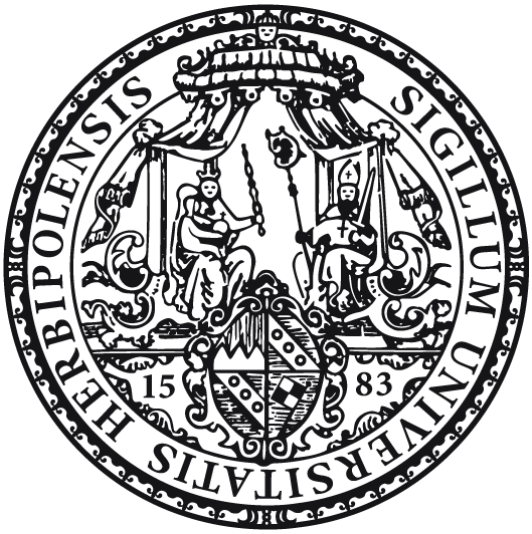
The Venus flytrap

Role of oxylipins in trap performance of *Dionaea muscipula*



Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Julius-Maximilians-Universität Würzburg

vorgelegt von

Martin Frederik von Rüden

aus Ehringshausen

Würzburg, 2022

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender: …….

Gutachter: Prof. Dr. Rainer Hedrich

Gutachter: Dr. Arthur Korte

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Remember to look up at the stars
and not down at your feet.

Stephen Hawking

Summary

A part of the plant kingdom consists of a variety of carnivorous plants. Some trap their prey using sticky leaves, others have pitfall traps where prey cannot escape once it has fallen inside. A rare trap type is the snap-trap: it appears only twice in the plant kingdom, in the genera *Aldrovanda* and *Dionaea*. Even Charles Darwin himself described *Dionaea muscipula*, the Venus flytrap, with the following words “This plant, commonly called Venus' fly-trap, from the rapidity and force of its movements, is one of the most wonderful in the world”. For a long time now, the mechanisms of *Dionaea*’s prey recognition, capture and utilization are of interest for scientists and have been studied intensively.

Dionaea presents itself with traps wide-open, ready to catch insects upon contact. For this, the insect has to touch the trigger hairs of the opened trap twice within about 20-30 seconds. Once the prey is trapped, the trap lobes close tight, forming a hermetically sealed “green stomach”.

Until lately, there was only limited knowledge about the molecular and hormonal mechanisms which lead to prey capture and excretion of digestive fluids. It is known that the digestion process is very water-consuming; therefore, the interplay of digestion-inducing and digestion-inhibiting substances was to be analyzed in this work, to elucidate the fine-tuning of the digestive pathway. Special attention was given to the impact of phytohormones on mRNA transcript levels of digestion-related proteins after various stimuli as well as their effect on *Dionaea’s* physiological responses.

Jasmonic acid (JA) and its isoleucine-conjugated form, JA-Ile, are an important signal in the jasmonate pathway. In the majority of non-carnivorous plants, jasmonates are critical for the defense against herbivory and pathogens. In *Dionaea*, this defense mechanism has been restructured towards offensive prey catching. One question in this work was how the frequency of trigger hair bendings is related to the formation of jasmonates and the induction of the digestion process. Upon contact of a prey with the trigger hairs in the inside of the trap, the trap closes and jasmonates are produced biosynthetically. JA-Ile interacts with the COI1-receptor, thereby activating the digestion pathway which leads to the secretion of digestive fluid and production of transporters needed to take up prey-derived nutrients. In this work it could be shown that the number of trigger hair bendings is positively correlated with the level and duration of transcriptional induction of several digestive enzymes/hydrolases.

Abscisic acid (ABA) acts, along with many other functions, as the plant “drought stress hormone”. It is synthesized either by roots as the primary sensor for water shortage or by guard cells in the leaves. ABA affects a network of several thousand genes whose regulation prepares the plant for drought and initiates protective measurements. It was known from previous work that the application of ABA for 48 hours increased the required amount of trigger hair bendings to achieve trap closure. As the digestion process is very water-intensive, the question arose how exactly the interplay between the jasmonate- and the ABA-pathway

is organized, and if ABA could stop the running digestion process once it had been activated. In the present work it could be shown that the application of ABA on intact traps prior to mechanically stimulating the trigger hairs (mechanostimulation) already significantly reduced the transcription of digestive enzymes for an incubation time as short as 4 h, showing that already short-term exposure to ABA counteracts the effects of jasmonates when it comes to initiating the digestion process, but does not inhibit trap closure. Incubation for 24 and 48 hours with 100 µM active ABA had no effect on trap reopening, only very high levels of 200 µM of active ABA inhibited trap reopening but also led to tissue necrosis. As the application of ABA could reduce the transcription of digestive hydrolases, it is likely that *Dionaea* can stop the digestion process, if corresponding external stimuli are received.

Another factor, which only emerged later, was the effect of the wounding-induced systemic jasmonate burst. As efficient as ABA was in inhibiting marker hydrolase expression after mechanostimulation in intact plants, the application of ABA on truncated traps was not able to inhibit mechanostimulation-induced marker hydrolase expression. One reason might be that the ABA-signal is perceived in the roots, and therefore truncated traps were not able to react to it. Another reason might be that the wounding desensitized the tissue for the ABA-signal. Further research is required at this point.

Inhibitors of the jasmonate pathway were also used to assess their effect on the regulation of *Dionaea´s* hunting cycle. Coronatine-O-methyloxime proved to be a potent inhibitor of mechanostimulation-induced expression of digestive enzymes, thus confirming the key regulatory role of jasmonates for *Dionaea´*s prey consumption mechanism.

In a parallel project, the generation of in vitro cultures from sterilized seeds and single plant parts proved successful, which may be important for stock-keeping of future transgenic lines. Protoplasts were generated from leaf blade tissue and transiently transformed, expressing the reporter protein YFP after 24 h of incubation. In the future this might be the starting point for the generation of transgenic lines or the functional testing of DNA constructs.

Zusammenfassung

Ein Teil des Pflanzenreiches besteht aus einer Vielfalt fleischfressender Pflanzen. Einige fangen ihre Beute mit klebrigen Blättern, andere haben Grubenfallen, aus denen die Beute nicht mehr entkommen kann, wenn sie erst einmal hineingefallen ist. Ein seltener Fallentyp ist die Klappfalle: Sie kommt im Pflanzenreich nur zweimal vor, in den Gattungen *Aldrovanda* und *Dionaea*. Charles Darwin selbst beschrieb *Dionaea muscipula*, die Venusfliegenfalle, als "eine der schönsten Pflanzen der Welt". Die Mechanismen der Erkennung, des Fangs und der Nutzbarmachung von Beutetieren durch *Dionaea* sind seit langem von Interesse für die Wissenschaft und wurden intensiv untersucht.

Dionaea hat weit geöffnete Fallen, die bei Kontakt Insekten fangen können. Dazu muss das Insekt innerhalb von ca. 20-30 Sekunden zweimal die Triggerhaare der geöffneten Falle berühren. Sobald die Beute gefangen ist, schließen sich die Fallenhälften fest und bilden einen hermetisch verschlossenen sogenannten „grünen Magen“.

Bis vor einigen Jahren gab es nur wenige Informationen über die molekularen und hormonellen Mechanismen, die zu Beutefang und Sekretion von Verdauungsflüssigkeiten führen. Es ist bekannt, dass der Verdauungsprozess sehr viel Wasser verbraucht; daher sollte in dieser Arbeit das Zusammenspiel von verdauungsauslösenden und verdauungshemmenden Substanzen untersucht werden, um die Feinabstimmung des Verdauungsweges aufzuklären. Ein besonderes Augenmerk wurde auf den Einfluss von Phytohormonen auf die mRNA-Transkriptzahlen von Verdauungsproteinen nach verschiedenen Stimuli sowie auf deren Auswirkungen auf die physiologischen Reaktionen von *Dionaea* gelegt.

Jasmonsäure (JA) und ihre mit Isoleucin konjugierte Form, JA-Ile, sind ein wichtiges Signal in pflanzlichen Signaltransduktionsprozessen. In der Mehrzahl der nicht-karnivoren Pflanzen sind Jasmonate entscheidend für die Abwehr von Herbivoren und Pathogenen. In *Dionaea* wurde dieser Abwehrmechanismus für den offensiven Beutefang umstrukturiert. Eine Frage in dieser Arbeit war also, wie die Häufigkeit der Triggerhaarberührungen mit der Bildung von Jasmonaten und dem Verdauungsvorgang miteinander in Verbindung steht. Beim Kontakt von Beute mit den Triggerhaaren im Inneren der Falle schließt sich diese, und es werden durch Biosynthese Jasmonate gebildet. JA-Ile interagiert mit dem COI1-Rezeptor und aktiviert so den Verdauungsweg, der zur Sekretion von Verdauungsflüssigkeit und zur Produktion von Transportern führt, welche zur Aufnahme von aus Beute gewonnenen Nährstoffen benötigt werden. In dieser Arbeit konnte gezeigt werden, dass die Anzahl der Triggerhaarberührungen positiv mit der Höhe und der Dauer der Transkriptionsinduktion mehrerer Verdauungsenzyme bzw. Verdauungshydrolasen korreliert.

Abscisinsäure (ABA) fungiert neben vielen anderen Funktionen als pflanzliches „Trockenstresshormon“. Es wird entweder von Wurzeln als primärem Sensor für Wassermangel oder von Schließzellen in den Blättern synthetisiert. ABA beeinflusst ein Netzwerk von mehreren tausend Genen, deren Regulation die Pflanze auf Dürre vorbereitet

und entsprechende Schutzmaßnahmen einleitet.
Aus früheren Arbeiten war bekannt, dass die 48-stündige Inkubation einer *Dionaea*-Falle mit ABA die erforderliche Anzahl an Triggerhaarberührungen erhöhte, die für einen Fallenschluss notwendig sind. In der vorliegenden Arbeit konnte gezeigt werden, dass das Aufbringen von ABA auf intakte Fallen vor der mechanischen Stimulierung der Triggerhaare (Mechanostimulation) die Expression von Verdauungsenzymen bereits bei einer Inkubationszeit von nur 4 Stunden signifikant reduzierte. Das zeigte eindeutig, dass die kurzzeitige Einwirkung von ABA bereits die Effekte von Jasmonaten blockiert, wenn es um den Beginn des Verdauungsprozesses geht, aber keinen Einfluss auf den Fallenschluss hat. Eine Inkubation für 24 und 48 Stunden mit 100 µM aktiver ABA hatte keine Auswirkung auf das Wiederöffnen der Falle, nur sehr hohe Konzentrationen von 200 µM aktiver ABA hemmten das Wiederöffnen der Falle, führten aber auch zu Gewebenekrose. Da ABA die Transkription der Verdauungsenzyme reduzieren konnte, ist es wahrscheinlich, dass *Dionaea* den Verdauungsvorgang stoppen kann, wenn entsprechende externe Signale empfangen werden.

Ein weiterer Einflussfaktor, welcher erst später erkannt wurde, war die Auswirkung des verwundungsbedingten, sprunghaften systemischen Anstiegs der Jasmonatkonzentration auf die Wirkung von extern aufgegebenen Phytohormonen. So wirksam ABA bei der Hemmung der Markerhydrolasen-Expression nach Mechanostimulation in intakten Pflanzen war, so konnte diese Inhibition nach Anwendung von ABA auf abgeschnittenen Fallen nicht mehr beobachtet werden. Ein Grund könnte sein, dass das ABA-Signal in den Wurzeln wahrgenommen wird und daher abgeschnittene Fallen nicht darauf reagieren konnten. Ein anderer Grund könnte sein, dass die Verwundung das Gewebe für das ABA-Signal desensibilisiert hat. An dieser Stelle besteht weiterer Forschungsbedarf.

Ebenfalls wurden Inhibitoren des Jasmonat-Weges verwendet, um ihre Wirkung auf die Regulation des Beutefangzyklus von *Dionaea* zu untersuchen. Coronatine-O-methyloxim erwies sich als wirksamer Inhibitor der durch Mechanostimulation induzierten Expression von Verdauungsenzymen und bestätigte damit die zentrale regulatorische Rolle von Jasmonaten für den Beutefangmechanismus von *Dionaea*.

Ein parallel laufendes Projekt war die Erzeugung von in vitro-Kulturen aus sterilisiertem Saatgut und einzelnen Pflanzenteilen, das sich als sehr erfolgreich erwies, was für die Erzeugung zukünftiger transgener Linien wichtig sein kann. Ebenfalls wurden Protoplasten aus Blattgewebe erzeugt, diese wurden transient transformiert und exprimierten YFP nach einer Inkubationszeit von 24 Stunden. In Zukunft könnte dies der Ausgangspunkt für die Generierung transgener Linien sein und der Funktionsüberprüfung von DNA-Konstrukten sein.

Table of contents

1. Introduction

The surface of our planet is covered by roughly 70 % oceans and 30 % land mass. Most parts of the land mass are, to some degree, covered by plants, therefore most humans call them an everyday sight. Thinking about plants, one usually ponders about peaceful sessile beings that do not actively try to harm someone. Hunting plants are known to mankind mostly from folktales and movies like “Little Shop of Horrors”. Therefore, the thought “How fast is it, and does it bite?” is usually nothing that comes to mind first when thinking about a plant. However, a small number of exceptional plants have developed the ability for rapid movement… and carnivory. The following work sheds light on some very interesting features of *Dionaea muscipula*, the Venus flytrap, with the focus on the regulation of prey digestion and its hormonal control.

1.1 Evolution of a carnivorous life style

1.1.1 Origins and definition of carnivory

To answer “what is a carnivorous plant” is not as easy as it may seem and a matter of discussion. One straightforward definition of carnivory is given by Barry Rice in his article “Reversing the Roles of Predator and Prey” in the book “All Flesh is Grass” (Seckbach and Dubinsky 2011) in three key points:

1. Clear adaptions to capture prey are present. Such adaptions may include specialized structures and also enhancements to improve the luring and capture of prey.
2. A mechanism is present by which prey [animals] are degraded into a form that can be assimilated by the plant. It may be enzymes produced by the plant, decomposition by bacterial activity, or other organisms in a mutualist relationship with the plant.
3. A pathway is available that allows nutrients to be absorbed into the plant, thus contributing to the plant´s competitive and reproductive fitness […].

Plants that fulfill only part of these criteria are called para- or semi-carnivorous. The question “why” plants developed carnivory is best explained by the following quote: “Plant carnivory may be regarded as a niche evolutionary strategy that is best suited to exploit marginal habitats where few competitors can survive. Most carnivorous plants are unable to adapt to radically different environments and many only populate ecologically isolated areas where there is little influx of competition” (Bailey and McPherson, 2012).

As the definition shows, the release and the uptake of prey-derived nutrients is an important feature for the carnivorous lifestyle. In contrast to the common plant which acquires almost all inorganic nutrients required for maintaining cellular and metabolic functions and growth by means of root uptake, carnivorous plants have unlocked new ways for nutrient harvesting and gained access to nutrient matters unavailable to the majority of plant life.

Non-carnivorous nutrient uptake in roots

For non-carnivorous plants, the soil is the major source of nutrients. Nitrate (NO_3^-) for example is the major nitrogen (N) source, together with ammonium (NH_4^+) and urea ($CO(NH_2)_2$). The NO_3^- uptake capacity of a plant depends on the functional properties of transporters in the root, the density of functional transporters at the plasma membrane of root cells and the surface and architecture of the root system. Nitrate uptake is facilitated in the roots by two specialized transport systems, a High Affinity Transport System (**HATS)** which allows nitrate absorption at low external concentrations (< 0.5 mM) and a Low Affinity Transport System (**LATS)** which allows absorption at high external concentrations (> 0.5 mM), as well as by four families of specialized transporters (NITRATE TRANSPORTER 2 (**NRT2,** Krapp et al., 2014), NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY (**NPF**, Léran et al., 2014), CHLORIDE CHANNEL FAMILY (**CLC**, Barbier-Brygooetal., 2011) and SLOW ANION ASSOCIATED CHANNEL HOMOLOG (**SLAC/SLAH**, Negi et al., 2008, Maierhofer et al., 2014)), then distributed to the rest of the plant via the xylem. Nitrate is then reduced to nitrite by nitrate reductases (**NR**) and further to ammonium by nitrite reductases (**NiR**) before it is incorporated into amino acids (AA) (Noguero and Lacombe, 2016).

