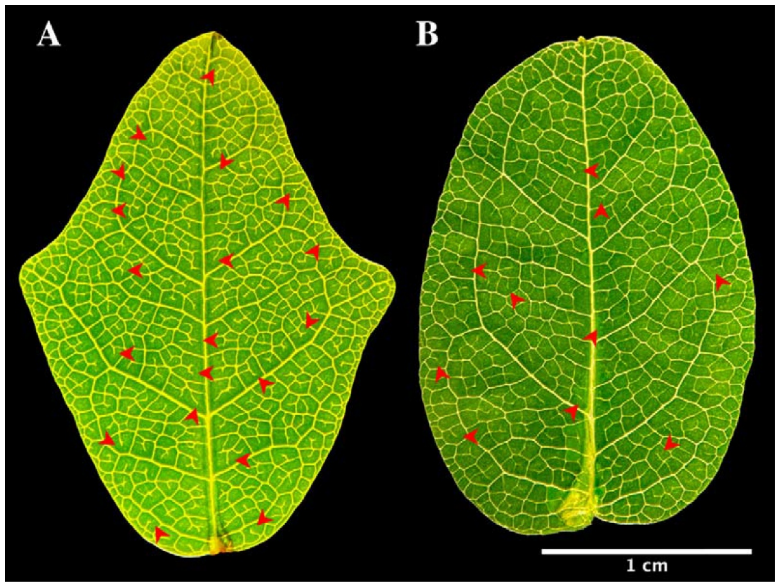

Figure 1. Leaf shapes in Boquila trifoliolata. (a) Non-mimic leaf, with three lobes, dense vascular network. (b) Mimic leaf, with a single lobe in the apex, less dense vascular network. Red asterisks shows examples of free-ending veinlets.

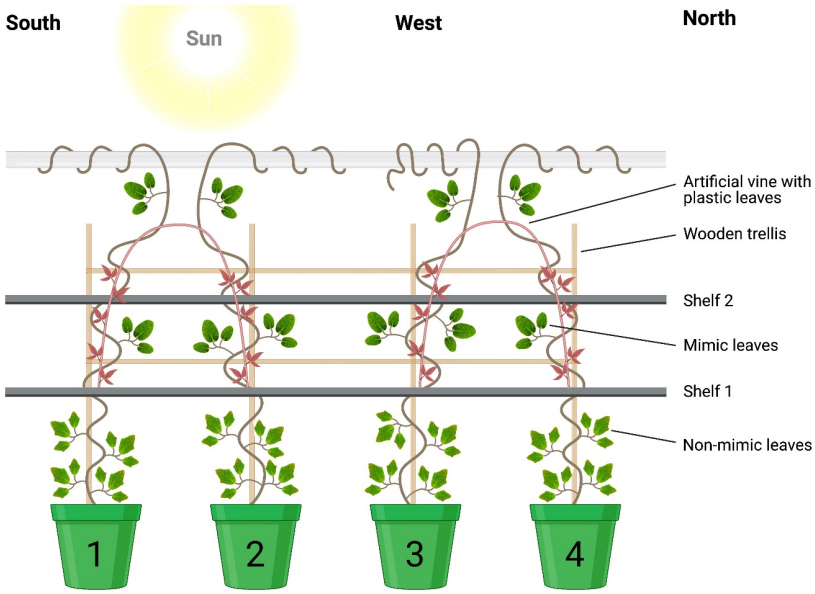

Figure 2. Experimental design. Four *Boquila trifoliolata* plants lined up side-by-side in front of a window and the artificial model vine plant with plastic leaves (red). Leaves below shelf 1 is the non-mimic (control) leaves. Leaves above shelf 1 is the mimick leaves. Created with BioRender.com.

Moreover, we used a leaf recognition algorithm for quantification of leaf forms (for details, see the Materials and Methods section).[9] We have observed that the middle-age and old non-mimic leaves had significantly greater leaf area, perimeter, length, and width than the mimic leaves (Figure 5). Regarding young leaves, we observed significant differences in leaf widths. By establishing a relationship between these four parameters, we calculated different variables. We observed that the non-mimic leaves had greater rectangularity.[9] Regardless of their age, leaf shapes took on a more uniform rectangle-like forms (Figure 6). Furthermore, when we compared the ratio between leaf diameter and leaf length (form factor), we observed that the young middle-aged and old leaves of the non-mimic plants had this ratio higher (Figure 6). However if we compared the ratio between length and diameter (aspect ratio), we found that mimic leaves had higher values, which means a more slender shapes (forms).

These last two parameters (aspect ratio and form factor) show us that mimic leaves are generally longer rather than wider, indicating that they are more similar to the elongated plastic leaves that were placed next to the Boquila plants, as

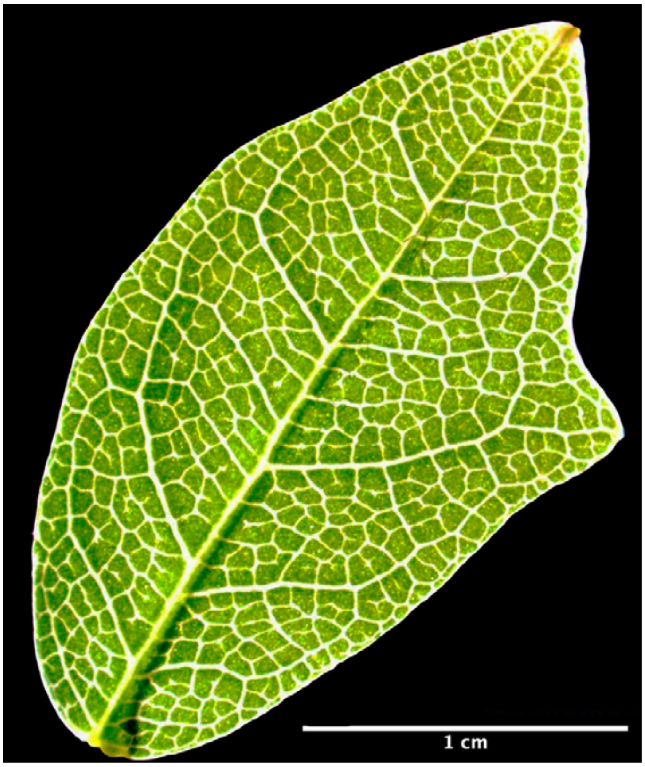

Figure 3. Single lobe mimic leaf. Mimicry attempt to the plastic leaves of artificial host plant.

a model of host plant. The non-mimic leaves showed similar values for lengths, having their form factor values close to 1 (similar width and length values result in the form factor values close to 1), the more similar the leaves are in length and width. Corroborating these data, we obtained rectangularity, showing us that the non-mimics are more roundish in shape, in comparison to the slender mimic leaves.

The mimicry began just below the artificial vine (between shelfs 1 and 2) and when more leaves were facing the model leaves, it seemed to affect the detail of mimicry. This suggests that the lower leaves sample details of the leaves next to them and pass the obtained information to the next set of growing leaves. New leaves are formed in the mimic shape and young leaves grow larger in that shape. This suggests that lower leaves play some roles in the leaf mimicry.

An interesting aspect was observed about the venation pattern when we analyzed the leaves under binocular microscopy. It was observed that non-mimic leaves had more free-ending veinlets, represented by tiny veinlets having their extremities ending freely in the leaf mesophyll[10] (red arrow heads in Figure 1). Greater amounts of the free-ending veinlets were observed in non-mimic leaves in young leaves as well as middle-aged and old leaves (Figure 7). It is well known that the development and patterning of the veins progresses in a basipetal direction (from the leaf apex toward the base); therefore, the more advanced stage of the venation networks can be found at the leaf apex than at its base.[10,11] In comparison to the non-mimic standard leaves, mimic leaves show lower numbers of free-ending veinlets and less dense vascular networks (Figure 1). This feature is an indication of high auxin

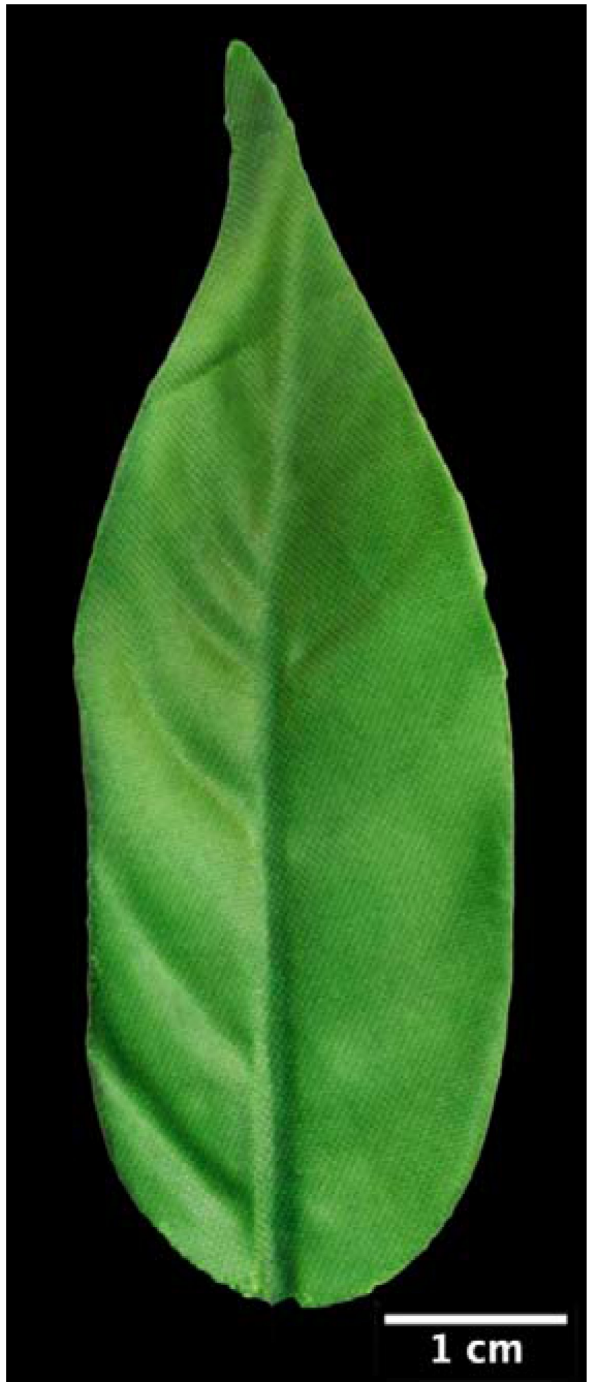

Figure 4. Plastic leaf of a model artificial plant with longitudinal shape.

concentrations at the leaf margins, suggesting that perhaps these leaves have altered patterns of auxin biosynthesis and polar auxin transport.[10,11] This can be interpreted as an attempt to modify their leaf shape, trying to mimic the plastic leafs.