Carnivorous nutrient-uptake

In carnivorous plants, nutrient uptake is, in addition to uptake by the roots, facilitated by specialized organs. As most carnivorous plants live in swamp or bog areas, some nutrients may be completely absent from the soil. Using the example of *Dionaea*, the plant possesses a reshaped lamina, the snap-trap. The inner surface of the trap-lobes is covered with secretory glands which can excrete a digestive fluid but do also possess a plethora of transporters for the uptake of nutrients obtained from the digested prey (Scherzer et al., 2013; Gao et al., 2015; Böhm et al., 2016b).

1.1.2 Evolutionary development of carnivory

Number of carnivorous species

There are currently an estimated 380.000 plant species on earth (IUCN 2016). Thereof, depending on the source, between 583 (Givinish, 2015), 636 (Ellison and Gotelli, 2009), 730 (Bailey and McPherson, 2012) and 810 (Ellison and Adamec, 2018) plant species are known to be carnivores, but new species are discovered every year (Adamec et al., 2021).

Carnivory is present in the plant kingdom almost all over the world, having multiple points of origin. These independent developments are reflected in the great number of varieties of carnivorous trapping mechanisms. Carnivory in the plant kingdom has evolved independently at least six times in five angiosperm orders (Ellison and Gotelli, 2009). The means those plants developed to capture prey are quite diverse. Seven genera consist of pitcher plants (*Cephalotus, Heliamphora, Nepenthes, Sarracenia, Darlingtonia, Paepalanthus, Brocchinia*), six genera have sticky leaves, also known as flypaper or adhesive traps (*Drosera, Pinguicula, Byblis, Roridula, Drosophyllum, Triphyophyllum*). Only two carnivorous genera (*Aldrovanda* and *Dionaea*) have snap traps (rarely referred to as “bear traps”). The genus *Genlisea* is the only one having lobster-pot-type traps; its members are also named the corkscrew plants. *Utricularia*, also known as the bladderworts, are the sole representatives of their trap type, a “suction trap” which, after triggering, pulls the prey inside the trap using the pre-tensed bladder walls (Lloyd, 1942; Bailey and McPherson, 2012).

As it is demonstrated in the family tree presented by Ellison and Gotelli (2009, **Fig. 1.1**), the genus *Droseraceae (with Dionaea, Drosera* and *Aldrovanda)* is a member of one monophyletic group, together with the *Nepenthaceae, Drosopyllaceae* and *Dioncophyllaceae* families.

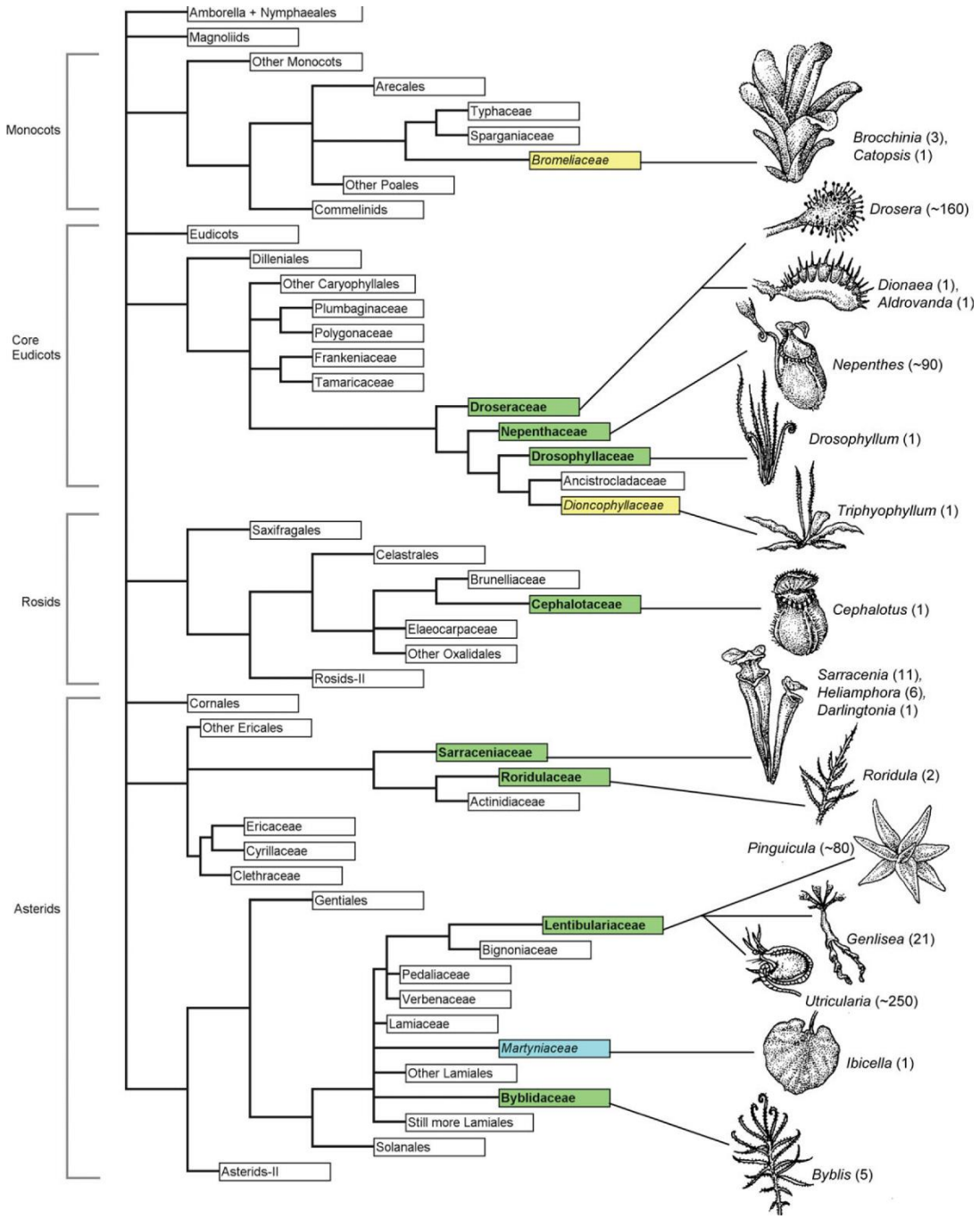


Fig. 1.1 The phylogeny of carnivorous plants in the angiosperm class
Ellison and Gotelli (2009) presented the shown descriptive family tree of the carnivorous plants within the angiosperm class. Exclusively carnivorous families are represented in green, families with one or two carnivorous genera are depicted in yellow, and the *Martyniaceae* with the possibly carnivorous *Ibicella* is represented in blue. The number of carnivorous species in each species is given in parenthesis. Representative trap types are depicted for each genus. Branch length do not indicate relationship status.

1.1.3 Benefits and costs of carnivory

Advantage over competitors

It has been observed that carnivorous plants mainly occur in nutrient-deficient (e.g. Nitrogen-deficient) sites, like swamps or bogs. As mentioned above, carnivorous plants are able to populate evolutionary niches that have been previously inaccessible to plant life, therefore minimizing competition with other plants (Givinish et al., 1984), as the slow growth rate of carnivorous plants puts them in a disadvantage over other plants in the competition for sunlight (Roberts and Oostings, 1958; Schulze et al., 2001).

Costs of carnivory

The building and maintenance of carnivorous structures - such as active trapping mechanisms - are nutrient- and energy-consuming processes. In addition, it often means less leaf material available for photosynthesis. Pavlovic et al. showed that during digestion, especially during the generation of action potentials (APs), the net photosynthesis rate within the trap is reduced, but not in the leaf blade of *Dionaea* (Pavlovic et al., 2010; Pavlovic et al., 2011). But these costs are repaid in full by the advantages gained. As, for example, *Dionaea* occurs in bright, wet spots, photosynthesis of the remaining leaf material is more than sufficient to compensate for the loss generated by the photosynthetic-inefficient trap, and the nutrients gained by carnivory compensate for the energy required to build and maintain the trapping apparatus (Givnish et al., 1984; Ellison and Gotelli, 2009).

1.2 *Dionaea muscipula* – The Venus flytrap

1.2.1 History, phylogeny and morphology

The scientific name, *Dionaea muscipula*, is derived from Dione, the Greek goddess of love (*Dionaea*, lat. "the daughter of Dione"), and the word mousetrap (lat. muscipula). The Latin word for mouse is "*mus*", the Latin word for fly "*musca*" and "capere" for catching. The use of the word mousetrap is a reference to the snapping mechanism which resembles a snap shutting mousetrap, as the Latin word for flytrap is "*muscacipula*". The English version of the name, Venus flytrap, is partly due to the fact that Venus, the roman goddess of love, is the roman version of the Greek goddess Aphrodite, who is the daughter of Dione, and partly due to the "beautiful appearance of its milk-white flowers and the elegance of its leaves" (Ellis 1768; Barthlott et al., 2007; Bailey and McPherson, 2012). Other indecent meanings of the name should not be explored at this point but are discussed at length by Bailey and McPherson.

After the first botanical descriptor, John Ellis, the full name is sometimes cited as *Dionaea muscipula* J. Ellis, although it is debated that the full name should be cited as *Dionaea muscipula* Solander ex J. Ellis, as Daniel Solander examined and described *Dionaea muscipula* as early as 1765, but did not publish it, and was credited by John Ellis in publication (Ellis, 1768; Nelson, 1989).

First description and history

When it was first described in the western world, *Dionaea* triggered fascination among scholars. Charles Darwin even described it with the words "This plant, commonly called Venus' fly-trap, from the rapidity and force of its movements, is one of the most wonderful in the world" (Darwin, 1875). Among the earliest records is a report from 1760 written by Arthur Dobbs, Governor of North Carolina, who described *Dionaea* as the "Fly Trap Sensitive" (Bailey and McPherson, 2012).

Phylogenetic classification of *Dionaea muscipula*

The Venus flytrap belongs to the clade Eudicots, is generally placed in the order *Caryophyllales (as "*non-core *Caryophyllales")*, the family of the *Droseraceae*, the genus *Dionaea* and the species *Dionaea muscipula*. It is the only member of its genus (Heubl et al*.,* 2006; Ellison and Gotelli, 2009; Walker et al., 2017). Recent research based on analysis of *Dionaea´*s pigments suggests that the *Droseraceae* family should be sorted in the *Nepentales* order rather than in the *Caryophyllales* order (Henarejos-Escudero et al., 2018).

Although the *Dionaea* genus is considered to be monotypic, the variety of shape, coloration, and size within this genus is astonishing. Plants with different appearance (called "cultivar") are collected and grown by breeders all over the world and are registered with the International Carnivorous Plant Society (ICPS). Today there are 130 individually registered

cultivars (ICPS, accessed 01.01.2022). *Dionaea* has a diploid set of chromosomes (Veleva et al., 2017), with the number of chromosomes of *Dionaea muscipula* is reported with 32 to 33 (32 by Veleba et al., 2017; 33 analysed in root samples, Hoshi and Kondo, 1998) with an estimated haploid genome size of approx. 3 Gbp (Jensen et al., 2015, Veleba et al., 2017; Palfalvi et al., 2020), a GC content of 43.9 % (Veleba et al., 2017) and 21,135 predicted genes (Palfalvi et al., 2020). With respect to the transcriptome, the closest related plants known today are the tomato (*Solanum lycopersicum*) with 59,8 % similarity and the grapevine (*Vitis vinifera*) with 53,8 %, which can be explained by the small number of transcriptomes sequenced so far (Jensen et al., 2015).

Natural habitat

Dionaea originates in the southeast of North Carolina and in the northeast of South Carolina (USA), in a 120 km radius around the city of Wilmington. *Dionaea's* main habitats are open, wet, sandy and nutrient-poor (especially low in phosphorous and nitrogen content) swamp or savannah areas with an average temperature range from -9 °C to + 37 °C (WolframAlpha.com, downloaded 21.10.2016). Due to its slow growth, *Dionaea* depends on periodical wildfires to ensure that it is not overtopped and overgrown by other species, which happens about 3-5 years after the fire when the vegetation recovers. The higher grasses reduce the probability of catching larger prey, therefore reducing the uptake of nitrogen (N) from the insects to 46 % and increasing the dependency on N-supply from the soil (Schulze et al., 2001). After a wildfire, new *Dionaea* leaves appear as soon as two weeks after burning (Roberts and Oostings, 1958).

***Dionaea´s* dietary preferences**

To compensate for the nutrient-poor soil, *Dionaea* obtains up to 75 % of the required nitrogen from insect prey (Schulze et al., 2001). *Dionaea´*s insect diet is diverse, with the main part consisting of *Formicidae* (ants) and *Araneae* (spiders), and smaller fractions *Coleoptera* (beetles), *Diptera* (flies) and *Orthoptera* (grasshoppers and crickets), and even smaller fractions consisting of *Hemiptera* (true bugs) and *Lepidoptera* (moths/butterflies) (Ellison and Gotelli, 2009).

Pollination and the pollinator-prey-conflict

It has been shown that there is very little overlap between *Dionaeas* prey and its pollinators. It is hypothesized that this is due to the spatial separation of the traps and the flowers via the long flower stalk, thereby pollinators don´t come in close proximity to the snap-trap while flying from flower to flower, and the color difference between trap and flower as well as other factors, like the release of volatile organic compounds, are also thought to be involved (Youngsteadt et al., 2018). As the pollinator-prey-conflict (Juniper et al., 1989) is of importance for *Dionaea muscipula*, it is of interest to note that the main pollinators for *Dionaea muscipula* were found to be *Hymenoptera* (mainly bees) and *Coleoptera*, followed by *Diptera*, *Araneae*, *Hemiptera, Lepidoptera* and *Orthoptera*. Except for one crab spider, the 10 most abundant flower-visiting taxa never occurred in traps (Youngsteadt et al., 2018).

Plant description and trap architecture

A *Dionaea muscipula* plant (**Fig. 1.2**) consists of a rosette of leaves surrounding a flower stalk with a length of approximately 30 cm (during florescence). At the base of the leaves there is a white, fleshy bulb-like rhizome with lateral roots of black color (Bailey and McPherson, 2012), the black color appears regardless of whether the plant is grown in soil or in nutrient media (own observation). The lateral root system that originates at the rhizome is responsible for water uptake and can provide sufficient nutrients from nutrient-rich soil. In nutrient-poor soil,

the plant depends on an animal diet to gain required nutrients (Böhm et al., 2016a). The leaf itself is divided into three parts, the first is the leaf blade, (the lamina) at the proximal end, further referred to as **petiole**, the second is the connection between the petiole and the trap**,** and the third part is the bilobed snap-trap (the reshaped lamina at the distal end, further referred to as **trap**). The two trap lobes are connected via the midrib. At the adaxial surface of the trap there are usually 3 trigger hairs on each trap lobe protruding from the epidermis, arranged triangularly (Darwin, 1875; Pavlovic et al., 2010). If an insect touches either one trigger hair twice or two or more trigger hairs once within 20 s (Brown, 1916), the trap snaps shut in a time between 100 ms (Forterre et al., 2005) and 300 ms (Volkov et al., 2007). The inside of the trap is covered by digestive glands, on average 58 glands/mm², roughly 37.000 glands/trap which are responsible for secreting the digestive fluid containing the lytic enzymes and the subsequent nutrient uptake (Escalante-Pérez et al., 2011). Each digestive gland consists of 3 layers of cells, the outer layer (L1), the inner layer (L2) and the endodermoid layer (L3). The outer rims of the trap are covered by nectary glands via which starving traps release volatile organic compounds (VOC) resembling the smell of fruits and flowers to attract prey (Kreuzwieser et al., 2014; Bemm et al., 2016). The inner parts of the trap contain large amounts of anthocyanin, with cyanidine-3-glucoside being the most prominent coloring agent (Gregorio and DiPalma, 1966). From the margins of each trap lobe several marginal spikes protrude (also referred to as marginal lashes, spines or teeth; Darwin, 1875; Bailey and McPherson, 2012). The marginal spikes have been found to be of utter importance for medium-size prey capture, as removal greatly reduced trapping success in medium-sized crickets. Davis et al. found that removing the marginal spikes decreased the rate of prey capture success for moderate-sized cricket prey by 90 % (Davis et al., 2019).

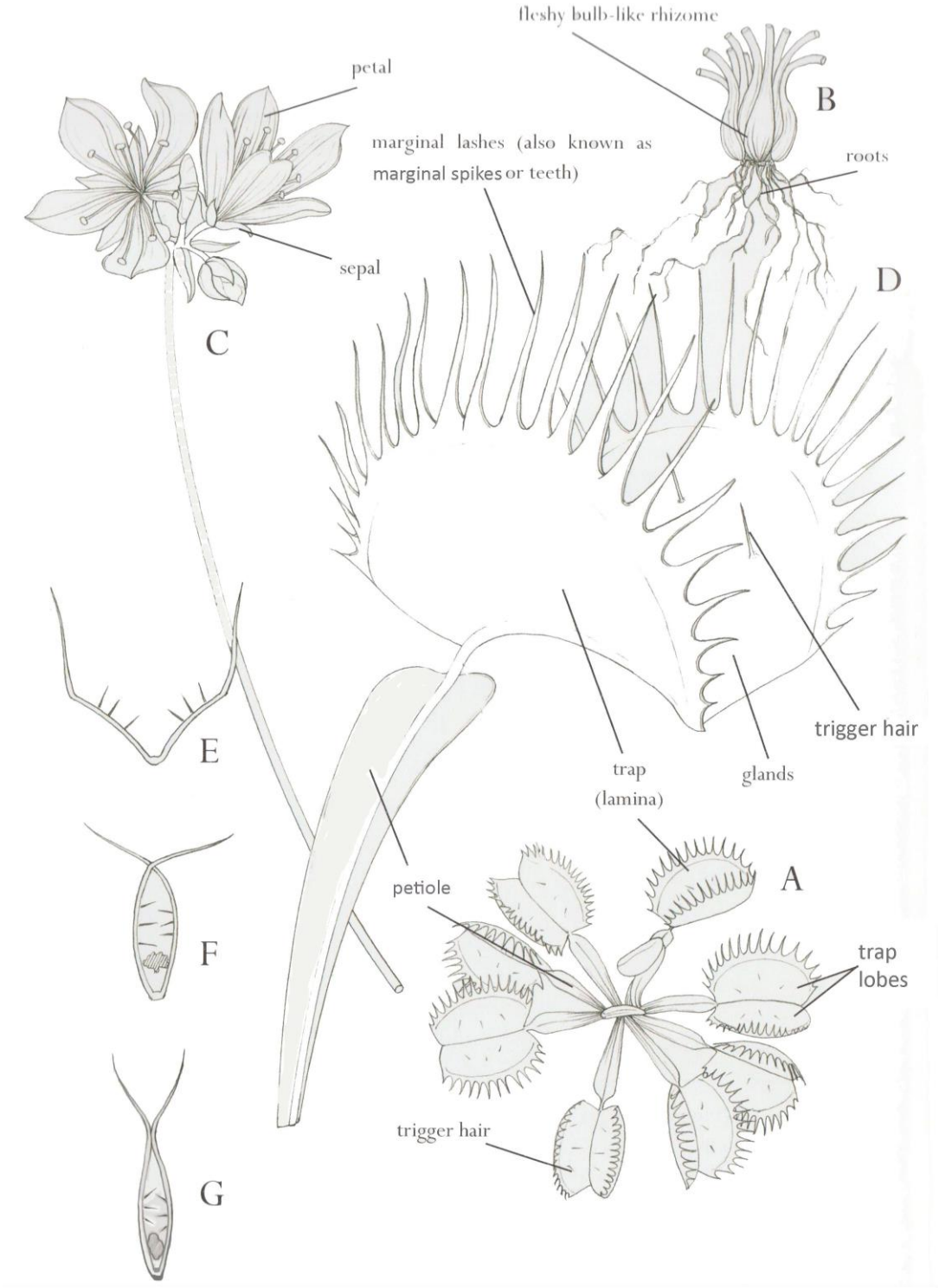


Fig. 1.2 The basic morphology of *Dionaea muscipula*

A shows the morphology of the upper parts of a *Dionaea* plant, **B** the bulb-like rhizome and the roots at the base of the leaves, **C** the flower stalk and the flowers, **D** a trap with trigger hairs, marginal spikes and **E-G** show the open stage of a trap, the closed stage and the locked stage (E-G, named after Volkov et al., 2011; Image altered from Bailey and McPherson, 2012. Reproduction with kind permission of Steward McPherson, Redfern Natural History Productions, Dorset, United Kingdom).

1.2.2 The hunting cycle of *Dionaea muscipula*

Trigger mechanism, action potential and trap closure

When a prey enters the trap and touches the trigger hairs, electrical signals, so-called action potentials (APs) are elicited. Immediate trap closure is triggered by two subsequent action potentials within 20 - 30 s (Brown 1916; Volkov et al., 2011; Suda et al., 2020). The trigger hair transmits the touch sensation to the mechanosensors at the trigger hair base where the AP is generated. The trigger hair base consists of mechanoreceptor cells which, when stimulated by the mechanical deformation, translate the touch sensation into an all-or-nothing AP that spreads over the entire trap (Iosip et al., 2020).

Generally, an AP consists of four phases, first the depolarization phase after the threshold to elicit the action potential has been reached, followed by the repolarization phase and the hyperpolarization phase, and finally the recovery of the resting potential (See **Fig. 1.3**).

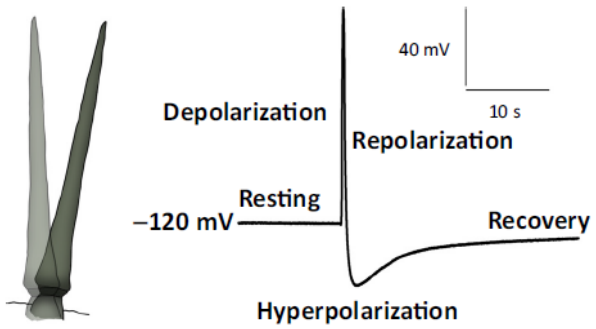


Fig. 1.3 *Dionaea muscipula* action potentials
The resting potential of a *Dionaea* mesophyll cell is about -120 mV to -140 mV. Once the threshold of -100 mV is reached, an action potential (AP) is elicited which first reaches about -20 mV in the depolarization phase, then hyperpolarizes to about -150 mV before recovering. The usual duration of a *Dionaea* AP is about 2 seconds and can be elicited every 2 seconds (reviewed in Hedrich and Neher 2018; Hedrich and Fukushima 2021).

The exact mechanism of the AP generation in *Dionaea* has still to be explained in detail, but it is most likely that depolarization is based on Cl^- and Ca^{2+} influx and the repolarization on K^+ and H^+ efflux. Interestingly, many cell types in the trap tissue, not only the mechano-receptor cells surrounding the trigger hair, are able to generate APs, for example cells in the upper and lower epidermis and the assimilation parenchyma (Brown 1916; Hodick and Sievers, 1988; Krol et al., 2006; Escalante-Pérez et al., 2011; Volkov et al., 2011; Böhm et al., 2016a; Hedrich and Neher, 2018; Hedrich and Neher, 2021). It was concluded that Na^+ is not involved in the depolarization or repolarization process (Böhm et al., 2016b).

It has been shown that the surrounding temperature has an effect on the number of mechanical stimulations required for trap closure. Between 15 °C and 25 °C, two mechanostimulations are sufficient to elicit trap closure. At higher temperatures between 35 °C and 40 °C, one mechanostimulation is sufficient (Darwin, 1875; Brown and Sharp, 1910; Volkov et al., 2008a). The trap closing speed is also temperature-dependent. At 20 °C, the trap closed twice as fast as at 36 °C (Volkov et al., 2008a).

It has been observed that trigger hair bending leads to an elevation of cytosolic Ca^{2+} levels, which decreases again over time. If two trigger hair bindings occur in a time of approximately

30 seconds, the trap closes rapidly. Escalante-Pérez et al., (2011) recorded Ca^{2+} spikes in gland cells after more than three trigger hair bendings by injecting the calcium indicator dye FURA-2 in the gland cells, and Suda et al., (2020) were able to show the calcium transients in more detail by generating transgenic *Dionaea muscipula* plant lines which expressed the calcium sensor protein GCaMP6f, which contains a cpEGFP fluorescent moiety. Suda et al. could show that one trigger hair bending already elicits an increase of cytosolic Ca^{2+} levels, starting from the bent trigger hair and spanning over the entire trap over both trap lobes, but did not cross the connection between the trap and the petiole. After the second trigger hair bending, the Ca^{2+} levels rose even higher and trap closure was elicited. These Ca^{2+} levels could not be detected in pigmented gland cells, but could be picked up in unpigmented gland cells after the first and also after the second stimulus (Suda et al., 2020).

Jaffe (1973) speculates about the existence of an ATPase in the midrib of the *Dionaea* trap that is involved in trap closure and that either the addition of ATP or environmental conditions which increase the ATP level of the plant increase the trap closing speed. He states that the midrib of an open trap contains large amounts of ATP, and that this value is decreased by approximately 30 % after stimulation and trap closure have occurred. According to Jaffe, the addition of 100 µM exogenous ATP for 30 min increased trap closing speed as well as trap reopening speed, the incubation of the trap in 100 % CO_2 reduced the closing speed significantly, whereas the incubation in 100 % O_2 increased it significantly.

The petiole, the outside of the trap and the marginal spikes are covered in rosette - like stellate trichomes with an overall diameter of about 80 µm, with the central spindle consisting of eight radially arranged cells with nuclei. Those stellate trichomes have been reported to act as touch sensors. When the outer trap was gently struck with a steel wire, after about 8-10 strokes an action potential was fired within the trap, after 3-4 subsequent strokes a second AP was fired and the trap closed. It was also reported that by gently striking the outside of the trap or the marginal spikes with a brush, the number of APs required to elicit trap closure was significantly reduced (1.4 stimuli after conditioning the trap on the marginal spikes, $p< 0.001$, and 1.6 stimuli after conditioning the outside of the trap ($p<0.001$), compared to 2 / 2.1 stimuli in unconditioned traps) (DiPalma et al., 1966). It may be assumed that these results are caused by the effects of slow wave potentials (SWP, or variation potentials) and their effect on cytosolic Ca^{2+}-levels (Nguyen et al., 2018). If sufficient SWPs have increased the cytosolic Ca^{2+}-levels, then sometimes less than two trigger hair displacements were sufficient to elicit trap closure.

Each individual trap can perform only a limited number of digesting processes, and besides that, only a certain number of closure and openings. Jaffe (1973) reports the maximal number of closure and openings with 2-3, Davis et al., (2019) report the number with a maximum of 4. The reported maxima of digestion processes performed by an individual trap before necrosis sets in ranges between 2 and 4 (Darwin, 1875).

Hydroelastic curvature model

The mechanism of the Venus flytrap's rapid trap closure has been subject to some hypotheses. The acid growth hypothesis is one of them, stating that the rapid trap closure involves irreversible cell enlargement (irreversible expansion of the outer epidermis involved in closure and irreversible expansion of the inner epidermis involved in reopening) by massive ATP-dependent H^+ transport (Williams and Bennett, 1982). They report that after trap closure, the central outer epidermis of the trap lopes expanded by approx. 28 %, but not the inside. During reopening, the inner epidermis is reported to expand by approx. 12.3 %, while no changes were observed on the outer epidermis. In addition, Williams and Bennet infiltrated traps with different buffer solutions. An acetate buffer with 30 mM buffering capacity above pH 4.50 almost did not affect the trap closing, whereas an acetate buffer with 30 mM buffering capacity above pH 4.75 inhibited the trap closure slightly. Buffer infiltration of an acetate buffer with 30 mM buffering capacity above p.H 5.0 resulted in only slight trap lobe movement after long and continuous stimulations, and infiltration of the trap lobe with a buffer with 50 mM buffering capacity paralyzed the traps. Interestingly, the buffer-infused plants required a much higher number of stimuli, with pH 4.50 11 ±5 stimuli were required to elicit 50 % trap closure, with pH 4.75 60 ±24 stimuli were required.

This hypothesis is today considered not very likely to be true due to the time required for all the necessary physiological changes (e. g. acidification of the cell, loosening of the cell wall). It is doubtful that these changes can be achieved in the very short time which is required for the trap to close.

The current, generally accepted model is the “hydroelastic curvature model”, according to which the trap lobes consist of outer and inner hydraulic layers where different hydrostatic pressures can build up. The open, convex state of the trap is the state that needs energy to be accomplished and therefore contains hydroelastic energy, due to the hydrostatic pressure differences between the outer and inner layers of the lobe, which are thought to be two distinct (upper and lower) hydraulic layers. After two trigger hair displacements, aquaporins facilitate the quick rush of water from the inner hydraulic layer (located near the inside of the trap with the glands) to the outer layer (located near the outside of the trap, opposite of the side with the glands), thereby facilitating rapid change of the trap lobe state from convex to concave, inducing rapid trap closure (Volkov et al., 2008a; Volkov et al., 2014; personal communication Prof. Volkov). This model is backed up by experimental proof in which ion channel and aquaporin blockers and uncouplers were shown to either decrease or increase the trap closing speed (Volkov et al., 2008b).

The hunting and digesting cycle – The green stomach

When trap closure is elicited, the trap snaps shut in a fraction of a second (0.1 s to 0.3 s, Forterre et al., 2005; Volkov et al., 2007). In the first minutes, the two trap lobes have not closed tightly (See **Fig. 1.4**, second stage) allowing small prey not worth the effort of digestion to escape through the interlocking marginal spikes. Larger prey is denied exit of the trap by the spikes and tries to escape, thereby eliciting more APs. This stage is called the "closed stage". If more than 5 APs are generated, the trap lobes seal hermetically during the next 1-2 hours, thus forming the "green stomach". This stage is also called the "locked stage". About 12 h after prey capture, visible secretion of digestive fluid can be observed, and the digestion process continues for 5-10 days, depending on the size of the prey (Escalante-Pérez et al., 2011; Volkov et al., 2011; Bemm et al., 2016). The active secretion and digestion process can be seen by the formation of a digestive pocket (Fagerberg and Howe, 1996).