It appears that over the months, *B.*trifoliolata plants improved their mimicking of the plastic host plant significantly (Figure 8). The mimic leaves doubled in size from one analysis to the next (first analysis December 2020, second analysis

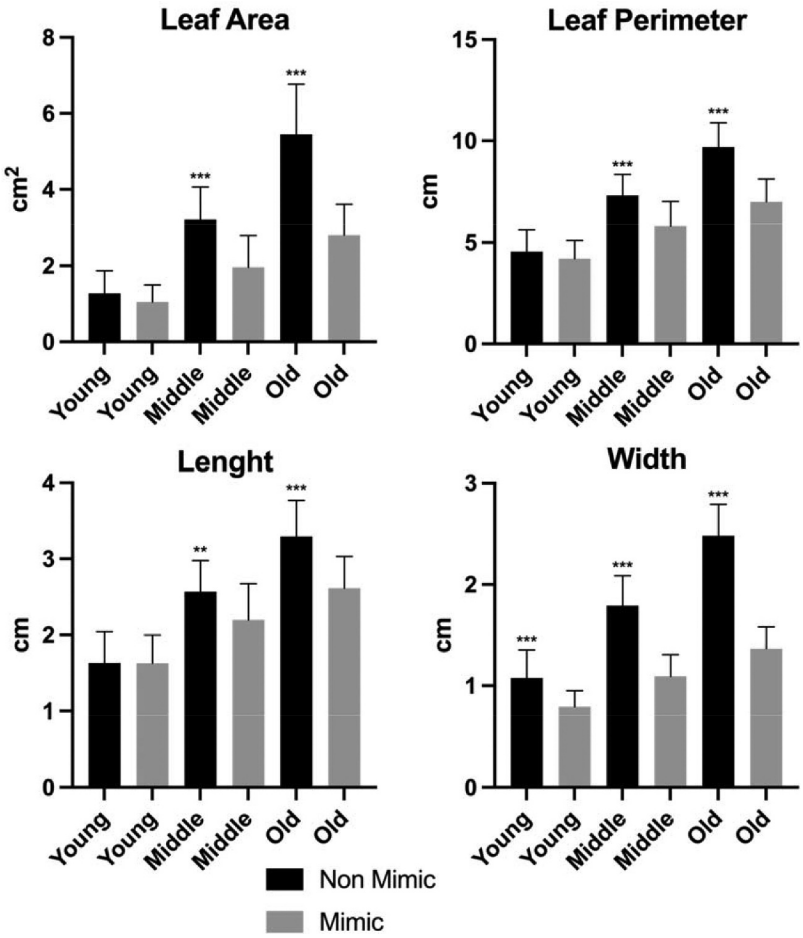

Figure 5. Morphometric analysis of *Boquila trifoliolata* leaves. Black bars correspond to non-mimic leaves (control), without contact with plastic leave. Gray bars correspond to mimic leaves, with close contact with plastic leaves. Leaves were classify into young, middle and old regarding their age. Measurements performed in 16 biological repetitions and two-tailed Student's t-test was used to identify significant differences between mimick and non-mimic leaves. P-values<0.05 were considered significant (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represent standard deviation.

June 2021) and the form factor has reduced significantly, approaching the form factor of the plastic leaves having slender shapes (form factors close to the value 1). This improved ability of *B.*trifoliolata plants to mimic shapes and sizes of plastic leaves implicates learning and memory processes in plant mimicry.

Leaf mimicry attempts have been observed on all shoots growing near the artificial model (host) plant. Some mimicking leaves are not perfect in their mimicry, similarly to their attempts at serrated leaves in nature.[1] Perhaps due to the uneven edges on the artificial plant, all leaves in contact with the artificial vine have a markedly different shape than the non-mimic leaves below the shelf. Our results showed that leaves of *B. trifoliolata* mimic artificial leaves, changing their shape to a more longitudinal shape devoid of lobes. This goes in the opposite direction of the two hypotheses proposed by Gianoli & Carrasco-Urra 2014, which speculated that the leaves of Boquila could pick up airborne chemicals released by other trees or use genes from its host via parasite or microbe. Our present analysis favors *plant vision* based on plant-specific leaf ocelli.[4,5]

Outlook and perspectives

Up to this point, the leading explanation for leaf mimicry in the *B. trifoliolata* has been volatile signaling and horizontal gene transfer.[1,3] Volatile signaling and horizontal gene transfer in plants have been proposed.[12,13] However, since the *B. trifoliolata* can mimic leaves when not in contact with the host plant makes this unlikely and hard to test. Volatile signaling does show promise and can be easily tested, as in a recent study has shown that *Cuscuta racemose* can choose between different hosts plants at a certain distance.[14]

Recent research into plant perception and communication has provided new surprising details into the life of plants enjoying not only ability of communication through chemical volatiles but also perception of acoustic signals.[13,15,16] Moreover, research done on the visual capabilities of algae and protists clearly suggest vision already in unicellular organisms.[17–23] Experimental testing of the ocelli-based *plant vision*, as it was done by Harold Wager,[4] would be the logical next step in our quest for understanding the plant sensory complexity.

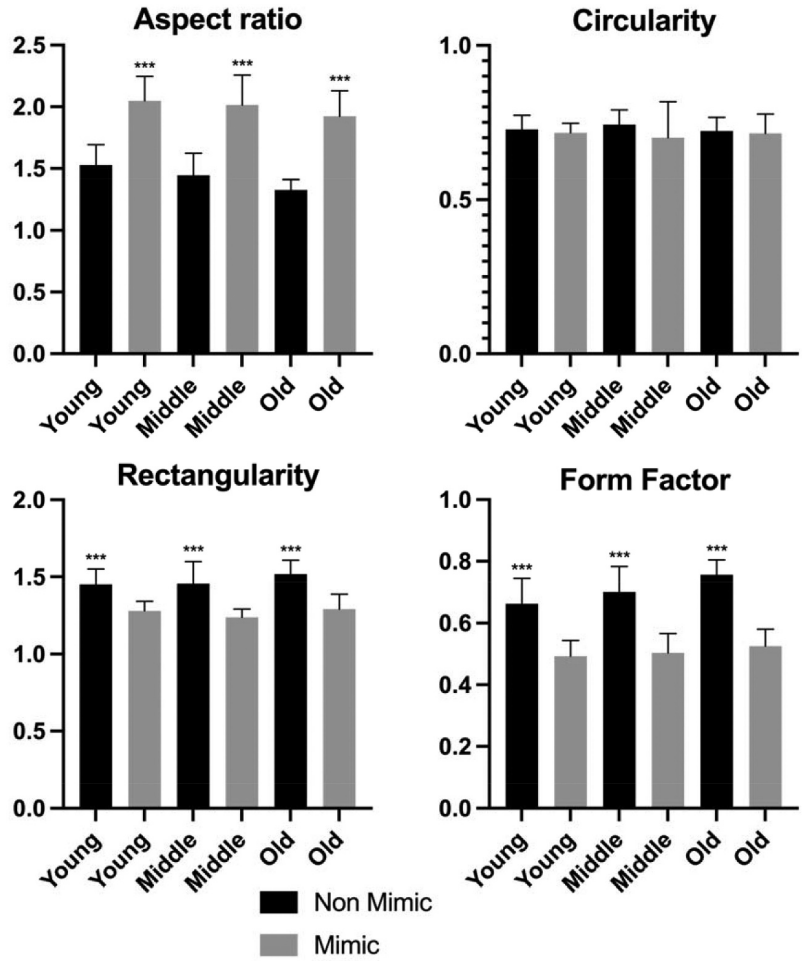

Figure 6. Morphometric analysis of *Boquila trifoliolata* leaves. Aspect ratio is the ratio of leaf length and width. Circularity describes the difference between a leaf and a circle. Rectangularity describes the similarity between a leaf and a rectangle. Form factor is the ratio between leaf width and length. Measurements performed in 16 biological repetitions and two-tailed Student's t-test was used to identify significant differences between mimick and non-mimic leaves. P-values<0.05 were considered significant (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represent standard deviation.

Currently, in a cooperation with the group of Prof. Maximilian Weigend, we are growing several Boquila plants in the Botanical Garden of the University of Bonn. These plants will allow us to perform these critical experiments in our future studies.

Materials and methods

Plant material and growth conditions

Boquila trifoliolata plants were purchased from a local store placed in Port Townsend Washington and arrived in 15.24 cm pots. Shortly after arrival plants were reported in 25.4 cm pots filled with high nutrient potting soil with a pH of 6.3, 0.30% nitrogen, 0.45% phosphate, 0.05% potassium, and 1.00% calcium. The plants were watered with distilled water (approximately 236 ml) until they reached field capacity every other day to keep the soil moist. A stone humidifier was placed near the plants to maintain a higher humidity. The experiment was conducted in Magna, Ut, USA (40°42′N, 112°06′W) during the period from September 2019 to October 2020. The plants were placed in front of a large west facing window. The first leaves sample for analysis was collected in December 2020 and the second sample was collected in June 2021.

Each plant was assigned a number and placed on a growing rack. Two artificial vines were placed above the plants on a wooden trellis. During the winter, the plants grew quickly through the leaves showed poor mimicry of the artificial plants leaves. The original plant that we had did not show good evidence of mimicry until the spring and summer. We decided to continue the experiment and see if there were better results in the warmer months.

Experimental design

The plants were lined up side by side in front of the window through which they received sunlight coming from the west direction. Above the plant pots, two opaque shelves (shelf 1 and shelf 2) were placed to keep the lower parts away from the artificial vine and plastic leaves. Two White Wisteria Garland artificial vines were purchased from a local store, and the flowers were removed so only the silk leaves would remain.