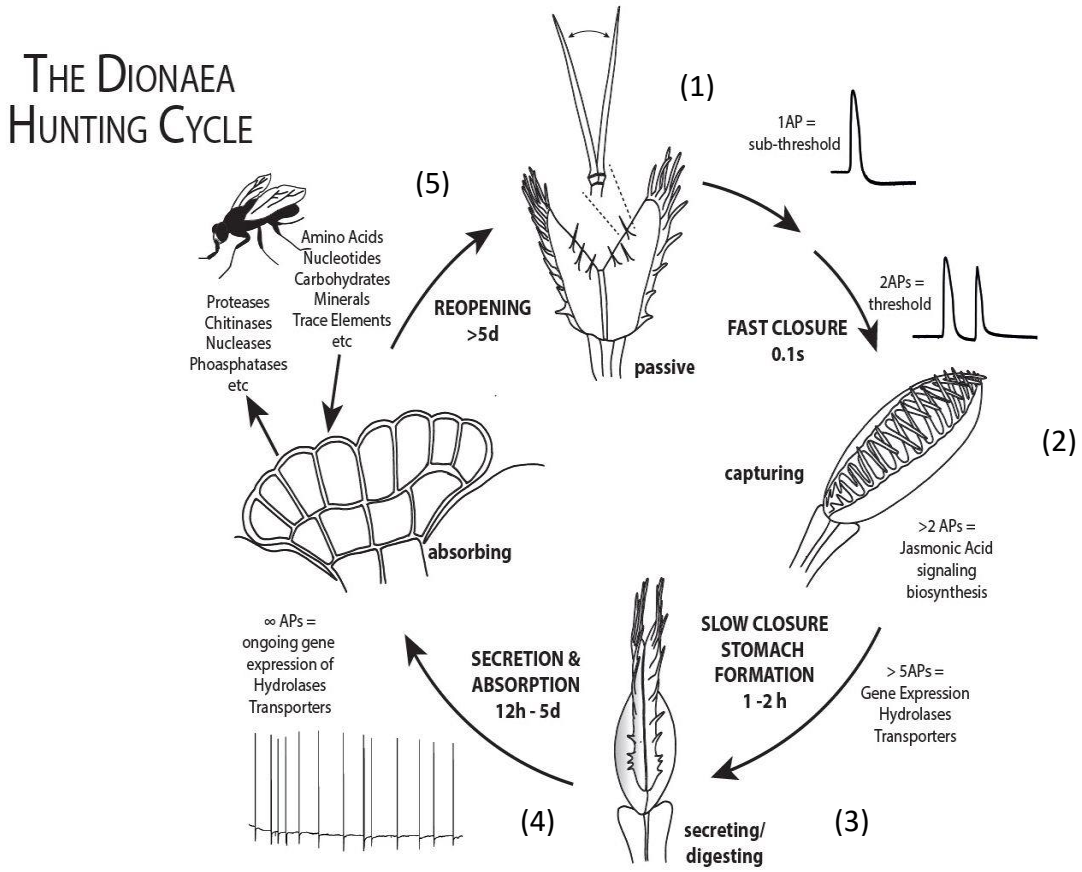


Fig. 1.4 The *Dionaea* hunting cycle In the open state, the trap is pre-tensed and ready to snap shut in a fraction of a second. The fast closure **(1 to 2)** is triggered by touching of the trigger hairs **(1)** if the internal closing threshold (generally 2 APs) is reached. If the trap is closed **(2)**, more than 2 APs are required to activate the JA pathway. This is a safeguard against energy waste if trap closure was initiated by accident (e.g. a falling leaf). If an insect is captured, it struggles to escape and triggers more APs. More than 5 APs are required to initiate the formation of the green stomach **(3)** and the expression of digestion-related transcripts. In the secreting/digesting – phase **(4)** the trap lobes have sealed hermetically, and the onset of secretion can be observed as soon as 12 h after trap closure. The trap secretes a plethora of digestive enzymes to access the nutrients contained within the captured prey. As these nutrients are made available, transporters in the glands absorb and deliver them to deeper tissue layers of the trap. When the digestion process has concluded **(5)**, the trap reopens and is ready to catch its next prey (modified after Bemm et al., 2016).

“The plant that counts” – Plant response to different number of contacts

It has been shown that 2 APs usually suffice to induce trap closure. After prey capture, levels of OPDA, a member of the jasmonate family and the precursor of the phytohormone jasmonic acid (JA) rise twofold within 30 minutes (Escalante-Pérez et al., 2011). The expression of COI1 (CORONATINE INSENSITIVE 1, the receptor for JA-Ile) and its co-receptor JAZ1 (JASMONATE ZIM DOMAIN 1) are also affected. After 2 APs are elicited, COI1 expression is reduced by approx. 60 % compared to resting traps, whereas JAZ1 expression is induced 5.5-fold. The JAZ1 induction indicates the mechanical activation of the JA-pathway. 3 APs suffice to induce gland activity by eliciting a calcium spike and 5 APs are sufficient to trigger a peak in DmJAZ1-expression as well as expression of a significant number of genes encoding prey-degrading hydrolases (Escalante-Pérez et al., 2011; Böhm et al., 2016a).

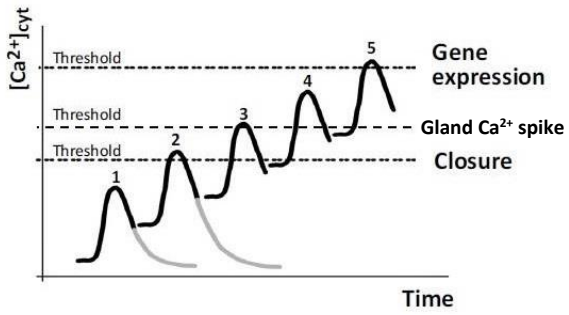


Fig. 1.5 The number of elicited action potentials influences trap behavior
It has been demonstrated that 2 APs are usually sufficient to induce trap closure, 3 APs induce a pronounced rise in gland cell cytoplasmic Ca^{2+} level, and 5 APs activate gene expression (modified after Hedrich and Neher, 2018; with data from Escalante-Pérez et al., 2011))

Digestive fluid – Composition and regulation of secretion

If the trap closure has been triggered by an insect, it is encased by the closed trap lobes. While struggling to escape, it repeatedly touches the trigger hairs and triggers APs. If the number of APs reaches the threshold, secretion of the digestive fluid is initiated.

The pH-value of the secreted fluid varies over time. Escalante-Pérez et al. (2011) reports a pH shift from approx. pH 4.4 at 12 h after secretion onset (secretion elicited by coronatine treatment) to pH 3.4 after 2 weeks. Sickel et al. (2019) reported a pH value of digesting plants (secretion elicited by insect) between pH 3 and pH 4.5 3 days after secretion onset, with traps reopening after 8-9 days, and a pH between 3.5 and 4.5 (secretion elicited by coronatine treatment), with traps reopening 19 to 29 days after the coronatine stimulus.

The digestive fluid contains numerous digestive enzymes, namely peroxidases, nucleases, phosphatases, phospholipases, a glucanase, chitinases, cysteine proteases, aspartic proteases, and a serine carboxypeptidase. Proteases and peroxidases were found to be large protein families in the digestive fluid. As many of those enzymes were pathogenesis-related proteins, it is suggested that the plant turned defense-related processes into a digestive system (Schulze et al., 2012).

The marker transcripts as indication for activation of the digestion process

After analyzing the fluid content of the green stomach, three transcripts coding for hydrolases stood out because they were present so abundantly (Schulze et al., 2012). In subsequent studies, their transcripts were used as "digestion marker hydrolases" (also referred to as "marker transcripts") to estimate the intensity of the activation of the digestion process:

VF CHITINASE I

The enzyme VF CHITINASE I is mostly required for the enzymatic breakdown of the insects´ chitin-based shell. Chitin, an insoluble linear polymer, similar to cellulose in its chemical structure and composed of the polysaccharide β-(1,4)-linked N-acetyl-glucosamine (GlcNAc), occurs in three different crystalline forms: α-, β-, and γ-chitin. Arthropods, the Venus flytrap´s major prey, are covered in a cuticle of α-chitin. In contrast to chitin, whose existence is mainly restricted to the insect and fungi kingdom, chitinases are much more common. In plants, for example, they play a role in defense against fungal and herbivore attack. VF CHITINASE I mainly exhibits endochitinase activity, thus cutting internal bonds in chitin chains and producing low molecular mass multimers of GlcNAc. It has a maximum activity range between pH 4 and 5, which is consistent with the measured pH level within the digestive fluid. The working temperature range is between 0 °C and 90 °C, with 40 % activity at 20 °C, 50 % at 30 °C, 60 % at 40 °C and maximum activity at 50 °C, reflecting the temperature conditions of *Dionaea*'s habitat. The transcript is 942 bp long and encodes for a protein with 314 amino acids with a calculated molecular mass of 33.4 kilodaltons (kDa) and is therefore similar to other plant chitinases known to play a role in pathogen defense (25-35 kDa). The sequence contains many cysteine residues, which is probably the cause for the compact structure and hence provides protection from auto-proteolytic degradation, as well as a high content of proline in exposed loop regions (7.8 % proline as compared to an average of 4.7 % proline in the majority of enzymes) and one glycosylation. VF CHITINASE I is probably assisted by two additional chitinases present in the digestive fluid (Merzendorfer, 2006; Schulze et al., 2012; Paszota et al., 2014). The level of expression of VF CHITINASE I is proportional to the number of mechanical stimulations and can only be detected after stimulation (either via mechanostimulation or via exogenous jasmonate/COR application). Only trace amounts can be detected in unstimulated traps, the major transcription takes place in stimulated *Dionaea* glands (Paszota et al., 2014; Böhm et al., 2016a).

SAG 12

In analogy to AtSAG12, Dionaea´s senescence-associated gene 12 (SAG 12) was found via the version 1.1.2 of the Carnivorome transcriptome browser (Tbro, Ankenbrand et al., 2016), resulting in reference comp232698_c0, which is similar to AT5G45890, GenBank: KT223141). SAG 12 very likely reflects a cysteine protease, although its biochemical function has not been described yet. SAG 12 is encoded by an mRNA transcript consisting of 1035 bp and encodes for a protein with 344 amino acids. In Schulze et al., 2012 it was referred to as Dionain-3 and

was among the most abundant proteins in the digestive fluid (Schulze et al., 2012; Böhm et al., 2016a).

SCPL 49

The serine carboxypeptidase-like enzyme SCPL 49 (Tbro reference comp_214244_c0.0, Tbro version 1.1.2, similar to AT3G10410, GenBank: KT223142) is relatively abundant in the digestive fluid of *Dionaea muscipula*. It belongs to the S10 family of serine proteases and to the plant serine carboxypeptidase III group. Serine caboxypeptidases cleave only the C-terminal peptide bond in polypeptides and have serine residues in their active site (reviewed in Breddam, 1986). Proteins belonging to the S10 family of serine proteases are only active at acidic pH-level. It is encoded by an mRNA transcript with 1467 bp and contains 488 amino acids (Schulze et al., 2012; Böhm et al., 2016a).

Nutrient uptake and transporters within the snap-trap

The main function of the three aforementioned marker transcripts of digestion induction (VF CHITINASE I, SAG 12 and SCPL 49) is, exemplary for the whole of secreted lytic enzymes within the digestive fluid, to process the prey nutrients by degrading the chitin polymer coat encasing the prey´s "flesh", followed by the proteins, nucleic acids, glycans and lipids into their respective monomers and to dissociate the nitrogen, phosphate and sulphate side groups for uptake by the plant via the snap-trap (Hedrich, 2015). This uptake is facilitated by specialized transport proteins (Böhm et al., 2016a).

Numerous of these transporters are already known and well characterized, for example **DmKT1** (KT = **K**$^+$ **t**ransporter) and **DmHAK5** (**HAK** = **H**igh-**a**ffinity **K**$^+$ transporter) for the uptake of the essential plant macronutrient potassium (K^+) and the **am**monium-**t**ransporter **DmAMT1** for the uptake of ammonium/NH_4^+ as well as the ion channel **DmHKT1** for sodium (Na^+) uptake. These transporters are localized in the gland-cell complexes, and at least DmHKT1 is expressed in the glands upon trigger hair bending, the number of transcripts being positively correlated with the number of mechano-electric stimulations (Scherzer et al., 2013; Scherzer et al., 2015; Böhm et al., 2016a; Böhm et al., 2016b). In contrast to most root-localized AMTs known from other (non-carnivorous) plants, DmAMT1 expression in the gland cells is not induced by the presence of ammonium (Scherzer et al., 2013). When comparing *Arabidopsis* and *Dionaea* transcriptome data, Bemm et al., (2016) found out that the *Dionaea* transcriptomic profile of insect-induced active traps during insect capture and digestion closely resembles that of non-carnivorous plants facing herbivore attack or wounding as opposed to fungal or bacterial infections.

Reopening of the trap

After the digestion process is completed, the trap reopens, leaving an empty husk of the insect behind to be washed away by wind and rain and is ready to trap the next prey (BBC, 2009; Escalante-Pérez et al., 2011; Bemm et al., 2016).

According to Fagerberg and Howe (1996), the reopening process of *Dionaea muscipula* traps can be subdivided in four consecutive steps (**Fig. 1.6**)

(1) Sealed stage: the last stage of trap closure before the trap begins to reopen
(2) De-appressed stage: characterized by a convex bulge in the upper region of the trap
(3) Release stage: in which the bulge region moved closer to the trap margins, initiating lobe separation, while the marginal spikes remained intertwined but begin to disentangle
(4) Fully opened stage: the trap lobes assumed a morphology similar to that of a non-stimulated trap.

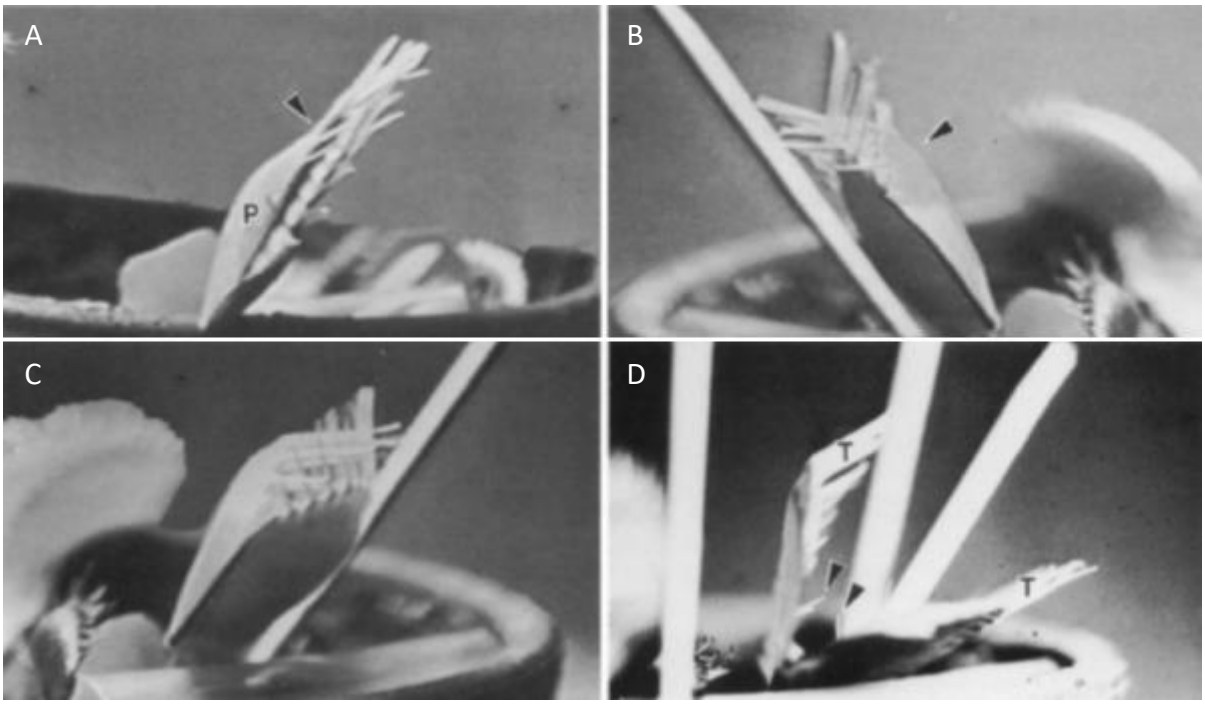


Fig. 1.6 Distinct steps in the reopening process of *Dionaea muscipula* traps
Fagerberg and Howe (1996) subdivide the reopening process of *Dionaea muscipula* traps in four steps. The first step is the **sealed stage** (A) with appressed margins (arrow) and the digestive pocket (P) of the trap lobe. The second stage is the **de-appressed stage** (B), where a bulge (arrow) has formed. The third stage is the **release stage** (C), where the margins of the trap are separated and the marginal spikes are beginning to disentangle. The fourth stage (D) is the **fully opened stage**, where the trap lobes (T) have unfolded to near pre-stimulation morphology.

According to Fagerberg and Howe, the axis of the marginal spikes changes after a trap closure process. According to this publication, prior to the first trap closure the axis of the marginal spikes lies in plane of the trap lobes, and after trap closure and reopening the axis of the marginal spikes lies no longer in the plane of the lobe, an indication that the trap has been closed at least once (See **Fig. 1.6 A, D**).

1.2.3 Phytohormones in plants – The interplay of jasmonic acid and abscisic acid

"Phytohormones are endogenous molecules occurring naturally in plants at very low concentrations. They do not have any nutritional function but act as signaling compounds that promote and influence plant development and physiology" (Sauer et al., 2013). Additionally, they "coordinate plant responses to stress, acting as bridges between the sensing process and the physiological responses" (de Ollas et al., 2015). Due to the lack of hormonal glands in plants, which are specialized to produce and secrete hormones, as they exist in animals, phytohormones in plants are generally produced by different tissues:

- Jasmonic acid is produced in shoots, in seedlings, in response to wounding and its synthesis is initiated in the chloroplasts (Katsir et al., 2008b). Different molecules of the jasmonate group, e.g. 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile) and methyl JA (MeJA), have been reported to be bioactive molecules in the JA-pathway (de Ollas et al., 2015).
- Abscisic acid (ABA) is produced in the roots, the terminal buds and the chloroplasts in the guard cells (Finkelstein, 2013; Vishwakarma et al., 2017).

There are many more phytohormones, for example auxins, cytokinins, ethylene, nitric oxide and gibberellins, but due to the scope of this thesis, they shall not be discussed further at this point.

The regular hunting cycle of *Dionaea* is initiated by mechanical stimulation of the trigger hairs, followed by rapid trap closure and ongoing trigger hair activation by the encased prey, followed by the activation of the Jasmonic acid (JA) pathway, the expression of hydrolases and transporters necessary for digestion, finally leading to secretion of the digestive fluid and uptake of the nutrients derived from the digested prey. The exogenous application of OPDA/MeJa, JA-Ile or coronatine (COR), the molecular mimic of JA-Ile, effectively short-circuits the hunting cycle, circumventing the need for mechanical stimulation and directly triggers secretion of lytic enzymes, followed by slow trap closure (Escalante-Pérez et al., 2011; Bemm et al., 2016).

1.2.3.1 Hormonal regulation of trap behavior and plant performance - Jasmonic acid/Jasmonates

Jasmonates are derived from phospholipids and have varying effects on plant physiology, from inducing growth over regulating plant defense gene expression in response to wounding/herbivory to reproductive development. They play a crucial role in regulating defense responses to a large range of biotic aggressors. Other processes where jasmonates are involved are lateral and adventitious root formation, seed germination, leaf senescence, glandular trichome formation and embryo and pollen formation (Wasternack and Strnad, 2016) as well as the response to UV radiation and reproductive development (Katsir et al., 2008b). As reviewed in Koo and Howe (2009), mechanical tissue damage is sufficient to trigger a rapid and transient JA/JA-Ile burst in the damaged tissue, but wounding can also activate de novo JA synthesis in undamaged parts of the plant. This is known for well-studied model organisms like *Arabidopsis thaliana*, *Nicotiana benthamiana* and *Solanum lycopersicum*. For *D. muscipula*, the physiological responses have to be analysed further.

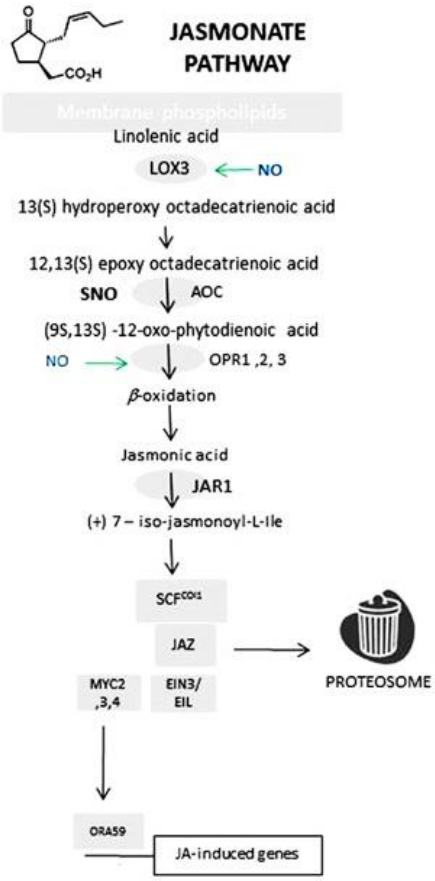


Fig. 1.7 The Jasmonate pathway
The diagram shows the conversion of Linolenic acid over several steps to JA-Ile, the bioactive form of JA (modified after Mur *et al*., 2013). In *Dionaea*, elevated 12-oxo-phytodienoic acid (OPDA) can be detected as fast as 30 minutes after trap closure (Escalante-Pérez et al., 2011).

Starting with linolenic acid as a precursor, over several steps OPDA is produced in the chloroplasts and transformed to JA in the peroxisome via ß-oxidation (See **Fig 1.7**). It is further metabolized in the cytosol by JAR1 to (+) 7 – iso – jasmonoyl-L-isoleucine (JA-Ile). JA-Ile interacts with the COI1-protein, a part of the Skp-Cullin-F-box-complex (SCF^{COI1}) which targets the jasmonate ZIM-domain (JAZ)-protein. JAZ is acting as a transcriptional repressor and gets degraded after COI-JAZ-interaction via the 26S proteasome. Thereby, a range of transcription factors, like EIN3/EIL and MYC2, 3 and 4 are released from transcriptional repression which finally leads to the activation of JA-responsive genes. The amplitude and duration of JA responses are controlled in large parts by intracellular JA-Ile levels (Sheard et al., 2010; Wasternack and Xie, 2010; Mur et al., 2013; Koo et al., 2014). Inactivation of JA-Ile has been found to be associated with members of the cytochrome P450 94 – family in *A. th* by oxidation of JA-Ile to 12OH-JA-Ile and 12COOH-JA-Ile (Koo et al., 2014) as well as modification via glycosylation, hydroxylation and glycosylation (Katsir et al., 2008b; Mur et al., 2013; Farmer et al., 2014; Ahmad et al., 2016; Wasternack and Strnad, 2016). COI1 (CORONATINE INSENSITIVE 1) is the JA receptor, together with JAZ1 they

build a receptor complex. JA-Ile binds to COI1, this binding leads to the formation of the COI1-JAZ complex. Thereby, JAZ1 is ubiquitinated and degraded (Katsir et al. 2008b; Gfeller et al., 2010; Pauweis and Gossens 2011; Gimenez-Ibanez et al., 2015; Böhm et al., 2016a).

It is well known for a long time that coronatine, a phytotoxin that is produced by the plant pathogen *Pseudomonas syringae*, is able to mimic Jasmonates and to activate the jasmonate pathway (Weiler et al., 1994). It causes stomata to reopen after closure in response to the detected pathogen-attack as a means of entrance for the pathogen into the host tissue (Melotto et al., 2009). Coronatine is structurally very similar to a distinct stereoisomer of JA-Ile, (+)-7-iso-JA-L-Ile [(3R,7S)], which is described as the most bioactive form of JA-Ile (Wasternack and Xie, 2010). COR binds to COI1 with a high affinity (K_d ~20 nM; -78.33 kJ mol^{-1} average binding free energy), whereas JA-Ile binds to COI1 with -47.68 kJ mol^{-1} average binding free energy, which shows that COR binds much stronger to COI1 than JA-Ile (Katsir et al., 2008a; Yan et al. 2009). If sprayed on the open trap of *Dionaea*, coronatine triggers secretion of the digestive fluid even in the absence of mechanostimulation, followed by slow trap closure, thereby short-circuiting the regular “hunting cycle”. Secretion of the digestive fluid after a single treatment with coronatine lasts for up to 14 days (Escalante-Pérez et al., 2011; own observation). Reopening of the trap after coronatine-treatment can take much longer than after prey-induced trap closure and digestion, it can take up to 30 days, or the traps even wither before reopening (Sickel et al., 2019).

In *D. muscipula*, it is known that mechanical stimulation and trap closure by prey elicits a rise in OPDA levels 30 minutes after prey capture. It was also observed that after the exogenous COR-application on traps, OPDA levels rose as well (Escalante-Pérez et al., 2011).

On the other hand, it was shown that the spray application of the synthetic inhibitor coronatine-O-methyloxime (COR-MO) effectively suppresses JA-induced gene expression of JAZ1/COI1 as well as expression of the marker hydrolases after mechanostimulation (Monte et al., 2014; Böhm et al., 2016a). There are several known inhibitors known to address the jasmonate pathway. Monte et al., (2014) described the development of a ligand-based antagonist of jasmonate perception, namely coronatine-0-methyloxime (**Cor-MO**), a derivative of COR. In *A. thaliana*, COR-MO binds to the COI1-receptor, as well as JA-Ile or COR, but instead of activating, COR-MO is preventing COI-JAZ – interaction, JAZ-degradation and the effects of JA-Ile or COR on several JA-mediated responses. It is also effective in reversing the effects of JA-Ile- or COR-treatments. **Phenidone** inhibits lipoxygenase (LOX), thus preventing the conversion of linolenic acid to 13(S)-HPOT, which is a precursor of MeJA (Cucurou et al. 1991) and **Neomycin** inhibits the accumulation of JA-Ile by increased turnover to 12-hydroxy-JA-Ile, that way inhibiting downstream expression of JA-Ile-responsive genes (Vadassery et al. 2014). These three inhibitors were used in this thesis on *Dionaea muscipula* (see 3.1.7).

1.2.3.2 Abscisic acid (ABA) – The drought-stress-hormone

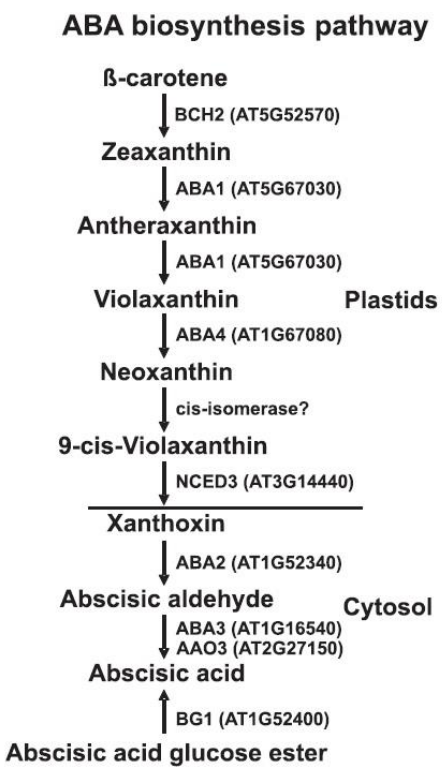


Fig. 1.8 The different steps of biosynthesis of ABA
The diagram shows the different steps of ABA-biosynthesis in *A. thaliana* in relation to the place of synthesis, starting from the precursor ß-carotene in the plastids to the final steps in the cytosol, with required enzymes and the known respective AGI codes given in brackets (Bauer et al., 2013).

Another hormone, known as the drought-stress-hormone, is abscisic acid (ABA). Its biosynthesis pathway in *A. thaliana* is well understood. ABA is a monocyclic sesquiterpenoid derived from ß-carotene and is produced in roots, the terminal buds and the in the plastids of the guard cells. Its metabolism begins with the plastidic MEP pathway (named for the first committed molecule, 2C-methyl- D-erythritol-4-phosphate), and after being modified over several steps (See **Fig. 1.8**), with the 9-cis-Violaxanthin as the last intermediate product in the plastids, the final modification takes place in the cytosol, with Xanthoxin as the first intermediate product in the cytosol, from which Abscisic acid (ABA) is formed via abscisic aldehyde. The last step in the ABA biosynthesis in *A. thaliana* requires molybdenum as a cofactor, and mutations in the cofactor ABA3, a molybdenum cofactor (MoCo) sulfurase which is required for correct MoCo function in *A.th*, together with abscisic aldehyde oxidase (AAO3) result in ABA-deficient plants (Xiong et al., 2001). In *Dionaea*, a high-affinity molybdate transporter (MOT1) was found in glands, which is consistent with the requirement of molybdenum as a cofactor in the catalytic center of several plant metabolic key enzymes, for example ABA synthesis (Bemm et al., 2016). It might therefore be that ABA synthesis in *A. thaliana* and *D. muscipula* are partly similar.

The naturally occurring and biologically active form of ABA is (+)-(2-cis,-4-trans)-ABA (or *S*-(+)-ABA). It can easily enter cells across plasma membranes, but membrane-localized transporters also exist. Local active ABA levels reflect a balance of ABA biosynthesis and inactivation by turnover or conjugation, further modified by compartmentation and transport. ABA leads, among many other effects (there are thousands of genes that are regulated by ABA in various contexts, Finkelstein, 2013; Yoshida et al., 2014) to stomatal closure in plants to prevent water loss. If exogenously sprayed upon *A. th.* guard cells, 1080 genes were up- and 470 downregulated (Bauer et al., 2013).

ABA biosynthesis is directly correlated with plant/tissue water status (de Ollas et al., 2015), and ABA plays, among others, a crucial role in response to abiotic stress, for example drought stress.

As reviewed by Finkelstein (2013) and Zhang et al., (2015), and published in Dittrich et al., (2019), in *A.th*. a network of proteins of the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) family has been identified as potential ABA receptors. The PYR/PYL/RCAR family together with protein phosphatases type-2C (PP2Cs), Snf1 (Sucrose-non-fermentation1)-related kinases subfamily2 (SnRK2s) and downstream substrates constitute the core ABA signaling network (Zhang et al., 2015).

In *A.th*, ABA is formed mainly in the event of limited cell water availability (Hauser et al., 2011). If the plant experiences a water-shortage via soil drying, this is perceived by the roots, probably by the PYR/PYL/RCAR-family. The roots then synthesize ABA, and this is delivered to the different plant parts via the xylem. Additionally, guard cells are able to synthesize ABA on their own after the contact with xylem-derived sulfate, which seems to be a chemical signal for drought that induces stomatal closure, via the activation of QUAC1/ALMT12 and/or the induction of guard cell ABA synthesis (de Ollas and Dodd, 2016; Malcheska et al., 2017; Dittrich et al., 2019). The increased ABA content leads to a broad variety of plant responses, for example increased production of waxes to reduce transpiration via the leaf surface (here ABA activates an ABA-inducible transcription factor) or increase of osmolyte production like sugars and alcohols to maintain cell turgor (Seki et al., 2007; Seo and Park 2011). In *A.th*, incubation of abaxial epidermal sections with 10 µM ABA for 2 h resulted in reduction of stomatal aperture (=stomatal closure) from approx. 4.5 µM to approx. 1.5 µM, so by roughly 66 % (Suhita et al., 2004).

1.2.3.3 Interaction of Jasmonates and abscisic acid

As described by de Ollas and Dodd in 2016, ABA accumulates in roots and shoots even under optimal conditions, but this is caused by any cellular turgor-decrease. It is synthesized in many biosynthetic steps, but NCED seems to be the key biosynthetic enzyme in the pathway because its transcription is correlated to the plant´s water status. The interconnection between JA- and ABA-correlated molecular responses in common model organisms like *A. thaliana* and *Oriza sativa* is summarized in **Fig. 1.9.** For *D. muscipula*, the physiological responses have to be analysed further.