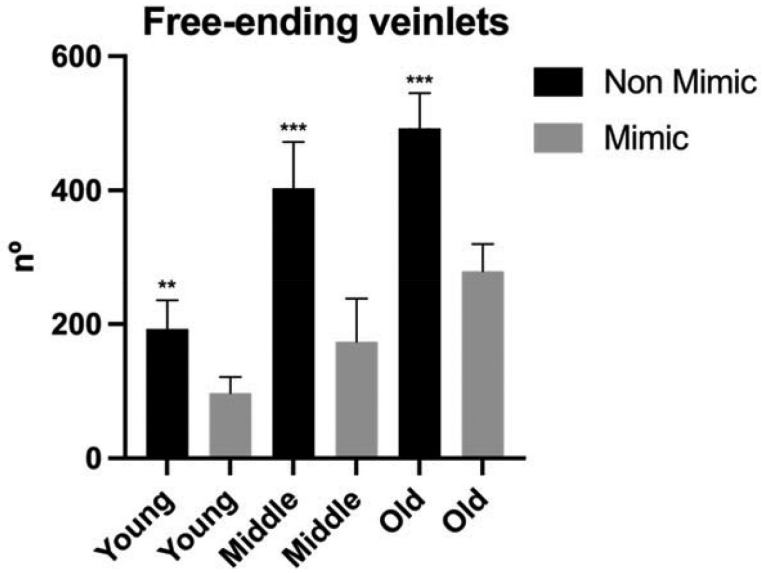

Figure 7. Quantification of the free-ending veinlets. Number of free-ending veinlets per leaf. Black bars correspond to non-mimic leaves (control), without contact with plastic leave. Gray bars correspond to mimic leaves, with close contact with plastic leaves. Leaves were classify into young, middle and old regarding their age. Measurements performed in 4 biological repetitions and two-tailed Student's t-test was used to identify significant differences between mimick and non-mimic leaves. P-values<0.05 were considered significant (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represent standard deviation.

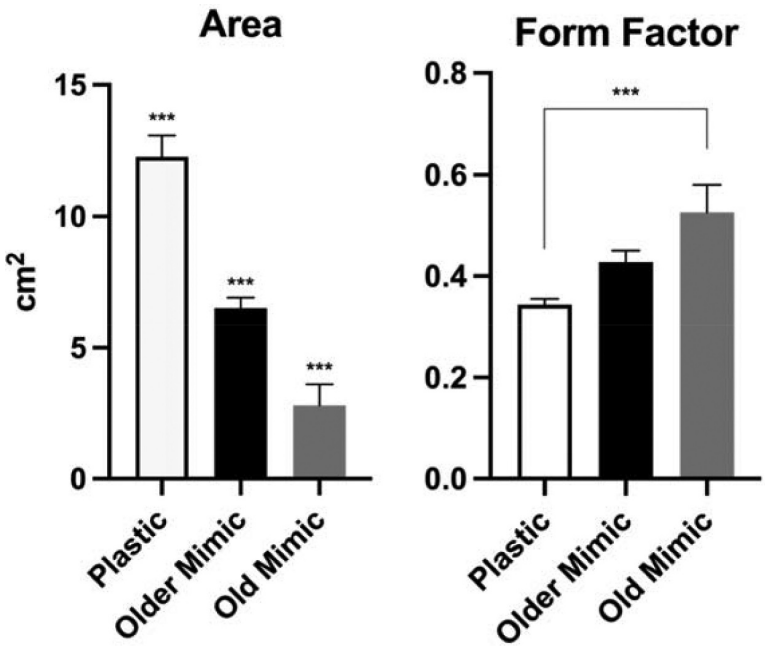

Figure 8. Leaf area and form factor (ratio between leaf width and length) of plastic leaves, old mimic leaves and older mimic leaves (leaves one year older). All three groups showed significant differences in leaf area. Only the plastic and old mimic groups showed a difference between each other, the other interactions showed no significant differences. The data were submitted to one-way analysis of variance (ANOVA) and the mean values were compared by Tukey test (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represent standard deviation.

The artificial vines were covered with fake leaves (Figure 4) and placed 28 cm above the top of the pots containing *B. trifoliolata*, so the artificial vine with fake leaves were not visible below the shelf 1, Figure 2 (The plastic plants are in red in Figure 2 for easy discrimination between the mimic leaves. However, in reality, the plastic leaves are green, as shown in Figure 3). As the plants grew, wires were placed adjacent to growing shoots to guide then toward the artificial vines if they did not attach to the trellis. Plants were observed daily with notes taken of new shoot growth and wires were added as needed to bring the new shoots closer to the artificial leaves.

Leaf morphology analysis

The plants were classified into three groups in relation to the leaves age;

Young: juvenile, newly formed leaves;
Middle: middle-aged leaves;
Old: fully formed leaves.

In addition, we classified the leaves into two additional groups that were compared to each other: mimic group and non-mimic group.

The leaves were analyzed based on four basic geometrical features:[9]

Leaf area (*A*): total leaf area, calculated in pixels of the entire leaf with the help of the Plugin LeafJ;[24]

Perimeter (*P*): the leaf perimeter was calculated by counting the pixels consistent with the leaf margin;

Length (*L*): distance between the two terminals of the main vein;

Width (*W*): the longest distance between two points that intersect the straight line of length at a 90 degree angle.

Using the four basic geometrical features, we define four digital morphological features, used for leaf analysis:

Aspect ratio: is defined as the ratio of length to width;

$$\frac{L}{W}$$

Circularity: describes the difference between a leaf and a circle, according to the following equation:

$$\frac{4\pi A}{P^2}$$

Rectangularity: describes the similarity between a leaf and a rectangle, according to the following equation:

$$\frac{LW}{A}$$

Form Factor: ratio between width and length.

$$\frac{W}{L}$$

The free-ending veinlets were analyzed using photos taken with a binocular microscope at 0.8x magnification. The photos were analyzed and the free-ending veins were counted using ImageJ software (Cell counter analyzer plugin). A minimum of 6 leaves were used for each group.

The largest leaves of the branch containing three leaves were removed, then these leaves were photographed with a camera (Canon EOS 1000D, Canon Inc., Tokyo, Japan) and binocular microscope (Leica MZ FL III with Leica DFC 290, Leica Microsystems, Wetzler Deutschland). The images were analyzed with Adobe Photoshop 2021 (22.3.0, Adobe Inc., San José, CA, USA) and Fiji ImageJ (LeafJ Plugin[24]).

Data analysis

Data were analyzed using the statistical software GraphPad Prism (9.1.0, GraphPad Software, San Diego, CA, USA). All data were obtained from 16 biological repetitions and two-tailed Student's t-test was used to identify significant differences between mimic and non-mimic leaves. One-way analysis of variance (ANOVA) was used to identify significant differences of plastic leaves from older and old mimic leaves and the mean values were compared by Tukey test (***P < .001; **P < .01; *P < .05). The error bars reported in all graphs represent standard deviation.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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13. Appendix 6

Chapter 6

“Algal Ocelloids and Plant Ocelli”

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Perspective

Algal Ocelloids and Plant Ocelli

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Abstract: Vision is essential for most organisms, and it is highly variable across kingdoms and domains of life. The most known and understood form is animal and human vision based on eyes. Besides the wide diversity of animal eyes, some animals such as cuttlefish and cephalopods enjoy so-called dermal or skin vision. The most simple and ancient organ of vision is the cell itself and this rudimentary vision evolved in cyanobacteria. More complex are so-called ocelloids of dinoflagellates which are composed of endocellular organelles, acting as lens- and cornea/retina-like components. Although plants have almost never been included into the recent discussions on organismal vision, their plant-specific ocelli had already been proposed by Gottlieb Haberlandt already in 1905. Here, we discuss plant ocelli and their roles in plant-specific vision, both in the shoots and roots of plants. In contrast to leaf epidermis ocelli, which are distributed throughout leaf surface, the root apex ocelli are located at the root apex transition zone and serve the light-guided root navigation. We propose that the plant ocelli evolved from the algal ocelloids, are part of complex plant sensory systems and guide cognition-based plant behavior.

Keywords: algae; cyanobacteria; eyes; eyespots; ocelloids; ocelli; plants; roots; shoots; vision

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1. Introduction

Vision in animals is incredibly diverse and it evolved multiple times independently [1–3]. Despite a great diversity of visual organs, an eye can be defined as the existence of a cornea and/or lens which focuses the light towards a sensory region, such as eye retina or other light-sensitive structures and tissues, with photo-responsive proteins transforming the light signal first into electrical and then into chemical signals [4–6].

In 1905, Gottlieb Haberlandt proposed the plant ocelli concept for leaf epidermis in which the upper epidermal cells resemble convex or planoconvex lens, converging light rays on the light-sensitive subepidermal cells [7]. The Haberlandt plant ocelli theory is not surprising if we consider that various organisms such as bacteria, algae, and fungi (as discussed below) have cells with similar light-sensing properties. However, plant ocelli theory was almost forgotten and only recently revived [8,9]. Supporting this leaf epidermal ocelli scenario, leaf epidermis cells, with the exception of stomata guard cells, do not generate photosynthetic chloroplasts, although they have the best position with respect to the amount of light they receive.

This concept was recaptured some 70 years later when young seedlings of tropical vine *Monstera gigantea* were reported to localize and suitably support host trees using growth towards darkness termed *skototropism*—the directional movement of plant organ towards darkness [10]. Due to observations, and apart from other theories, Strong and Ray (1975) found skototropism to be the relevant mechanism in the finding of host trees by the Monstera vine. They provided evidence that shoot skototropism is an independent mechanism. Nevertheless, they assumed it to be a modification of negative phototropism. Additionally, they reported a negative effect of increasing distance and a positive effect of increasing host stem diameter on the shoot skototropism. Importantly, the larger a potential host tree is and the closer it is located to the vine seedling, the stronger the skototropic response will be [10].

2. Chlamydomonas Algal Eyespot: Rhizoplast and Rootlet Connections

The green alga *Chlamydomonas reinhardtii* also has a subcellular eyespot apparatus. Algal eyespots are anchored at the Chlamydomonas cell periphery via so-called D4 bundles of microtubules, organized by the basal body (Figure 1). In addition, an important—but often neglected—feature of Chlamydomonas is the rhizoplast, which is a contractile centrin-based structure connecting basal bodies of flagella with the nuclear surface [11–13]. These so-called rhizoplasts or fibrous flagellar roots anchor nuclei to the flagellar or ciliar basal bodies [14–19]. The eyespot of Chlamydomonas is anchored to the D4 rootlet, extending from the peripheral flagellar basal bodies into the cell interior [20–22]. Intriguingly, similarly to the scenario with the ocelloids of the warnowiid dinoflagellates discussed below, these algal eyespots are also assembled from putatively symbiotic components. Besides the chloroplasts, there is cellular evidence suggesting that the nucleus–basal body–flagellum/cilium complex is of symbiotic origin, representing the guest cell of the host–guest cell consortium [23,24].