It has been described by de Ollas et al., (2015) that in drought-stressed *A. th*-roots accumulated JA leads to a JA-Ile-buildup. This buildup and the JA-Ile-COI1-interaction was reported to be essential for ABA-biosynthesis and -accumulation in drought-stressed roots. It has been further described that JA deficiency in *jar1* mutants can diminish ABA accumulation. The induction of the transcription factor MYC2, which is induced by ABA as well as by JA, seems to rely on the JA receptor COI1 (Lorenzo et al., 2004). ABA is responsible for inducing stomatal closure in the drought stress response, but when ABA or MeJA were exogenously applied to detached leaves, they both elicited stomatal closure (Suhita et al., 2004). In contrast to this

finding, applying MeJA to isolated epidermal strips of ABA deficient mutants (*aba2-2*) proved ineffective to elicit stomatal closure, as well as when wild-type plants were pretreated with fluridon (FLU), an ABA-biosynthesis inhibitor. ABA could elicit stomatal closure in all these cases. Therefore, it is hypothesized that MeJA might act by increasing ABA biosynthesis (Hossain et al., 2011).

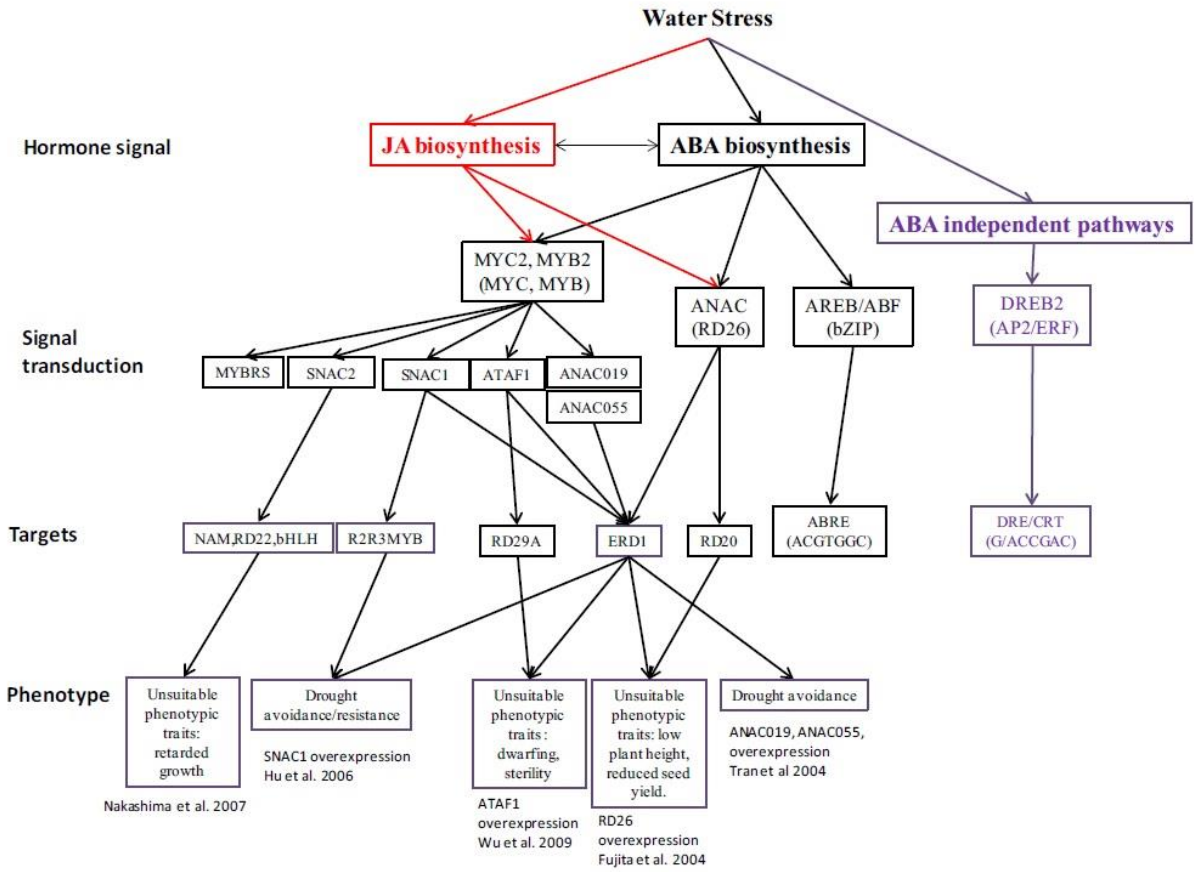


Fig. 1.9 Summary of signaling pathways related to JA–ABA interaction in response to drought
In the common model plants like *Arabidopsis thaliana* and *Oryza sativa*, it is known that water stress can enhance JA accumulation as well as ABA levels. This plant response map shows how the hormonal signaling networks are intertwined. Special attention shall be drawn to the fact that JA- and ABA-biosynthesis have an effect on the same signal transduction pathways, for example MYC/MYB or ANAC (ABA-responsive NAC genes, Bu et al., 2008).
(de Ollas and Dodd 2016, image used under the CC 4.0 license http://creativecommons.org/licenses/by/4.0).

When sprayed on *Dionaea muscipula* trap tissue, it was found that ABA has a negative effect on the trap sensitivity: if treated with exogenously sprayed ABA for 48 h, the trap closing threshold was elevated to mostly 3, sometimes 4-6 and even 10 APs required to induce trap closure (Escalante-Pérez et al., 2011). The exact interaction of the ABA- and the JA- pathway in *Dionaea* has still to be elucidated. In *A. th*, several interactions of the JA- and the ABA-pathway have been discovered. It was reported in Pan et al., (2020) that several JAZ repressors

of JA signaling physically interact with ABSCISIC ACID INSENSITIVE3 (ABI3), a critical transcription factor that positively modulates ABA signaling, and that JAZ proteins repress the transcription of ABI3 and ABI5.

Wounding and hormonal response

Herbivorous insects induce JA- and ABA-dependent pathways, and herbivory leads to elevated JA-levels (Vos et al., 2013, Wasternack and Hause, 2013). As described above, the hormonal defense system of non-carnivorous plants in reaction to an infection or an attack from a pathogen or an herbivore is similar to the hormonal reaction of *Dionaea muscipula* upon prey capture, including OPDA as a precursor for JA/JA-Ile-elevation. (Escalante-Pérez et al., 2011; Wasternack and Hause, 2013; Bemm et al., 2016). In *Dionaea*, insect-activated traps displayed a high transporter activity together with a massive wounding response compared with non-stimulated tissues, and the genes which where differentially expressed were mainly enriched for processes controlled or triggered by JA and ABA. Genes encoding the key enzymes of JA biosynthesis such as LOX2 (LIPOXYGENASE 2), AOS (ALLENEOXIDE SYNTHASE), and OPR3 (OXOPHYTODIENOATE-REDUCTASE 3) were highly induced, as well as the ABC-transporter PXA1 (PEROXISOMAL ABC-TRANSPORTER 1), mediating the import of the JA precursor OPDA (12-oxo-phytodioneic acid) into peroxisomes, and peroxisomal enzymes of the beta-oxidation chain generating JA from OPDA were stimulus-induced. JAR1 (JASMONIC ACID RESISTANT 1), which finally converts JA into its physiologically active form JA-Ile, appeared specifically induced by insects. It was shown that *Dionaea´s* transcriptomic profile during insect capture and digestion closely resembles that of *A.th* plants facing herbivore attack or wounding (Bemm et al., 2016).

1.3 Aim of this work

The production and maintenance of active traps requires nutrients and energy, and the trapping process itself is very energy- and water-consuming. Therefore, *Dionaea* has developed safeguard mechanisms which prevent futile trap closure (triggered for example by inanimate matter) or the onset of secretion triggered by unprofitable prey. Many resources and efforts have been dedicated to unravel how *Dionaea* developed these abilities, and how they work in detail.

The main focus of this work was to uncover the regulation of the trapping mechanism and prey digestion of *Dionaea muscipula*. It was already known from previous work that the exogenous application of the drought-stress-hormone ABA on the trap resulted in reduced trap-triggering sensitivity and elevated the threshold of required trigger hair bendings for trap closure from 2 to levels between 3 and 10. It was also known that the exogenous application of COR short-circuited the *Dionaea* "hunting cycle", resulting in direct secretion of the digestive fluid followed by slow trap closure. It was apparent by these observations that ABA and JA-Ile are physiological antagonists.

Based on these facts, the following questions arose to be answered within this thesis:

1. How many mechanical stimulations are required for a constant activation of the digestive fluid secretion, represented by marker transcript expression? Is there a spatio-temporal component (how many stimulations and when) built in the safeguard mechanism?
2. When the digestion process has started, is it irreversible or can it be stopped again?
3. What are the effects of JA-Ile (or COR as its mimic) and ABA on *Dionaea*'s physiology? Can one counteract the effects of the other?

In order to answer those questions, various conditions including mechanostimulation, hormone treatment and inhibitors were examined with regard to the effect on marker transcript expression, which was measured by quantitative real-time PCR (qPCR). To elucidate the physiological effects of JA/COR and ABA on the onset of secretion, the gas exchange measurement technique was employed. To get complementary results, the two techniques were used in conjunction.

Work done in parallel to the above-mentioned points were the establishment of a way to generate multiple plants from a single mother-plant using sterile-culture techniques, and the generation of transgenic protoplast. The generation of genetically identical plants for stock-keeping reasons and the possibility for genetic modifications are important to further investigate regulatory circuits and metabolic processes.

2. Material and Methods

2.1 Material

2.1.1 Devices

Device	Company
Acquity BEH C18 VanGuard, 2,1 x 5 mm, 1,7 µm	Waters GmbH, Eschborn
Acquity BEH C8 VanGuard, 2,1 x 5 mm, 1,7 µm	Waters GmbH, Eschborn
Acquity-UPLC™	Waters GmbH, Eschborn
Quattro Premier Triple Quadrupol mass spectrometer	Waters GmbH, Eschborn
AccuBlock Digital DryBath	Labnet (Edison, USA)
BioZero BZ-8011 (Microscope)	Keyence, Neu-Isenburg
Centrifuge 5180R	Eppendorf, Hamburg
Centrifuge 5424	Eppendorf, Hamburg
Centrifuge 5430R	Eppendorf, Hamburg
Digital pH – meter 646	Knick International, Berlin
DM6000 CS (Confocal LSM)	Leica Microsystems, Wetzlar
EasyCast Electrophoresis System B1A	ThermoFisher Scientific, Dreieich
Ebq 100 (electronic ballasts) with 75 W Xenon Lamp	Lightning and Electronics, Jena
Experion Automated Electrophoresis System	BioRad Laboratories, München
EZ4 HD (Binocular)	Leica Microsystems, Wetzlar
Ikamag REO (Heating and magnetic stirrer)	Ika Labortechnik, Staufen
Innova 4230 Refrigerated Incubator/Shaker	New Brunswick / Eppendorf, Hamburg
Intas UV-Imager	Intas, Göttingen
Kern 440 (Digital scale)	Kern u. Sohn GmbH, Balingen-Frommern

Kern ABS 220-4 (Analytical scale)	Kern u. Sohn GmbH, Balingen-Frommern
L46 (Lab shaker)	GLW, Würzburg
Mastercycler epgradient S Realplex^2 (PCR cycler)	Eppendorf, Hamburg
Mastercycler personal (PCR cycler)	Eppendorf, Hamburg
Minispin plus (Centrifuge)	Eppendorf, Hamburg
Model G28 (Bacteria shaker/Incubator)	New Brunswick /Eppendorf, Hamburg
Leica MZFLIII + DFC500 mit C-Mount (Microscope)	Leica Microsystems, Wetzlar
NanoDrop 2000c Spectrophotometer	ThermoFisher Scientific, Dreieich
Novaspec III (Spectrophotometer)	Amersham Biosciences (Amersham, GB)
NuAire Classic II (Clean bench)	Zapf Instruments e.K, Sarstedt
PDS 1000/HE Biolistics (Particle delivery system)	BioRad Laboratories, München
PlateFuge Microcentrifuge	Benchmark Scientific (Edison, USA)
Polymax 1040 (Horizontal shaker)	Heidolph Istruments, Schwabach
Power Supply P25	Biometra, Göttingen
Primus 96 plus (PCR cycler)	MWG-Biotech, Ebersberg
Programmable Rotor Mixer RM Multi-1	StarLab, Hamburg
Thermo Shaker TS-100	PeqLab/VWR Life Science, Erlangen
Vapor Pressure Osmometer 5500	Schlag GmbH, Bergisch Gladbach
VortexGenie 2	Scientific Industries (New York, USA)

2.1.2 Chemicals, reagents and other material

2-Propanol	Roche Diagnostics, Mannheim
ABA = 2-cis,4-trans-abscisic acid	Sigma-Aldrich, München
Acetic acid	Fluka, Neu-Ulm
Agarose	Invitrogen, Karlsruhe
Ampicillin	Appligene, Heidelberg
Bacto Yeast Extrakt	Carl Roth, Karlsruhe
Bacto-Trypton	Becton & Dickinson, Heidelberg
Bovine serum albumin/Albumin fraction V (BSA)	Carl Roth, Karlsruhe
BSA = Bovine serum albumin	Sigma-Aldrich, München
Calcium chloride	Merck, Darmstadt
Cellulase R 10	Yakult Pharmaceuticals (Tokyo, Japan)
Celaflor Roundup Alphee	Scotts Celaflor GmbH, Mainz
Chloramphenicol	Sigma, Taufkirchen
Chloroform	Carl Roth, Karlsruhe
Coronatine	Sigma-Aldrich, München
Coronatine-O-methyloxime (COR-MO)	Prof. W. Boland (Max Planck Institute for Chemical Ecology) Jena
CTAB = Hexadecyltrimethylammoniumbromide	Sigma-Aldrich, München
DECP = Diethylpyrocarbonate	Sigma-Aldrich, München
Desoxynucleotid triphosphate (dNTPs)	Carl Roth, Karlsruhe
Diethylether	Carl Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, München
Disodium hydrogenphosphate	Carl Roth, Karlsruhe
Ethanol	Carl Roth, Karlsruhe
Ethylenediamine tetraacetic acid (EDTA)	Carl Roth, Karlsruhe

Etylacetate	Carl Roth, Karlsruhe
Fluorescein-Diacetate (FDA)	Thermo Scientific, Schwerte
Formic acid	Carl Roth, Karlsruhe
Glucose	Merck, Darmstadt
Glycogen, RNA grade	Thermo Scientific, Schwerte
HPLC Gradient Grade H_20	Carl Roth, Karlsruhe
IBA = Indole-3-butyric acid	Duchefa Biochemie (Haarlem, Netherlands)
Isopropanol	Roche Diagnostics, Mannheim
Kanamycin	Sigma-Aldrich, München
Lithium chloride	Sigma-Aldrich, München
Magnesium chloride	Carl Roth, Karlsruhe
Methanol	Carl Roth, Karlsruhe
MS = Murashige and Skoog Medium	Duchefa Biochemie (Haarlem, Netherlands)
n-Heptan	Carl Roth, Karlsruhe
Neomycine sulfate	Carl Roth, Karlsruhe
Parafilm	Pechiney Plastic Packaging (Chicago, USA)
Pectolyase Y 23	Seishin Pharmaceuticals (Tokyo, Japan)
1-Phenyl-3-pyrazolidinone (= Phenidone)	Sigma-Aldrich, München
Polyoxyethylensorbitanmonolaurat (Tween 20)	Carl Roth, Karlsruhe
Potassium acetate	Carl Roth, Karlsruhe
Plant preservative mixture (PPM)	Plant Cell Technology (Washington, USA)
PVP 40 = Polyvinylpyrrolidone, MW 40.000	Sigma-Aldrich, München
PVPP = Polyvinylpolypyrrolidone	Sigma-Aldrich, München
Select Agar	Invitrogen, Karlsruhe

Sodium acetate solution (3 M), pH 5.2	Thermo Scientific, Schwerte
Sodium chloride	Carl Roth, Karlsruhe
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, München
Sodium dihydrogen phosphat	Carl Roth, Karlsruhe
TCEP = Tris(2-carboxyethyl)phosphine hydrochlorid	Sigma-Aldrich, München
Tris	Carl Roth, Karlsruhe
Triton X-100 (Octylphenolethylenglycoether)	Carl Roth, Karlsruhe

Further, unstated chemicals were purchased from the following companies:

Fluka (Neu-Ulm); Invitrogen (Karlsruhe); Merck (Darmstadt); Carl Roth (Karlsruhe); Serva

(Heidelberg); Sigma (Taufkirchen/Steinheim), and ThermoFisher Scientific (Different locations).