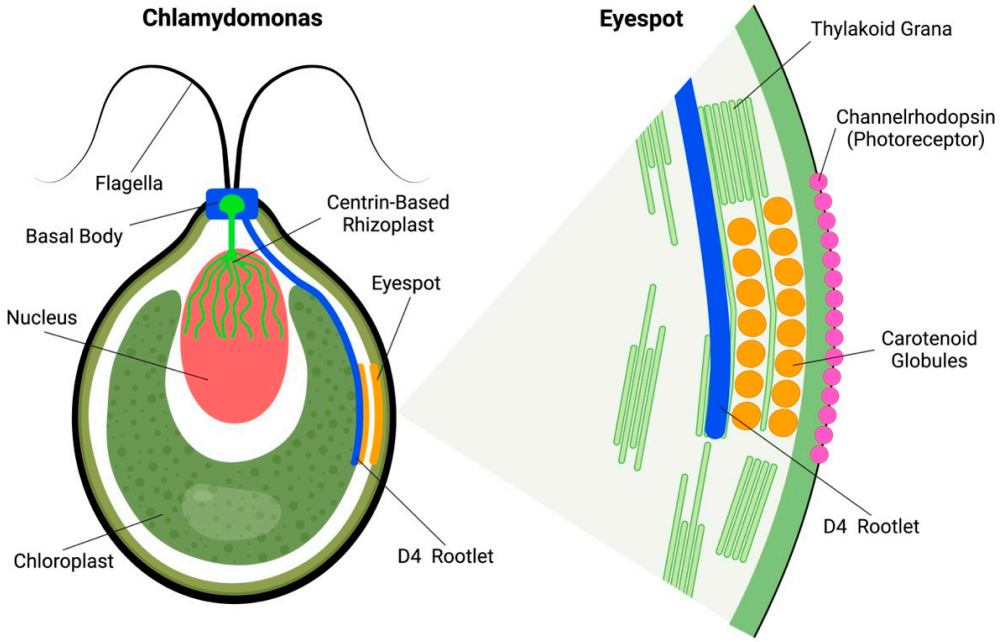

Figure 1. Algal Eyespot of Chlamydomonas. Chlamydomonas alga with two flagella associated with the basal bodies which intracellularly organize intracellular bundles of microtubules (known as rootlets) of which the D4 bundle anchors the eyespot. This eyespot is constructed from chloroplast thylakoid membranes and carotenoid globules, aligned under the plasma membrane which is enriched with photoreceptor channelrhodopsin. Besides the bundles of microtubules, the basal body also organizes the centrin-based contractile nucleo-basal body connector anchoring the nucleus. M4, M2 and D2 rootlets are not shown in this simplified scheme.

Chlamydomonas green algae have two vision responses. The first one is swimming in towards or away from light ray source, called phototaxis, depending on the total amount of reactive oxygen species (ROS) inside the cell [25,26]. The second is when they freeze for a few moments after receiving a strong light stimulus, followed by a backstroke, and then swimming normally in any direction. This second one is called photo-shock response: as the name implies, the algae stop their natural movement for seconds [27,28]. Under a microscope, it is easy to find the eyespots, as they are composed of orange carotenoid globules located under the plasma membrane enriched with photoreceptor proteins, channelrhodopsinsChR1 and ChR2 [29]. In green alga *Chlamydomonas reinhardtii*, the eyespot apparatus is composed of two layers of carotenoid globules (Figure 1) sandwiched between the thylakoid membranes of the chloroplast [28,30,31]. The eyespot apparatus is activated

through light stimuli, and afterwards controls flagella to accomplish phototaxic behavior [30]. An important aspect is that the light-induced eyespot electric currents activate and control the flagellar currents via the electric action potential-like transmission [32–35]. Rapid calcium influxes and bioelectric currents integrate sensory events at the eyespot with control of flagella beating and phototaxis [27,32,33,36].

Another algae protist that evolved a light-sensitive apparatus adapted for unicellular vision is *Euglena gracilis*. It shows two basic types of photo-movements in response to light stimuli, known as photophobic and phototactic behaviors. Similarly, as in the eyespot of Chlamydomonas, *Euglena gracilis* carotenoids are important for photo-movements. The plastids do not develop into chloroplasts due to the lack of chlorophyll synthesis [37,38]. Recent studies have reported that mutants, deficient in carotenoid production, lose their phototactic responsiveness [38]. Carotenoids are obviously essential for light perception of the Euglena eyespot. Similarly, as in Chlamydomonas, the eyespot of Euglena is associated with the microtubules-based flagella [37,39,40]. However, *Euglena gracilis* obtained their plastids much later via the secondary endosymbiosis and are evolutionary distant, belonging to Archaezoans [41]. Thus, it is not surprising that Euglena and Chlamydomonas rely on different photoreceptors in their ocelli.

3. From Algal Ocelloids to Plant Ocelli

In 1967, David Francis described an eyespot in *Nematodinium armatum*, describing lenses capable of focusing light rays and concentrating them into a structure called a pigment cup. This structure is supposed to be a light-sensitive retinoid and may have a role in image formation [42]. In 2015, further unexpected support for the plant ocelli theory of Gottlieb Haberlandt was provided with the surprising discovery of eye-like ocelloids in warnowiid dinoflagellates [43,44]. These planktonic unicellular organisms use symbiotic organelles which act as eye-like ocelloids. A mitochondria-based layer generates a cornea-like surface across a lens structure, whereas the retinal body of ocelloids develops from a membrane network formed from plastids (Figure 2). To verify these microscopically based findings, the scientists sequenced the DNA of a warnowiid retinal body, which had a substantially greater percentage of DNA originating from plastids than comparable samples from the total cell [43]. Warnowiid dinoflagellates are the only unicellular microbial organism having camera-type eye-like organs for camera-type vision-like modus [4,42–45].

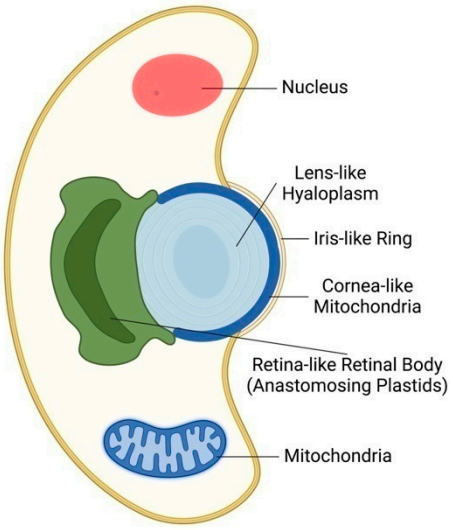

Figure 2. Algal Ocelloid of Dinoflagellates. Camera-like ocelloid of warnowiid dinoflagellates is composed of cornea-like mitochondrion enclosing hyaloplasm acting as lens and chloroplast-based retinal body. Similarly, as in the algal eyespot, the chloroplast plays the central role in the microbial vision. Adapted according [43].

4. Bacterial Vision: Cyanobacterium Synechocystis

The next surprising discovery followed one year later, when Schuergers et al. (2016) reported prokaryotic bacterial vision in cyanobacterium Synechocystis sp. PCC 6803 [46–49]. Here, the whole cell acts as a lens, focusing light on a small patch of the plasma membrane (Figure 3). A similar principle, in which the whole cell acts as a lens, was found also in eukaryotic volvocine algae [50]. Therefore, it should not be surprising if plant cells also rely on this feature via their ocelli. Importantly, biological evolution repeatedly uses all the successfully elaborated structures and processes which improve the organismal survival chances. Even the most complex organs of vision, such as animal and human eyes, represent the inherent part of the long evolutionary continuum. In the case multi-cellular volvocine algae, light-focusing roles of cells affect the adjacent cells in a manner which participates in morphological symmetries and colony behavior as relevant information [50]. In Synechocystis, light perception at the photosensitive patch of the plasma membrane electrically controls type IV pili-based motility apparatus [51] in such a manner that pili close to the light focal spot are inactivated, whereas pili on the opposite side of the cell (facing the light source) are active and allow movement towards the light source [46–49]. As cyanobacteria evolved more than three billion years ago, it is obvious that this ancient prokaryotic vision based on the type IV pili complex is a very successful solution to their environmental challenges [52,53].

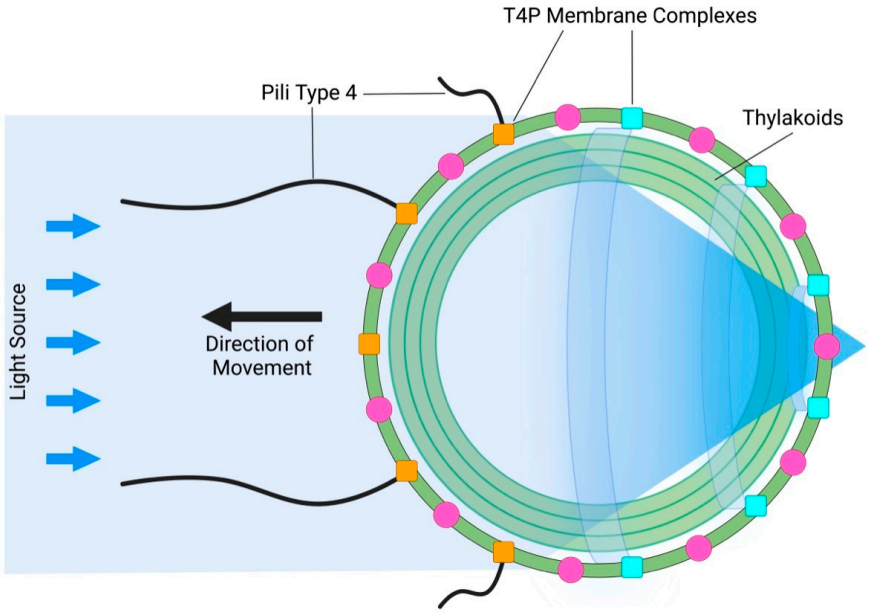

Figure 3. Bacterial Vision: Cyanobacterium Synechocystis. The whole cyanobacterial cell acts as a lens, focusing light beams on a small patch of the plasma membrane which controls the type-IV pili-based motility apparatus anchored in the plasma membrane via T4P complexes. Under the plasma membrane are thylakoid membranes. This model was adapted according to [49].

5. Plant Vision: *Boquila trifoliata*

Another example of an organism that can change its structures is the interesting plant *Boquila trifoliolata*, which can change its original three-lobed leaf shape into longitudinal leaves or any other shape, depending on the host plant next to its leaves. This is what the experiment by White & Yamashita (2021) illustrated [54]. The Boquila leaves were placed next to plastic leaves of non-living host plant, and the surprising result was that the Boquila mimicked the plastic plant leaves by changing leaf shape to a longitudinal shape, mimicking the plastic leaves of the non-living model plant. This experiment refutes two hypotheses proposed by other researchers. The first hypothesis was that horizontal gene transfer is mediated by the airborne microbes involved, thus allowing the Boquila to modify its leaves according to the leaves of the host plant. The second hypothesis was that

the Boquila modified its leaves following some volatile chemical signals released by the host plant. As the plastic leaves of non-living host plants were able to induce mimicking response in the Boquila, the hypothesis of horizontal gene transfer and the hypothesis of volatile substances can be dismissed. The plastic leaves might release some volatile substance under sunlight exposure, but these are biologically not relevant. This is very strong support for the proposal that plant-specific vision based on leaf ocelli is behind the mimicking responses of Boquila plants. This would also explain that the Boquila leaves can actively identify their surrounding environment, and modify not only leaf sizes and forms, but also color, leaf vein networks and other anatomical patterns. Future experimental research is needed to understand how all this can be accomplished.

6. Root Apex Vision: Root Skototropism

Although all roots of plants growing out in the nature are underground in darkness, they express all photoreceptors at their root apices [55]. While a dim light is not stressful for roots, they try to escape from stronger lights, which represent a stress factor for roots [56–58]. In order to avoid the direct illumination of roots in young seedlings grown in laboratory conditions using transparent Petri dishes, we have proposed the use of partially darkened dishes which allow us to keep roots in darkness [59–61]. Alternatively, the D-Root system was established as an alternative method to maintain roots in the shaded environment [62–64]. Surprisingly, roots grew even faster when grown within the D-Root system and our analysis revealed that this was due to steep light–darkness gradient provided by the D-Root system, which roots evaluate as a potent growth stimulant [65,66]. The process of speeding up the root growth under the steep light–darkness gradient of the D-Root system is based on the TOR complex activity, as its specific inhibition blocked this light escape tropism of illuminated roots [66]. Interestingly, roots placed in the illuminated portion of the shaded Petri dishes could recognize the dark portions of dishes, even when placed up to 2 mm from the light/darkness border (Figure 4). This implies some kind of root apex vision in the root apex skototropism response. The root apex ocelli proposed for this root skototropism are based on the blue-light phot 1 photoreceptor [55]. In contrast to diffusely distributed leaf epidermis ocelli, the root apex ocelli are assembled locally [67,68] at the root apex transition zone [69,70]. This position is optimal for the root apex vision, guiding the root apex navigation towards darkness [71].

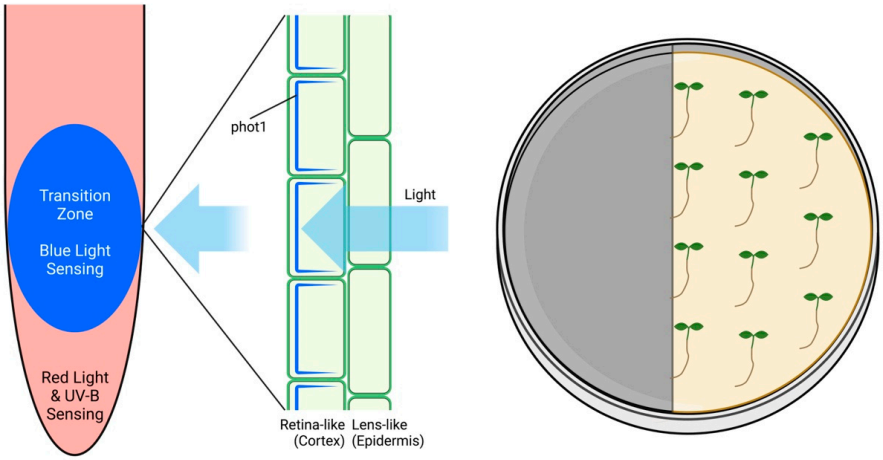

Figure 4. Root Apex Ocelli. Arabidopsis root apex expresses phot1 blue-light photoreceptor in cortex cells of the transition zone. The phot1 photoreceptors are arranged in the U-shape arrangements under the root epidermis cells which are devoid of phot 1 and are proposed to act as a lens cells, focusing the light on the underlying cortex cells. The root apex ocelli are proposed to allow root skototropism when roots grown within the illuminated portion of Petri dish can recognize the dark area and navigate the root growth towards it.

7. Conclusions and Perspectives

Vision via the whole organismal surface is known from some animals, such as cuttlefish and cephalopods [72,73]. Similarly, sea urchins and brittle stars have dispersed visual systems [74,75], all resembling the situation in plant leaf ocelli. Other lower animals have local eyes which resemble rather the root apex ocelli. Starfish have compound eyes at the arm tips [76,77]. Cnidarian medusae have eyes at the bases of their tentacles or on special sensory structures (rhopalia) which contain two lens-eyes flanked by two pairs of lens-less eyes [78]. Recent genetic studies have shown that the genes *Pax6*, *six1* and *six3* play key roles in the development of the eye in organisms from planaria to humans, arguing strongly for a monophyletic origin of the animal eye [79]. Nevertheless, there is no single regulatory gene in the formation of all animals. Diversity of vision in different animals must be based on gene expression as a tool and include the function of critical genes as mechanisms of the visual organ formation [79]. The hypothesis of phytochrome gene transfer from cyanobacteria, generating the first plastid in eukaryotes, paves the way for the presence of carotenoids in algae, which in turn are of extreme importance in eyespots [80]. Obviously, the leaf ocelli of plants conform well with algae and animal visual systems and represent obvious examples of convergent evolution. Root apex ocelli, based on the phot1 blue-light photoreceptor, represent another solution for the plant vision. It can be speculated that every cell with chloroplast has a cellular vision, resembling cells of cyanobacteria, algae, and plants. Albrecht-Buehler proposed 30 years ago that animal cells enjoy rudimentary vision [81–85] because they sense infrared wavelengths via their microtubules (Figure 5). This cellular vision is based on radial microtubules converging at their organizing centers (MTOCs), including centrosomes, basal bodies of cilia, and nuclear surfaces [86,87]. In future, it will be interesting to investigate the possible roles of microtubules in algal ocelloids and eyespots, as well as in plant leaves and root ocelli.

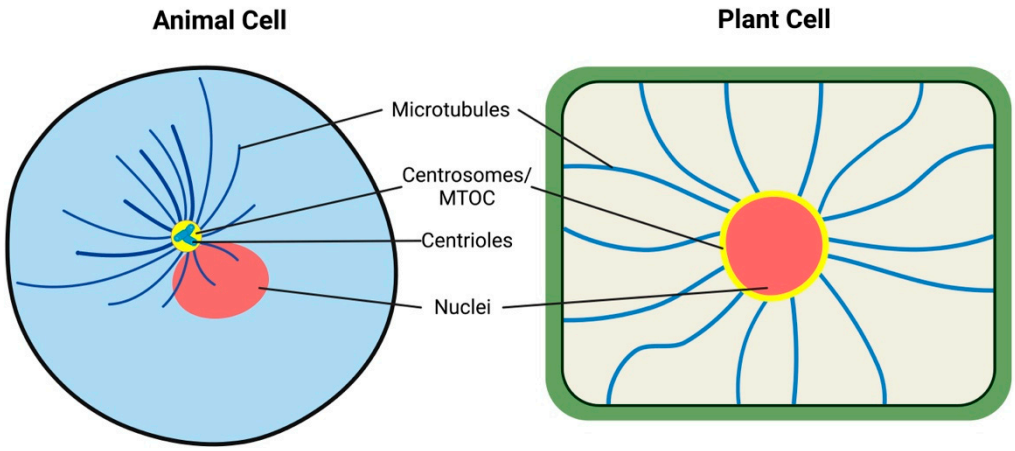

Figure 5. Microtubules-MTOC in Rudimentary Cell Vision of Eukaryotic Cells. Albrecht-Beuhler's rudimentary cellular vision is accomplished via microtubules conveying infrared wavelengths along microtubules towards the perinuclear centrosome of animal cells. In the plant cells, the centrosome is not corpuscular but is distributed diffusely along the whole nuclear surface.

In conclusion, it emerges that vision is an ancient sensory faculty which evolved some three billion years ago with the very first cyanobacteria. Evolution never discards successful innovations, and the algal and plant vision is based on that of chloroplasts too.

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14. Appendix 7

Chapter 7

"Root apex cognition: from neuronal molecules to root-fungal networks"

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Root Apex Cognition: From Neuronal Molecules to Root-Fungal Networks

František Baluška, Felipe Yamashita, and Stefano Mancuso

What we see is the blossom, which passes. The rhizome remains.
Jung (1963)

Abstract Plant roots are generally hidden from our sight, growing and living underground in alliances with symbiotic fungi. In order to find enough water and critical mineral nutrients, they explore large areas of soil with their root apices acting as plant cognition-based brain-like organs allowing them to use kin recognition, self/non-self recognition as well as swarm intelligence. Importantly, fungal hyphae integrate root systems into huge root-wide webs which allow not only the sharing of water and mineral nutrients, but also support long-distance chemical and electric signals. Roots use neuronal molecules such as glutamate and GABA supported by their specific receptors, as well as actin-based synapses and the plant-specific action potentials, to perform all their social activities and cognitive navigation for soil exploration.

1 Introduction

Plants conquered land in a tight co-evolution with symbiotic fungi, especially with the soil-borne members of the phylum Glomeromycota: arbuscular mycorrhiza (AM) fungi which teamed up with plant roots some 400 million years ago (Selosse and Le Tacon 1998; Redecker 2000; Selosse et al. 2015; Remy et al. 1994; Field et al. 2015; Hoysted et al. 2018). These so-called endomycorrhizal fungi were followed in evolutionary history by ectomycorrhizal (ECM) fungi, which grow as saprotrophs in soil and enter into mutualistic symbiosis with many trees by enveloping their root tips with mycelial mantles (Bonfante and Genre 2010; Genre et al. 2020). Whereas

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hyphae of the AM fungi enter root cells and form intracellular arbuscules, hyphae of the ECM fungi remain outside of root apex cells, forming Hartig nets and mantles surrounding the root apices (see Fig. 1 in Bonfante and Genre 2010; Genre et al. 2020). A unique feature of AM fungi is that the hyphae of their extraradical mycelium typically interconnect several root apices not only of the same plant, but also different plants of different species, forming 'common mycorrhizal networks' also known as the 'wood-wide-web' (Simard et al. 1997; Read 1997; Giovannetti et al. 2006; Beiler et al. 2010; Rog et al. 2020; Gorzelak et al. 2020). Besides plants specialized for either AM or ECM symbiosis, there are also so-called dual-symbiosis plants capable of associating their root apices with both the AM and ECM fungi (Brundrett and Tedersoo 2018; Teste et al. 2020).