2.2 Primers

2.2.1 qPCR primers

Table 2.2.1 List of all qPCR-primers with description

Name	Sequence (5'→3')	length [bp]	Temp.-range	Fragment [bp]	Tbro - component
(DmACT) Actin fwd	TCT TTG ATT GGG ATG GAA GC	20	50 -60	137	comp226979_c1_seq1
(Dm ACT) Actin rev	GCA ATG CCA GGG AAC ATA GT	20			
Dm SCPL 49 fwd	ACT TAA TCC GGG TAT CA	17	53 - 57	400	comp214244_c0.0
Dm SCPL 49 rev	AGG TCC ATA GGT ATT CA	17			
VF CHITINASE I fwd	GAA AGT TAT TAC GGT CG	17	50 - 57	295	comp234852_c1
VF CHITINASE I rev	CTT TAC CAC ACT CAA CG	17			
Dm SAG 12 fwd	CGC ATT CGA GTA TAT GA	17	53 - 62	386	comp231081_c0_seq1
Dm SAG 12 rev	CAA CAT TCC TTT GCA TC	17			
Dm AMT1 fwd 2	TTG CTA CCA AGA AAC AC	17	53 - 59	344	comp173079_c0_seq1
Dm AMT1 rev 2	TGA GTT GAT GTA AGG AG	17			
Dm COIp LC V2 fwd	TTA CGT CGG ACT GTA T	16	48-62	449	comp229648_c0_seq1
Dm COIp LC V2 rev	AAC TCT AAG CTA AGA CAT	18			
Dm JAZ1.1p LC fwd	GTG TTC AAC GAC TTC C	16	50-60	307	comp224086_c0_seq2
Dm JAZ1.1p LC rev	TTG TTA AGG TGT ATG GC	17			
Dm HAK5.4p LC fwd	ACT ATG TGT ATC GGA AG	17	52-58	386	comp217444_c1_seq1
Dm HAK5.4p LC rev	CTC CCG AAT GAA TAC C	16			

2.2.2 oligo dT-primer

The oligo dT-primers used to generate of cDNA are a blend of 25-mers with a variable 3′ anchor region:

5‘ - TTT TTT TTT TTT TTT TTT TTT TTT TVN - 3‘

V stands for A/C/G and N stands for A/C/G/T

2.3 Vectors

The pSAT-UBQ10prom::VenusYFP::mTurqouise2 – vector (created and provided by Dr. Kai Konrad, Julius-von-Sachs-Institut, Botany 1, Würzburg) was used to transfect *Arabidopsis* and *Dionaea* protoplasts. The vector is based on the pSAT-vector series created by Tzfira et al., 2005.

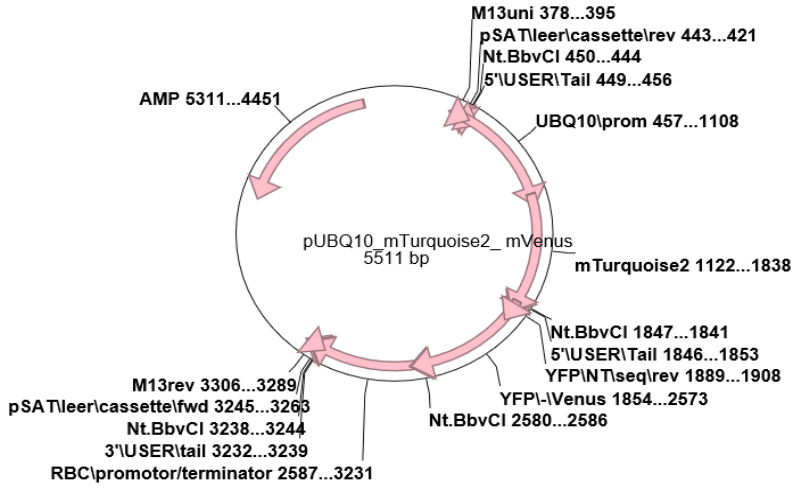


Fig. 2.3.1 Vector card of UBQ10::VenusYFP::mTurquoise2 in the binary vector pSAT

The pSAT-UBQ10::VenusYFP::mTurqouise2 – vector was used to transfect protoplasts. It has a length of 5511 bp and consists of a fusion between the fluorescent proteins mTurquoise2 and VenusYFP under the control of an UBQ10 – promoter.

2.4 Enzymes

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT)	Promega, Mannheim
RNaseA	Roche Diagnostics, Mannheim

Enzymes provided within or required for use of a kit are not listed separately.

2.5 Molecular biological reagents and kits

ABsolute qPCR Capillary Mix	ThermoFisher, Schwerte
Experion RNA HighSens analysis Kit	BioRad Laboratories, München

2.6 Software and internet databases

Transcriptome browser (Tbro)

Using various RNAseq data sets, the Tbro Transciptome browser was used to browse assembled *Dionaea* transcriptomes, focusing on the RNA expression profiles in various tissues after various stimulations. (Ankenbrand et al., 2015, Bemm et al., 2016).
http://tbro.carnivorom.com/

LightCycler® Probe Design Software 2.0 (Roche Applied Science)

The software was used to design suitable primers for qPCR analysis according to the manufacturer's instructions

https://lifescience.roche.com/en_de/products/lightcycler14301-probe-design-software-20.html

https://lifescience.roche.com/content/dam/RMS/lifescience/Documents/PDF/lc_probe_design_software_20.pdf

accessed 06.11.2018

Gas exchange data analysis

The data obtained using the gas exchange machine was analysed using the OriginPro2016Pro software.

2.7 Methods

2.7.1 Generation of RNase-free water using DEPC

RNases are RNA-degrading enzymes and ubiquitous in nature. To obtain RNase-free water for use in experiments with RNA, the water needed was mixed with the adequate amount of DEPC to obtain a 0.1 % (v/v) solution, e.g. 1 ml DEPC in 1 l of water. This mixture was stirred for at least 2 hours at room temperature (RT) during which RNases were inhibited by covalent modification of histidine, lysine, cysteine, and tyrosine residues (Wolf et al., 1970). Afterwards the DEPC-treated water was autoclaved to inactivate DEPC.

2.7.2 RNA extraction

RNA was extracted from plant material of the Venus flytrap that had been ground to powder using liquid nitrogen together with a mortar and a pestle. The mortar and the pestle were placed in a Styrofoam box, and the box and the mortar and pestle were pre-cooled with liquid nitrogen. Once this was achieved, the plant material was ground to a very fine powder, which was then transferred with a fine metal spatula to a reaction cup which lid has been sliced once using a sharp razor blade, so that expanding nitrogen from the plant material would not force it to open later on in the process. This mechanical breakdown of the cell walls was the first step in the isolation process.

The following buffer was used for the RNA extraction:

RNA – Extraction buffer	
• CTAB (w/v)	2 %
• Polyvinylpyrrolidone (=PVP) K25	2 %
• Tris-HCL pH 8.0	100 mM
• Na-EDTA pH 8.0	25 mM
• NaCl	2 M
• DEPC-H_20	ad. desired volume

700 µl extraction buffer were preheated to 65 °C. A small amount (tip of a spatula) of PVPP and 7 µl TCEP (1:100) were added. Roughly 100 mg of plant material was given to the vessel and vortexed immediately for 1 min to further break down the cell material. Afterwards the mixture was incubated at 65 °C for 10 to 15 min. Following this 700 µL chloroform:Isoamyl alcohol (24:1) were added and the vessel was inverted several times. To separate the phases it was then centrifuged at 10.000 x g for 20 min at 4 °C. Afterwards the upper aqueous phase was transferred into a new reaction vessel. For precipitation 175 µl LiCl (8 M) were added and the mixture was inverted several times. The precipitation occurred at 4 °C ON. Next the precipitated RNA was pelleted for 20 min at 13.000 x g and 4 °C. The supernatant was discarded and the pellet was resuspended in 100 µL DEPC-H_20, then incubated for 10 min at 65 °C. Afterwards another precipitation step using 0.1 Vol 3 M NaAcetate pH 5.2 (10 µL) and

2.5 Vol (250 µL) of 96 % EtOH was performed at -20 °C for at least 1 h. Following a centrifugation step at 13.000 x g at 4 °C for 20 min, the supernatant was discarded. 500 µl 70 % EtOH were added and the reaction vessel was inverted several times. After another brief centrifugation step at 13.000 x g at 4 °C for 5 min, the supernatant was discarded very exactly. The reaction vessel was now placed at 37 °C until the remaining EtOH was evaporated. The dry pellet was then resuspended in 40 µl of DEPC-H_20 and incubated for 10 min at 65 °C to quantitatively dissolve the RNA. The RNA was stored at -20 °C.

2.7.3 Analysis of RNA quality

Due to the extraction method used, extracted RNA from the Venus flytrap contained several unknown metabolites and therefore always had a distinct color (green to yellow), hence the determination of RNA quantity and quality using a spectrophotometer was not applicable. Instead, a BioAnalyzer (Experion Automated Electrophoresis System) was used. It was based on a LabChip system and could provide accurate details on Venus flytrap RNA quantity and quality using an intercalating dye. For reference and instructions see manufacturer´s information. The RNA dilution used in the BioAnalyzer was 1:50.

2.7.4 Degradation of genomic DNA using a DNase digest

In order to generate complementary DNA (cDNA) for analysis using qPCR, contaminating genomic DNA originating from the source material had to be removed from the RNA sample using a DNase digest. For a digestion assay, 1 µg of RNA was used. The DNase used was DNase I (AppliChem), for reference and instructions see manufacturer´s information. 0.5 µL RNase Inhibitor (Fermentas) were added, the total volume was adjusted to 10 µl using DEPC-H_20 (or a multiple of that, ingredients were scaled accordingly) and the digestion was performed at 37 °C for at least 30 min.

Following the digestion process, the remaining RNA had to be precipitated. Hence the assay was filled with DEPC-H_20 up to a volume of 100 µl. Then 0.1 Vol of NH_4-Acetate (5 M), 1 µg Glycogen (20 mg/ml), 2/3 Vol Isopropanol were added and the vessel was centrifuged for 20 min at 13.000 x g at 4 °C. After discarding the supernatant, 500 µl of 70 % EtOH were added, the vessel was inverted several times and then again centrifuged for 15 min at 13.000 x g at 4 °C. Thereafter the supernatant was removed and the resulting pellet dried at 37 °C. After complete desiccation, the pellet was resuspended in 7 µL DEPC-H_20.

2.7.5 Synthesis of complementary DNA (cDNA) using an mRNA template

For analyzing the isolated RNA further, the messenger RNA (mRNA) needed to be transcribed into cDNA to be analyzed in a qPCR reaction. For this,

• 6,7 µL of the DNA-free RNA (**See 2.7.4**) suspended in DEPC-H_20
• 2 µL 5x M-MLV Buffer
• 0,5 µl dNTP`s (10mM) and

- 0,4 µl oligo – deoxythymine (dT) 5’ CDS primer (100µM)

were added. As every strand of mRNA has a poly-A-tail, the oligo-dT 5´ - primer specifically binds to this tail and ensures only conversion of mRNA into cDNA. This mixture was heated for 2 min to 70 °C to denature secondary RNA structures. Afterwards, it was immediately put on ice. Then 0,4 µL of M-MLV reverse transcriptase were added and the reaction was performed at 42 °C for at least 1 h. Afterwards the cDNA was stored at -20 °C for further use.

2.7.6 Real-time quantitative PCR (qPCR)

The real-time quantitative PCR (qPCR) is a method to quantitatively analyse the expression of one or more specific genes by quantifying the mRNA abundance (i.e. the cDNA content in a sample which was generated from the mRNA) via the use of a fluorescent dye. The dye used in this reaction (SybrGreen I) intercalates in double-stranded DNA, then absorbs light at 494 nm and emits light at 521 nm. This fluorescence is detected after every elongation step. As the PCR reaction continues, more and more double-stranded DNA strands exist, hence more dye intercalates and a stronger fluorescence signal can be detected.

A qPCR reaction consists of three phases (early phase: signal beneath detection limit, log phase: exponential amplification, and plateau phase: suboptimal amplification due to reagent shortage (e.g. primers, dNTPs) and inhibiting reaction products), the correct quantification is only possible during the log phase (Applied Biosystems application note).

SybrGreen I also binds to single stranded DNA and RNA, but the fluorescence is much weaker. For all qPCR reactions, the ABsolute SYBR Green Capillary Mix (ThermoFisher) was used. For details and references see producers manuals.

For each assay the following substances were used:

ABsolute SYBR Green Capillary Mix	10 µl
Primer fwd	300 nM
Primer rev	300 nM
cDNA (dilution 1:20)	2 µl
H_20	Fill up to 20 µl
Total volume	20 µl

“LoBind” reaction vessels (Eppendorf) were used to minimize interaction of oligonucleotides with the vessel surface. Additionally, water with 10 ng/µl tRNA was used to saturate binding sites at the vessel surface.

2.7.7 Generation of a qPCR standard probe

For the correct calculation of the amount of cDNA in the assay standard probes were used. They consist of known concentrations of a specific DNA fragment of known length of the gene of interest (GOI) that has been generated via conventional PCR using the same primer pair as was used during the qPCR reaction. Standard probes were created in "LoBind" reaction vessels (Eppendorf) to minimize interaction of oligonucleotides with the vessel surface. Additionally, water with 1 % of 10 µg/µl tRNA was used to saturate binding sites at the vessel surface. The following standards were generated:

Standard probe	Concentration
1	10 ng / µl
3	100 pg / µl
5	1 pg / µl
7	10 fg / µl
8	1 fg / µl
9	0.1 fg / µl
10	0.01 fg / µl

In the qPCR reactions, 2 µl of the standard probes 7 to 10 were used to generate a baseline based on which the results of the reactions of interest were calculated.

2.7.8 Analysis of qPCR results

Using the standard probes with the known concentration of DNA, the qPCR cycler calculates the amount of SybrGreen I in each sample at the Ct. The following calculation was used

(1) $1\ \text{fg}\ 1000\ \text{bp dsDNA} = 910\ \text{molecules}$

(2) $\text{absolute amount of molecules} = \frac{910}{\text{fragment length [kb]}} * \text{SYBR Green I [fg]}$

(3) $x = \frac{\frac{910}{\text{fragment length [kb]}} * \text{SYBR Green I [fg]}}{\frac{910}{\text{reference gen [kb]}} * \text{SYBR Green I [fg]}} * 10\,000$

to get absolute results for later use. In formula (3) the obtained number of GOI molecules was normalized to 10.000 molecules of DmACT as a reference gene (TBro comp226979_c1_seq1, or GenBank:KC285589, Dm_00017292-RA, see **2.2.1**). The number 10.000 was chosen arbitrarily for optimal presentation of the results.

2.7.9 Mechanical processing of traps

Whenever an experiment required harvesting of a trap of the Venus flytrap, the trap was cut with a sharp razor blade at the base of the trap, immediately wrapped in labelled aluminum foil, shock-frozen in liquid nitrogen and stored at -80 °C until it was processed. When

processed, the trap was ground to powder, using liquid nitrogen and a pre-cooled mortar and pestle, and stored in a reaction vessel with pierced lid for pressure compensation.