2 Root Apex Transition Zone: Oscillatory Brain-Like Cognitive Organ in Soil Exploration

Evolution of roots in land plants was accomplished via root-fungal co-evolution when the first ancient plants succeeded in overcoming the difficult transition from sea to land (Taylor et al. 1995; Redecker 2000). This is obvious not only from paleontological records but also from the root-fungal symbiosis found in the earliest plant lineages of evolutionary ancient plants including Lycophytes, Liverworts and Hornworts (Rimington et al. 2020). Although it is generally accepted that the roots of vascular plants evolved later than their shoots (Raven and Edwards 2001), the lower capacity of roots to fossilize make this scenario less stringent. Furthermore, several extant plants lacking roots lost them secondarily, making it difficult to properly evaluate fossil plants lacking roots as this may also be the derived condition (Raven and Edwards 2001). Regardless, it is clear that the evolution of roots was accomplished in a stepwise manner with numerous progressive changes culminating in the generation of complex root systems found among contemporary flowering plants (Kenrick and Strullu-Derrien 2014; Hetherington and Dolan 2017, 2018; Hetherington et al. 2016; Fujinami et al. 2020).

In 1880, Charles Darwin suggested that the root apex acts as a brain-like organ, '*…brain being seated within the anterior end of the body, receiving impressions from the sense-organs, and directing the several movements*' (Darwin 1880; Baluška et al. 2006a, 2009a; Barlow 2006). This surprising claim received severe criticism from Julius Sachs, an influential contemporary botanist who accused Charles Darwin and his son Francis of performing flawed experiments in their country house (Heslop-Harrison 1980; de Chadarevian 1996; Ayres 2008). This dispute was a crucial crossroads in plant science, which was won by Julius Sachs not with scientific arguments but rather using his scientific political influence as leading figure in the field of plant physiology at that time. He asked his technical assistant Emil Detlefsen to repeat the experiments involving the surgical removal of maize root caps (originally reported by Ciesielski 1872) but he was not able to repeat this rather simple

experiment properly (Detlefsen 1881), even though he was a skilled assistant of Sachs. However, strong support in favour of Sachs also came from Julius Wiesner, professor of plant anatomy and physiology at the University of Vienna (Wiesner 1881, 1884a, b). Now we can only speculate what would have been the outcome for plant science if Julius Sachs and Julius Wiesner would have accepted that even experiments performed in a country house can produce good results. Later, Francis Darwin and Wilhelm Pfeffer published data confirming that maize roots, with the caps cleanly removed, are well-suited for experiments and that the allegedly flawed Down House root experiments outcompeted the laboratory experiments of Sachs and Detlefsen (Krabbe 1883; Heslop-Harrison 1980; de Chadarevian 1996; Ayres 2008; Kutschera and Briggs 2009). Currently, the removal of maize root caps is accepted methodology and removed root caps regenerate completely within 30–40 h (Juniper et al. 1966; Barlow 1974; Barlow and Sargent 1978; Barlow and Hines 1982; Bennet et al. 1985; Iijima et al. 2003; Feldman 1976). The roots of dicot plants such as pea and arabidopsis are also capable of root cap regeneration (Barlow and Hines 1982; Sena et al. 2009; Efroni et al. 2016). For example, when plant regeneration is accomplished using callus tissue then it occurs via root development pathways (Sugimoto et al. 2010, 2011).

In 1997, we succeeded at immunofluorescence labelling of F-actin cytoskeletons in the intact root apices of maize (Baluška et al. 1997a), the same model structure which caused the severe dispute between Sachs and Darwins in 1880. This was the first time the actin cytoskeleton was visualized not in protoplasts or isolated plant cells, but in cells organized intact within tissues of the root apex. Abundant F-actin meshworks were found to be associated with the non-growing end-poles/cross walls of the transition zone cells (Baluška et al. 1997a, 2000, 2003a). In 2003, we outlined the plant synapse concept for the first time (Baluška et al. 2003b, 2005). Our data showed that this F-actin-based recycling of vesicles, including cell wall components, especially pectins, allows for effective cell–cell communication in the root apex (Baluška et al. 2002, 2003a, b, 2005, 2009b). Later studies revealed that this endocytic vesicle recycling is linked with the polar auxin transport accomplished via PIN-based export of auxin out of cells in root apices (Šamaj et al. 2004; Mancuso et al. 2005; Baluška et al. 2009b, McLamore et al. 2010). The same situation was found also for the transition zone in *Arabidopsis thaliana* roots (Verbelen et al. 2006; Schlicht et al. 2006; Mancuso et al. 2007; Dhonukshe et al. 2009; Mettbach et al. 2017). Later it emerged that this is part of the actin-auxin oscillator that drives polar trans-cellular transport of auxin through plant tissues (Holweg 2007; Nick 2007; Nick et al. 2009; Baluška and Mancuso 2013a, b, c).

There are several critical features suggesting that the root apex transition zone represents the root *brain* as proposed by Charles and Francis Darwin in 1880 (Darwin 1880; Baluška et al. 2006a, 2009a). First of all, cells in this developmentally unique zone are not distracted by any obvious tasks. They are neither dividing nor rapidly elongating, which allows them to focus on sensory integration tasks. They are located in very close proximity to phloem unloading sites which means that they are flooded with abundant levels of sucrose (Complainville et al. 2003; Ross-Elliott et al. 2017). This is associated with high activities of cell wall invertase, an enzyme which cleaves

sucrose to hexoses (Hellebust and Forward 1962; Giaquinta et al. 1983; Roitsch and Gonzales 2004). Moreover, a high level of apoplastic sucrose induces osmotic stress which is relieved via induction of the fluid-phase endocytosis in cells close to phloem unloading sites (Baluška et al. 2004d). Another way to relieve this stress due to high sucrose levels is to synthesize large starch grains within the amyloplasts of the root apex transition zone cells (Fig. 6 in Baluška et al. 1993a and Fig. 2 in Baluška et al. 1993b).

This exceptional status of the transition zone cells allows them to focus mainly on cognitive tasks, resembling the situation of neurons of the central nervous system (CNS) seated within animal brains. Moreover, similar to CNS neurons, cells in the root apex transition zone also require greater levels of nutrient resources and oxygen (Baluška and Mancuso 2013a, b, c) in order to produce the ATP molecules necessary to drive the energetically demanding endocytic vesicle recycling and to support abundant and synchronized electrical spiking activities (Masi et al. 2009, 2015). This view is supported by a study reporting high cytosolic phosphate (Pi) concentrations in the transition zone for both epidermal and cortical cells of *Arabidopsis thaliana* root apices (Sahu et al. 2020). Pi is critical for ATP synthesis in mitochondria and for the synthesis of membrane phospholipids. In roots facing low levels of Pi in their environment, root caps act as the sensing organ which promptly stops root growth under Pi deficiency (Svistoonoff et al. 2007; Kanno et al. 2016). In this sensory circuit, the STOP1 transcription factor and ALMT1 anion/GABA (Ramesh et al. 2015, 2017, 2018; Žárský 2015; Kamran et al. 2020) act together to stop root growth (Abel 2017; Balzergue et al. 2017; Godon et al. 2019). ALMT1 also acts as a GABA receptor when, as in animal and human neurons, GABA lowers excitability of the plasma membrane (Žárský 2015).

There are intriguing similarities between animal brains and plant root apex *brains*: both enjoy uniquely protected as well as privileged locations within animal and plant bodies. Animal brains are protected mechanically within the skull, provided preferentially with nutrition and oxygen. Animal brains are free to perform only activities relevant to the control of cognitive behaviour of animals. Similarly, the Darwinian root-apex *brains* are positioned between the dividing cells of the root apical meristem and rapidly elongating cells pushing the whole root apex forward. In both maize and arabidopsis root apices, the size of the transition zone is similar to the size of the apical meristem, and unloading phloem elements define the basal border of the transition zone (Baluška et al. 1990, 1996a, 2001a, b; Verbelen et al. 2006). Finally, the brain is the only animal organ which is not in direct contact with blood. In fact, blood is toxic to neurons, and the blood–brain-barrier (BBB) effectively prevents direct contact of brain neurons with blood (Hagan and Ben-Zvi 2015; Righy et al. 2016; Abdullahi et al. 2018; Madangarli et al. 2019; Nian et al. 2020; Segara et al. 2021). Intriguingly, the etymological origin of the term neuron comes from the ancient Greek, meaning ‘vegetal fibre’ (Brenner et al. 2006; Mehta et al. 2020). More importantly, the allegedly unique features of neurons, formulated and popularized as the ‘Neuron Doctrine’ by Wilhelm Waldeyer in 1891 (Shepherd 1991; Jones 1994), are no longer considered to be so unique (Gold and Stoljar 1999; Guillery 2007).

Rather surprisingly, many so-called neuronal features are present in plant cells, especially in the transition zone of root apices (Baluška 2010). Recent advances in plant cell biology have revealed that plant cells, especially those located in the root apex transition zone, show almost all of the features which were defined, according the 'Neuron Doctrine', to be neuron-specific (Baluška 2010; Baluška et al. 2005, 2009a, b; Masi et al. 2009). As noted by Rainer Stahlberg, nerves in animals and vascular bundles in plants share analogous functions of conducting rapid electric signals (Stahlberg 2006a, b). Similar analogies to the cellular basis of plants and animals resulted in the acceptance of the Cell Theory. Therefore, it is puzzling that plant electrophysiology is considered to be esoteric (Alpi et al. 2007; Taiz et al. 2019). The most significant differences between plant and animal cells are associated with their different extracellular matrices, and their interactions with the plasma membrane and elements of cytoskeletal polymers (Reuzeau and Pont-Lezica 1995; Baluška et al. 2003b, Seymour et al. 2004; Halbleib and Nelson 2006; Campbell and Humphries 2011). For example, sodium is the major ion driving action potentials in animals but it is toxic for plants with pectinic cell walls (Feng et al. 2018; Verger and Hamant 2018), which rely instead on calcium fluxes (Hope 1961; Beilby and Coster 1979; Beilby and Al Khazaaly 2016; Hedrich and Neher 2018; Iosip et al. 2020). While plant cell walls pose additional problems for the excitability of plant cells and tissues, they also provide them with additional layers of signalling complexity (Baluška et al. 2003b; Ringli 2010; Wolf et al. 2012; Wolf 2017). Our discovery that cell wall molecules, such as calcium, boron cross-linked pectins and xyloglucans, are actively recycled from cell walls via endosomal vesicles (Baluška et al. 2002, 2009a, b; Dhonukshe et al. 2009) is crucial for our conceptual advancement of plant-specific synapses in the root apex transition zone.

3 Neuronal Molecules Relevant for Root Apex Cognitive Navigation and Soil Exploration

Plant root apices are supported via numerous molecules which were originally characterized as neuronal molecules. Among these, we will briefly discuss glutamate and GABA with their receptors, which control the electrical properties of the plasma membrane. Importantly, in both neurons as well as in plant cells, glutamate stimulates and GABA inhibits excitability of the plant plasma membrane. Although there are some differences in their receptors, especially with respect to GABA (Ramesh et al. 2015, 2017; Žárský 2015), the electrophysiological impacts on plasma membrane potentials and excitability are very similar. The same is true for another neurotransmitter, glutamate, in that the glutamate receptors of plants are very similar to those of animal brains (Weiland et al. 2016; Wudick et al. 2018; Qiu et al. 2020).

Evolutionary analysis even suggests that plant glutamate receptors might predate the animal glutamate receptors of the NMDA class which have a central role in the control of the brain's synaptic plasticity (Stroebel and Paoletti 2020). Importantly,

both glutamate and GABA shape action potentials (APs) in plants, partially through their control of voltage-gated potassium channels (Cuin et al. 2018; Adem et al. 2020; Koselski et al. 2020). Similar to the neuronal APs in humans and animals, plant-specific APs are also blocked by diverse anesthetics and this prevents the movements of plant organs (Yokawa et al. 2018, 2019; Pavlovič et al. 2020; Baluška and Yokawa 2021).

4 Synaptic Principles Relevant for Root Apex Cognitive Navigation

Root apex cells located in the transition zone are unique with respect to their cytoarchitecture, endocytic vesicle trafficking, arrangement of actin cytoskeleton elements, polar transport of auxin, and bioelectric activities of their plasma membranes. In 1987, we discovered that the actin cytoskeleton is organized via unique bundles of F-actin anchored at the cellular end poles (cross-walls) which are densely populated with plasmodesmata (Baluška et al. 2000, 2003a, b; Baluška and Hlavacka 2005). Later, the plant-specific myosin VIII was discovered in plants and was also localized abundantly to these cross-walls (Reichelt et al. 1999). It emerged that myosin VIII supports plasmodesmata structure and function, anchoring the F-actin cables at the cross-walls, and driving endocytosis and endocytic vesicle recycling (Baluška et al. 2000; Volkmann et al. 2003; Baluška and Hlavacka 2005; Golomb et al. 2008; Sattarzadeh et al. 2008; Haraguchi et al. 2014). Importantly, myosin VIII-based end-poles of cells in the transition zone assemble cell–cell adhesion domains which fulfil several synaptic criteria and support the brain-like status of the root apex transition zone (Baluška et al. 2005, 2009a, b; Baluška and Mancuso 2013a, b, c). Auxin emerges as acting not only as a plant hormone but also as a plant-specific neutrotransmitter-like molecule which is integrating sensory inputs into the context of root tropism outputs (Baluška et al. 2005, 2008, 2009a, b; Baluška and Mancuso 2013a, b, c; Schlicht et al. 2006; Baluška et al. 2008). Interestingly, the root apex transition zone acts as the specific target of aluminium toxicity (Sivaguru and Horst 1998; Kollmeier et al. 2000; Sivaguru et al. 1999, 2000, 2003a; Illés et al. 2006; Yang et al. 2014; Li et al. 2018). The central role of aluminium toxicity in the transition zone is especially relevant for the basipetal (shootward) flow of auxin driven via the PIN2 auxin efflux transporter (Kollmeier et al. 2000; Shen et al. 2008; Yang et al. 2014; Wu et al. 2014, 2015), and is mediated by the activity of plant glutamate receptors (Sivaguru et al. 2003b).

5 Transition Zone Energides in the Driver's Seat to Control Root Apex Navigation

One of the most prominent features of cells in the root apex transition zone is the fact that the nucleus is centralized and suspended in dynamic cytoplasmic strands organized by cytoskeletal polymers (Baluška et al. 1990, 1997a, 2000, 2001a, b, 2003a, 2006b, 2010). Whereas the F-actin bundles are organized conically between cellular end-poles and are the most prominent structure, the dense F-actin baskets that suspend the centrally positioned nuclei and perinuclear radiating microtubules are also important for the integral roles of these cells in sensory signal perception and integration, resulting in adaptive root tropisms (Baluška et al. 2004a, 2006a, b, 2009a, b, 2010; Baluška and Mancuso 2013a, b, c). The current version of the Cell Theory is facing skepticism due to the existence of multinuclear coenocytic (cell division not followed by cytokinesis) and syncytia (fusion of cells) cellular assemblies. In fact, almost all plant cells have free cytoplasmic channels known as plasmodesmata. We have extended and fully developed the Cell Body concept which was originally proposed by Daniel Mazia in 1993, and correlates well with the Energide concept of Julius Sachs from 1891 (Baluška and Barlow 1993; Baluška et al. 1997b, 1998, 2001b, 2004b, c, 2006a, b). The Energide-Cell Body is the smallest unit of cellular life originating from still unknown ancient and centrin-based archaea with microtubular flagella (Baluška and Lyons 2018, 2021). It is hypothesized that the cytoplasmic strands, supported by vibrating and oscillating F-actin cables and microtubules (Tuszyński et al. 2004; Cifra et al. 2010; Kučera and Havelka 2012), are transmitting sensory signals received at the plasma membrane to the central nuclei (Matzke et al. 2019). Similar neuronal synapse—nucleus communication is involved in the formation and maintenance of neuronal circuits (Saha and Dudek 2008; Cohen and Greenberg 2008). Action potentials seem to have originated from the repair of damaged plasma membranes of ancient cells and contributed to preservation and homeostasis of plasma membrane and cellular integrity (Goldsworthy 1983; Steinhardt et al. 1994; Brunet and Arendt 2016; Baluška and Mancuso 2019).

6 Changing Metaphor for Transition Zone Energide: From 'Bug in Cage' to 'Spider in Web'

In 2004, we proposed the metaphor *Bug in Cage* for the Cell Body/Energide enclosed by the plasma membrane and cytoplasm (Baluška et al. 2004b). The idea behind this metaphor was that the symbiotic evolutionary origin of the Cell Body/Energide implies its semi-autonomous nature and biological agency behind its organization and behaviour (Baluška et al. 1997b, 1998; Baluška and Lyons 2018, 2021). The Cell Body/Energides in the root apex transition zone cells are acting as navigators of root apices (Fig. 1, Baluška and Mancuso 2018) in their search for water and critical mineral nutrients and avoidance of toxic soil patches. They can act as kind of

sensitive radar for both acoustic and chemical cues (Falik et al. 2005; Schenk 2006; Gagliano et al. 2012a, b; Yokawa et al. 2014; Rodrigo-Moreno et al. 2017).

Our proposal here is that the Nuclei/Energides suspended within the cytoskeleton-supported cytoplasmic strands (Fig. 1a, b) of the root apex transition zone are perfectly suited to control the root apex navigation *akin* to navigators seated in the driver's seat (Fig. 1c). As the F-actin cables enclosing the nuclei are anchored at the root synapses (Baluška et al. 1997a, 2000, 2005, 2009b; Baluška and Hlavacka 2005), the Nuclei/Energide are optimally placed to navigate root apex trajectories. The most effective means to control root tropisms is to manipulate the onset of rapid cell elongation in a coordinated fashion across the root epidermis and cortex (Fig. 2). In the maize root apex, there are hundreds of cells located at the basal limit of the transition zone that are primed for rapid cell elongation. Their Energides give their

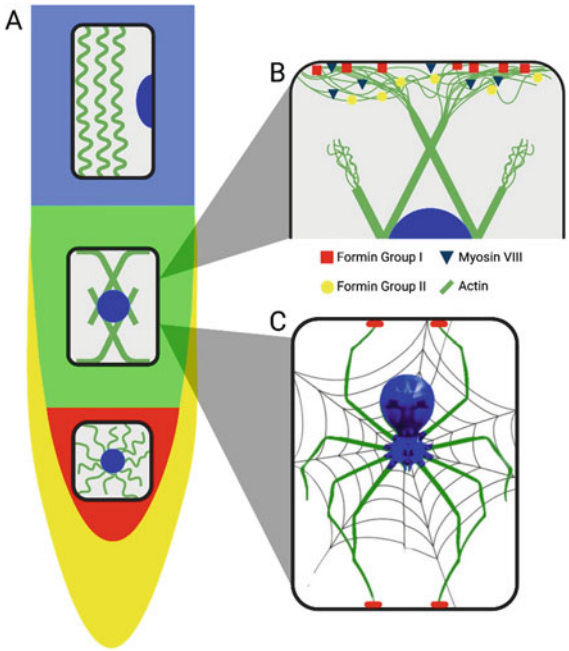

Fig. 1 Schematic Overview of the Root Apex Zones Relevant for Root Apex Navigation. **a** The root cap (yellow) encloses the apical meristem (red) and the transition zone (green). The zone of rapid cell elongation (blue) follows, which pushes all the other more apical zones forward. The nucleus (in blue) is enclosed by F-actin elements (in green) in the form of a meshwork (cells in meristem) or conical bundles anchored at the synaptic end poles (cells in transition zone). In cells of the rapid cell elongation zone, the nucleus is pushed to the cell periphery by the large central vacuole and relaxed F-actin bundles are organized longitudinally. **b** Detail of the two conical F-actin bundles organized at the synaptic cell periphery by actin-binding formins and myosin VIII. **c** Hypothetic scenario of root apex navigation via the transition zone Cell Bodies/Energides, depicted metaphorically in the form of a spider-in-web. For more details, see Baluška and Hlavacka 2005; Baluška and Mancuso 2013a, 2013b, 2013c, 2018; Baluška and Lyons 2018, 2021)

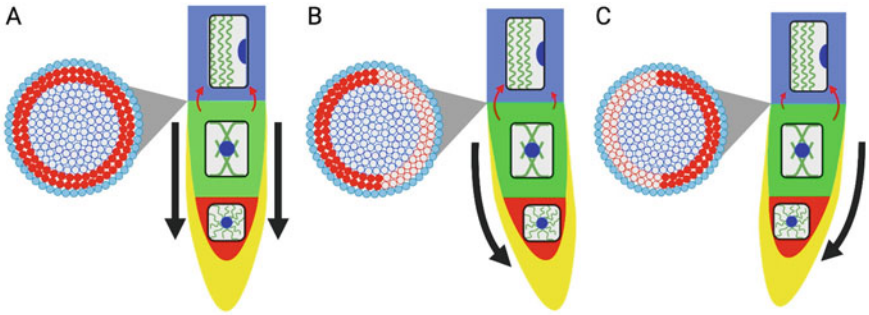

Fig. 2 Smart Border at the Basal Limit of the Transition Zone. The transition zone Cell Bodies/Energides control root apex navigation through their contacts at the synaptic end-poles of cells at the basal limit of the transition zone. This translates sensory perceptions into motoric root apex tropisms at this smart border between the basal limit of the transition zone. **a** If there is no relevant cue registered by the Cell Body/Energide, then all the transition zone cells are released into the rapid cell elongation zone in a coordinated fashion. **b, c** Differential release of cells from the transition zone into the rapid cell elongation zone allows root tropisms which are finely-tuned by relevant cues. The most critical cells for root apex tropisms are PIN2 expressing cells (shown as red circles) at the root periphery. **b** Repelling cues slow-down (small red arrow) the release of PIN2 cells (unfilled red circles in the root cross-section view) from the transition zone (green) into the region of rapid cell elongation (blue) at the opposite side of the root apex periphery. Attracting cues speed-up (large red arrow in the root cross-section view) the release of PIN2 cells (filled red circles) from the transition zone (green) into the region of rapid cell elongation (blue) at the opposite side of the root periphery

'yes' for the burst-like onset of the rapid cell elongation (Fig. 2) which is under the control of auxin, calcium, ethylene and actin-myosin forces (Baluška et al. 1993a, b, 1996a, 1997a, 2000, 2001a, b). On the other hand, microtubules are not involved in this developmental switch as maize root tropisms are completed with all microtubules depolymerized (Baluška et al. 1996b). In some way, the active Energides of the transition zone cells resemble spiders sitting within their webs (Fig. 1c), feeling web vibrations to inform them of the presence of prey, as well as of other relevant cues from their environment (Mortimer 2019; Mortimer et al. 2019). This sensitive cytoarchitecture would explain the surprising ability of growing roots to respond to specific acoustic signals via positive root phonotropism (Rodrigo-Moreno et al. 2017) or to recognize barriers from distance (Falik et al. 2005; Schenk 2006).

How could the Energide sense relevant sensory signals and integrate this information to control root cell elongation? Here the 'Plasma Membrane Control Centers' (Pickard and Ding 1993; Pickard 1994, 2013; Gens et al. 2000) and the 'Hechtian Growth Oscillator' (Lamport et al. 2014, 2018, 2020) concepts are relevant. For the root apex, important cues are water and critical minerals which, when perceived, are associated with changes in tension and vibrations of the cytoplasmic strands (Fig. 1). The contact of F-actin and myosin VIII with the critical plasma membrane domains can control the ion fluxes across the plasma membrane. Interestingly, the conical bundles of F-actin that enclose nuclei are straight and thick, as if under tension, in

the transition zone; in contrast, they instantly appear thin and wrinkled as root cells initiate their rapid cell elongation (Baluška et al. 1997a, 2000; Baluška and Hlavacka 2005).

Such sensitive and vibrating networks could allow effective perceptions from the root apex rhizosphere, including possible sound waves bouncing back from soil portions ahead of the growing root apices. For example, maize root apex generates sound waves in regular frequencies (Gagliano et al. 2012a, b). Analysis of growing roots of arabidopsis revealed that they are attracted by sound waves of 200 Hz which are close to the sound waves generated by streams of water (Rodrigo-Moreno et al. 2017). This root phonotropism can be expected to be useful for roots in their search for water (Rodrigo-Moreno et al. 2017; Fromm 2019). Acoustic root navigation, resembling bat echolocation, would also allow recognition of physical barriers in advance (Falik et al. 2005; Schenk 2006).

7 Evolution of the Root Apex Brain: From Ancient Roots Towards Complex Root Systems

In early root evolution, some 400 million years ago, ancient roots teamed-up with symbiotic AM fungi and have tightly co-evolved ever since (Pirozynski and Malloch 1975; Selosse and Le Tacon 1998; Selosse et al. 2015). Moreover, roots also attract specific bacteria which help roots to cope with diverse stresses. In order to control their rhizosphere, roots release large amounts of exudates and diverse infochemicals (Baluška and Mancuso 2020, 2021). These substances help them not only to develop the surrounding soil as their living niche but also to enjoy complex social lives with the roots of neighbouring plants (Baluška and Mancuso 2020, 2021). Roots are territorial (Schenk 2006; Novoplansky 2019). They discriminate self—non/self roots and apply the kin recognition (Bais 2018; Novoplansky 2019) in their behaviour (Baluška and Mancuso 2021). The root apex transition zone plays a central role in this social aspect of root life. Auxin transport via neurotransmitter-like modes based on synaptic-like vesicle recycling is critical aspect of root behaviour. In the evolution of roots, the auxin-transporting synapses (Baluška et al. 2005, 2008, 2009b) have been proposed to evolve from the ancient symbiotic synapses (Baluška et al. 2005; Kwon et al. 2008; Lima et al. 2009; Baluška and Mancuso 2013c).

Plants compete for light, water and mineral nutrients (Craine and Dybzinski 2013). In shoots, the shade avoidance syndrome is behind the light competition between neighbour plants (Smith and Whitelam 2007, Keuskamp et al. 2010; Martínez-García et al. 2010, 2014). In plant roots, fierce competition for water and critical minerals shapes root behaviour (Gersani et al. 2001; Schenk 2006; McNickle et al. 2009; Farrior 2019). Root apices apply their plant-specific perception, cognition and intelligence in order to succeed in their difficult task of finding sufficient water and mineral nutrients (Hodge 2009; Barlow 2010a, b; Gruntman et al. 2017; Baluška and Mancuso 2018; Fromm 2019; Novoplansky 2019; Parise et al. 2020). In plant evolution, roots

evolved from structurally and cognitively simple rhizoids up to the complex root systems of contemporary flowering plants which enjoy complex foraging behaviour. Plants use their root systems for plant-plant communication of sensory and stress cues (Falik et al. 2012; Elhakeem et al. 2018; Novoplansky 2019; Volkov and Shtessel 2020; Yamashita et al. 2021).

8 Root-Fungal Networks Control Underground Supracellular Life

Plant root evolution started with the earliest colonization of barren land with help from symbiotic AF fungi some 400 billions of years ago (Pirozynski and Malloch 1975; Remy et al. 1994; Heckman et al. 2001; Schüßler and Walker 2011; Feijen et al. 2018). Roots are hidden underground in the soil, leading to the prevailing view of plants as simply green organisms which flower when mature. As an example, the value of the largest living organism on Earth, the giant sequoia tree, is generally based on its shoot parts, while its root parts are ignored. However, the true nature of plants and trees is based on the fact that their roots are structurally and functionally connected through fungal hyphae networks. In some sense, these networks are analogous to our human invention of the internet because the latest advances suggest that they serve not only for exchange of nutrients and water, but also for chemical and electrical long-distance signaling (Simard et al. 1997; Song et al. 2010; Barto et al. 2012; Gorzelak et al. 2015, 2020; Sasse et al. 2018; Simard 2018; Volkov et al. 2019; Volkov and Shtessel 2020). Obviously, the true nature of plants is hidden underground, which would explain why plants are generally considered to be devoid of agency, cognition, and intelligence. The aboveground parts of plants, visible to us, are just support organs specialized for photosynthesis and sexual reproduction (Baluška and Mancuso 2021). Roots demonstrate kin recognition, self/non-self recognition and swarm intelligence (Baluška et al. 2010; Ciszak et al. 2012; Baluška and Mancuso 2018, 2020, 2021). They invest their carbon-based photosynthetic substances to control the rhizosphere microbiota communities and soil as a life-friendly biotop (Barlow 2010a, b; Barlow and Fisahn 2013; Novoplansky 2019; Baluška and Mancuso 2020, 2021). Future experimental studies will focus on the ecological, cognitive and electrophysiological aspects of the root-wide-web (Simard et al. 1997; Lee et al. 2013; Simard 2018; Giovannetti et al. 2006; Fukasawa et al. 2020; Volkov et al. 2019; Volkov and Shtessel 2020; Kokkoris et al. 2021) spanning large areas of the Earth surface. Unfortunately, these intact forest areas are shrinking and this has serious consequences for the life-friendly climate (Baluška and Mancuso 2020).

Circadian clocks have emerged as critical players in decoding sensory information obtained from the environment (Hearn and Webb 2020; Koronowski and Sassone-Corsi 2021), which is crucial for cognitive aspects of all organisms. With respect to plants, which live both above-ground (shoots) and below-ground (roots), the situation is unique (Baluška and Mancuso 2018, 2021; Lee et al. 2019). Although the shoot

clock was proposed to be the primary plant clock and the root clock is viewed as a simplified slave-like version of the shoot clock (James et al. 2008), recent studies revealed that the root clock coupling strength is extraordinary especially in the root apex (Gould et al. 2018; Maric and Mas 2020). Light can reach the root apices via internal tissues down to under-ground roots (Mandoli and Briggs 1984; Lee et al. 2016). This then allows them direct light-mediated entrainment of the root clock (Nimmo 2018; McClung 2018). As the AM fungi have their own circadian clocks (Lee et al. 2018, 2019), it can be expected that the huge symbiotic root—AM fungi networks are integrated via their supra-organismal circadial clocks (Lee et al. 2019). Similar trans-kingdom clocks are found in animals and humans (Thaiss et al. 2014; Page 2019). We can look forward to future studies in this newly emerging field of supra-organismal chronobiology.

9 Conclusions and Gaian Outlook

Land plants are decisive organisms with respect to the Earth's climate ever since they evolved from rather simple and small predecessors living in seas. The first terrestrial plants cooled the Ordovician Earth (Lenton et al. 2012). Their roots, in co-operation with symbiotic AM fungi, generated soil as a central habitat for terrestrial ecosystems (Rillig and Mummey 2006; van der Heijden et al. 2008). Ever since then, land plants have been integral in establishing and maintaining the climate of the Earth (Beerling 2019). Tree root systems are integrated and networked with the symbiotic fungal hyphae into huge super-organismal phenomenon known as wood-wide-web (Simard et al. 1997; Helgason et al. 1998; Giovannetti et al. 2006; Simard 2021). This wood-wide-web participates in homeostatic processes (Power et al. 2015) also known as the Gaia hypothesis proposed by James Lovelock in 1972 (Lovelock 1972, 1979, 2019: Lenton and van Oijeb 2002; Lenton and Latour 2018, Lenton et al. 2018). In this respect, although this seems to be counter-intuitive, plants are socially and cognitively active mostly underground as only roots, but not shoots, can enter into the long-lasting symbiotic interactions (Baluška and Mancuso 2018, 2020). There are examples of plants and even trees (Henschel and Seely 2000; Maurin et al. 2014) that live underground, and numerous myco-heterotrophic plants that are not green at all, obtaining all their food from fungal partners (Bidartondo 2005; Merckx et al. 2009). It is possible that future studies will reveal even more surprising connections between roots, fungal hyphae and microbial populations which control the terrestrial ecosystems and the Earth's climate. If we would like to solve the current climatic crisis and better understand the Earth's ecosystems, we should focus more on the underground life which is dominated by plant roots and their AM fungal partners. Here is where the key to our future life on the planet Earth is hidden.

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