2.7.10 Protoplast generation

Protoplasts are the smallest fundamental cellular entities, i.e. the contents of a living cell including the plasma membrane. In plants, protoplasts are generated by enzymatic degradation of the cell wall, resulting in spherical, living cells. Without the cell wall the protoplast is susceptible to changes in turgor pressure, which is why the osmolarity of the surrounding medium is crucial for the survival of the protoplast. The following solutions were used for the generation of *Dionaea* protoplasts:

Enzyme solution

For the first step, the following substances were mixed:

• Cellulase (Onozuka) RS	2 %
• Pectolyase Y-23	0,025 %
• Pepstatin A	50 µg
• MES-Tris pH 5.7	20 mM (preheated to 60 °C)

Enzyme powder and MES-Tris were carefully mixed using a spatula. The mixture was incubated at 60 °C for 10 minutes to inhibit proteases. After cooling down to RT, the following substances were added:

• Bovine serum albumin (BSA)	0,2 %
• KCl	40 mM
• $CaCl_2$	12.5 mM
• ddH_20	ad 400 ml

The osmolarity was checked using a vapor pressure osmometer and adjusted with Mannitol (powder) to 400 mOsmol/kg. The enzyme solution was stored at -20 °C.

Washing solutions

WI solution (400 mOsmol/kg)

MES-Tris pH 5.7	5 mM
KCl	20 mM

The osmolarity was checked and adjusted using Mannitol (powder).

W5 solution (400 mOsmol/kg)

• NaCl	100 mM
• $CaCl_2$	100 mM
• KCl	5 mM
• MES	1.5 mM

The pH was adjusted with KOH to 5.7, the osmolarity was checked and adjusted if necessary using Mannitol (powder).

PEG solution

• $CaCl_2$	0.27 M
• Mannitol	0.55 M
• PEG 4000 (Sigma)	1,11 g
• ddH_20	ad 3.6 ml

The PEG solution was heated to approx. 50 - 60°C and stirred using a magnetic stirrer until the PEG 4000 had dissolved. Thereafter it was cooled down to room temperature. PEG was used to make the cell membrane soluble for the DNA, excess use should be avoided as it would increase the risk of rupture for the membrane.

MMG – solution (15 ml)

• MES-Tris pH 5.7	4 mM
• $MgCl_2$	15 mM
• Mannitol	0.4 M

The osmolarity of this solution should be around 380 – 450 mOsmol/kg after mixing.

Generation of protoplasts

The enzyme solution was thawed and then filtered using a syringe-mounted membrane disc filter (0.45 µM pore size), and poured into a clean 10 cm diameter petri dish. For generating protoplast from leaf tissue, approx. 1-2 g of fresh green healthy leaf material was used. The midrib was cut out using a new, thin razor blade and discarded. The leaf was then cut in small stripes that were, directly after cutting, completely submerged in the enzyme solution. The petri dish was wrapped in aluminum foil and placed in a vacuum desiccator for 15 min to infiltrate the tissue with enzyme solution. Thereafter it was placed on a horizontal shaker for 4 h at 40 – 60 RPM. After the incubation period the solution was filtered through a pre-wetted (WI or W5 solution) mesh with 75 µM – 100 µM pore size in a 50 ml reaction tube using a funnel. Some WI or W5 solution was poured into the petri dish with the remaining plant material and incubated for 5 minutes. During this time remaining protoplasts swelled and were released from the plant material. The solution was again filtered through the mesh and the reaction tube was filled up to 50 ml. The tube was then centrifuged for 1-3 min with 40 – 60 x g with no acceleration and only minimal brake settings. The protoplast pellet was washed three times using 50 ml WI or W5 until the solution became clear. The protoplast pellet was resuspended in 1-4 ml of WI or W5 solution. It was incubated on ice or at 4 °C for 30 min. During this time the protoplasts assumed a spherical shape and sedimented at the bottom of the tube.

2.7.11 Protoplast transformation

From the sedimented protoplasts (**see step 2.7.10**) 200 µl – 1000 µl (depending on the cell density) were placed in a 2 ml reaction vessel with a round bottom and centrifuged at 100 x g for 1 min. The supernatant was discarded and the protoplasts were resuspended in 2 ml MMg–solution.

For each transformation assay, 200 µL protoplasts resuspended in MMg were used and 30 µg of DNA for single plasmids (2 x 15 µg for two plasmids) were added. This mixture was gently pipetted up and down three times using a 1000 µl pipette tip with the tip cut off. Then 220 µl PEG solution were added and the tube was shaken gently to mix. This mixture was incubated for 5-10 minutes at RT. After the incubation 1.6 ml of W5 solution were added, the vessel was shaken gently, then centrifuged for 1 min at 100 x g. The supernatant was discarded and 0.5 ml of W5 solution were added. The protoplasts generated in this way were then transferred into a 4 cm diameter petri dish. They were incubated for 24 hours in the dark, thereafter they were examined for fluorescence.

2.7.11.1 How to proceed if sentient life is accidently discovered in a forgotten petri dish in the back of a laboratory fridge

Starfleet General Order #1 – The Prime Directive

As the right of each sentient species to live in accordance with its normal cultural evolution is considered inviolable, no Starfleet personnel may interfere with the normal and healthy development of alien life and culture.

Such interference includes introducing superior knowledge, strength, or technology to a world whose society is incapable of handling such advantages wisely.

Starfleet personnel may not violate this Prime Directive, even to save their lives and/or their ship, unless they are acting to right an earlier violation or an accidental contamination of said culture. This directive takes precedence over any and all other considerations, and carries with it the highest moral obligation.

(Changed after Michael and Denise Okuda, "The Star Trek Encyclopedia: A Reference Guide to the Future", 2016, Harper Design / HarperCollins US, 4th ed., vol. 2, p. 180, ISBN 9780062371324; see also https://memory-alpha.fandom.com/wiki/Prime_Directive)

2.7.12 Sterile plant *in vitro* culture – Seed sterilization and plant growth

To generate sterile plant *in vitro* cultures, *Dionaea* seeds needed to be surface-sterilized. Therefore, the amount of seeds necessary was put in a reaction vessel together with 1.5 ml 70 % EtOH and placed in an overhead shaker for 1 h. Thereafter the EtOH was discarded and replaced with 1 ml of 5 % NaClO + 2 % Triton X-100 and the seeds were put back in the overhead shaker for a maximum time of 15 min. The next steps were performed under a clean bench. The liquid was discarded and the seeds were washed 5 times with sterile ddH_20. The seeds were then disseminated on a petri dish containing seed growth medium and incubated in a growth cabinet at 22 °C under long day conditions using 36 Watt/865 Hg Lumilux Coolwhite lights. After seedlings developed, they were transferred in a 6-well-plate containing seedling medium. Mature plants were kept in food-rated, transparent polypropylene cups.

Seed growth medium = Plant growth medium

• ½ MS-Powder + MES + Vitamins
• 2 % Sucrose
• 2 ml/l plant preservative mixture (PPM)
Adjust pH to 5.8 using KOH
• 1.5 % Agar (Kobe I)

Seedling medium

• 1/3 MS-Powder + MES + Vitamins
• 2 % Sucrose
• 20 ml/L Iron stock solution
Adjust pH to 5.8 using KOH
• 0.8 % Agar (Kobe I)
After autoclaving, add IBA to a final concentration of 0.5 µM/L

Iron stock solution

• $FeSO_4$ x 7 H_2O	0,7 g
• EDTA	0,95 g
Dissolve in 500 ml ddH_20, then autoclave. Store in dark and at 4 °C	

2.7.13 Sterile plant *in vitro* culture – Micropropagation

After a sterile plant culture had been established, the plants could be micropropagated. To propagate plants, a healthy green plant part (leaf with attached trap or a truncated trap) was placed on plant growth medium without addition of growth hormones. After about 8-12 weeks adventitious buds arose, developed into shoots and after approx. 16 weeks into small plantlets. These could be separated and grew into single mature plants.

2.7.14 Plant growth conditions

Dionaea muscipula plants were obtained from Cresco carnivora V.O.F., De Kwakel, Netherlands. Genotypes, age, feeding status and growth conditions of the plants prior to the arrival in the laboratory greenhouse were not known. Upon arrival, they were kept under 18 h light / 6 h dark conditions at a minimal temperature of 22 °C during daytime and 18 °C during nighttime. The minimal light intensity was 130 µmol × m ^{2} /s using a sodium vapor lamp (SON-T Agro 400W, Phillips).

Arabidopsis thaliana Columbia ecotype (Col-0) were self-grown from seeds in a climate chamber under 8 h light/16 h dark – conditions, at 22 °C during daytime and 16 °C during nighttime, with a stable relative humidity (rh) of 60 % and 150 µmol/s m light intensity generated by Osram L58W / 77 FLUORA and Philips TLD 58W / 840 lamps. 5 – 7 week old plants were used for protoplast generation.

2.7.15 Gas exchange measurements

Plants can tightly control their CO_2 – uptake/ H_20 - loss via adjustable pores called stomata (greek “stoma”: mouth, opening) (Geiger et al., 2011). The opening and closing of the stomata is accompanied by a change in humidity and CO_2 levels in the surrounding air. As described in Bauer et al. 2013, using two parallel water-cooled whole plant cuvettes with subsequent customized Infra-Red-Gas-Analyzer (IRGA), changes in water concentration in the air (relative humidity = rh) caused by opening or closing of the stomata could be detected. The air stream per cuvette was regulated to 1 L/min using mass flow meters, heated to 20 °C resulting in 50 % rh. CO_2 levels were set to 400 PPM. Illumination was provided by three LEDs, providing light at 655 nm at a photon fluence rate of 100 µmol m^2/s (Winger WEPDR3-S1 Power LED Star tiefrot 3W), at 455 nm at 8 µmolm^2/s (Philips, Luxeon, Royal Blue) and at 395 nm (Winger WEPUV3-S1 UV Power LED Star 395 nm). The three light beams were collected by 2 dichroic mirrors (Q525 LPXR and DCLP 425, Chroma, www.chroma.com) and guided through two fiber optics to the cuvettes (Bauer et al., 2013). In the experiments, *Dionaea* plants (leaf and trap) were cut at the base of the petiole and placed in a vessel filled with water to ensure constant water flow. Coronatine was sprayed on the traps and excess fluid was removed immediately.

If not mentioned otherwise, the traps have been kept open using folded paper clips. Afterwards the prepared plants were transported into the gas exchange measurement chamber and the chamber was locked. ABA was given through the locked chamber via a syringe and a tube to the water in the vessel, hence it was taken up via the leaf.

2.7.16 Mechanostimulation of traps

In order to elicit action potentials within the trap, a trigger hair has to be agitated. The agitation of a single trigger hair leads to the generation of one action potential (Böhm et al., 2016a). If several trigger hairs are agitated at the same time, only one action potential is generated (personal communication Dr. Scherzer, University of Würzburg, June 2014). Therefore, if a closed trap is squeezed gently between two fingers, all trigger hairs are agitated at once, eventually leading to the generation of only one action potential.

2.7.17 Student´s t-test

To prove or disprove if the mean values of two sample sets are significantly different from each other, they were tested using a two-tailed student´s t-test for independent samples under the assumption of a normal distribution and equal variance. These conditions were tested using the The spreadsheet application “Microsoft Excel 2013” was used for the calculations. The two samples were considered significantly different from each other if the resulting p-value was ≤ 0.05 (marked with one asterisk, *), strongly significantly different if the p-value was ≤ 0.01 (marked **), and highly significant if the p-value was ≤ 0.001 (marked ***) (Student, 1908).

2.7.18 Ultra Performance Liquid Chromatography (UPLC)

UPLC – measurements are used to identify and quantify non-exhalable substances in plants and the effect of different treatments on their levels *in planta*. Here, the abundance of different oxylipins such as OPDA, JA and JA-Ile as well as ABA and SA was assessed in response to various stimuli.

Dionaea traps were ground to powder using liquid nitrogen. A screw-capped vessel with two ceramic balls was pre-cooled in liquid nitrogen. About 200 µg – 400 µg of plant powder were added and the exact amount of powder was weighted for later use.

950 µl of Ethylacetate:Formic acid (99:1) were added to the plant powder and the vessel was mixed rapidly. 50 µl of internal standard (IS) (containing each of the following components at 1 ng/µl in acetonitrile: dihydroJA, [$^{18}O_2$] OPDA, JA-Norvaline, [D_4]SA and [D_6]ABA) was added and the assay was vortexed again. To further break down the plant material, the reaction

vessel was placed in a Tissue Lyser for 3 min with a frequency of 20 Hz. Afterwards it was sonicated for 2 min. Then the assay was centrifuged for 10 min at 14.000 x g at RT. The supernatant was transferred carefully into a new lid-capped reaction vessel without transferring solid material. The supernatant was evaporated in a SpeedVac (Christ, RVC 2-25 CD plus) at 50 °C. These steps were repeated twice without the addition of the IS.

At the end of the procedure, a solid layer could be seen at the bottom of the tube wall. This pellet was quantitatively resuspended using 30 µl of Acetonitrile (HPLC-grade). Afterwards 30 µl of H_20 (ddH_20) were added. The assay was vortexed briefly, then sonicated for 3 min. It was centrifuged for 10 min at 14.000 x g at RT, then 40 µl of the supernatant were transferred into a UPLC glass vial, which was stored at -20 °C until processing.

Measurements were performed by Dr. Markus Krischke from the department of pharmaceutical biology of the University of Würzburg, using Waters Quattro Premiere triple-quadrupole mass spectrometer (MS/MS) with an electrospray interface (ESI) coupled to a Waters Acquity utra-performance liquid chromatography (UPLC) setup, a BEH C18, 2.1mm×50mm, 1.7 µm particle size (Waters) – column and a BEH C18 (Waters) equipped with prefilter – precolumn. The following parameters were used for detection with a dwell time of 0.025 s per transition:

Compound	Transition	Cone voltage [V]	Collision energy [eV]	Retention time [min]	Peak width at half maximal peak height	Peak area
SA	137→93	17	17	2.97		
$[D_4]$SA	141→97	17	17	2.95	0.09	37899
ABA	263→153	26	14	3.10		
$[D_6]$ABA	269→159	26	14	3.09	0.07	19884
JA	209→59	19	17	3.58		
dHJA	211→59	19	17	3.91	0.07	5008
JA-Ile	322→130	24	18	4.13		
JA-Nval	308→116	24	18	3.83	0.16	43823
OPDA	291→165	22	26	5.11		
dnOPDA	263→165	22	26	4.42		
$[^{18}O_2]$OPDA	295→165	22	26	5.11	0.06	4553

3. Results

3.1 Molecular biological results related to transcriptional and hormonal regulation of *Dionaea muscipula*

As it has been demonstrated before, the Venus flytrap is able to "count" the number of APs elicited e.g. by insect prey and to modify its behavior accordingly. Counting can be understood in the sense that *Dionaea* is able to distinguish the number of APs elicited in the trap and to activate certain metabolic functions only after a certain number of APs has been recognized (Böhm et al., 2016a). In the same publication, it was also shown that the expression of the digestion marker transcripts, the hydrolases DmSAG12, DmSCPL49 and VF CHITINASE I, is positively correlated with the number of action potentials elicited within the trap.

3.1.1 Analysis of the effect of different numbers of mechanostimulation on hydrolase transcript expression

In the following experiment, the correlation between the number of APs and the duration of marker hydrolase expression was analysed in intact plants using the qPCR method (See **2.7.6**). Similar to already published procedures (Schulze et al., 2012), a metal ball was used as an insect mimic and placed into an open trap, and then trap closure was elicited mechanically. The ball was moved from the outside using a small magnet to imitate prey movement under various conditions:

Condition 1	control (no movement)
Condition 2	mechanical trap closure and three mechanostimuli during the first hour, once every 15 minutes **(Initial stimulation)**
Condition 3	as in condition 2 with a single additional mechanostimulation after 4 h **(Initial +1st additional stimulus)**
Condition 4	as in condition 3 with a second single additional mechanostimulus after 8 h **(Initial +1st and 2nd additional stimulus)**

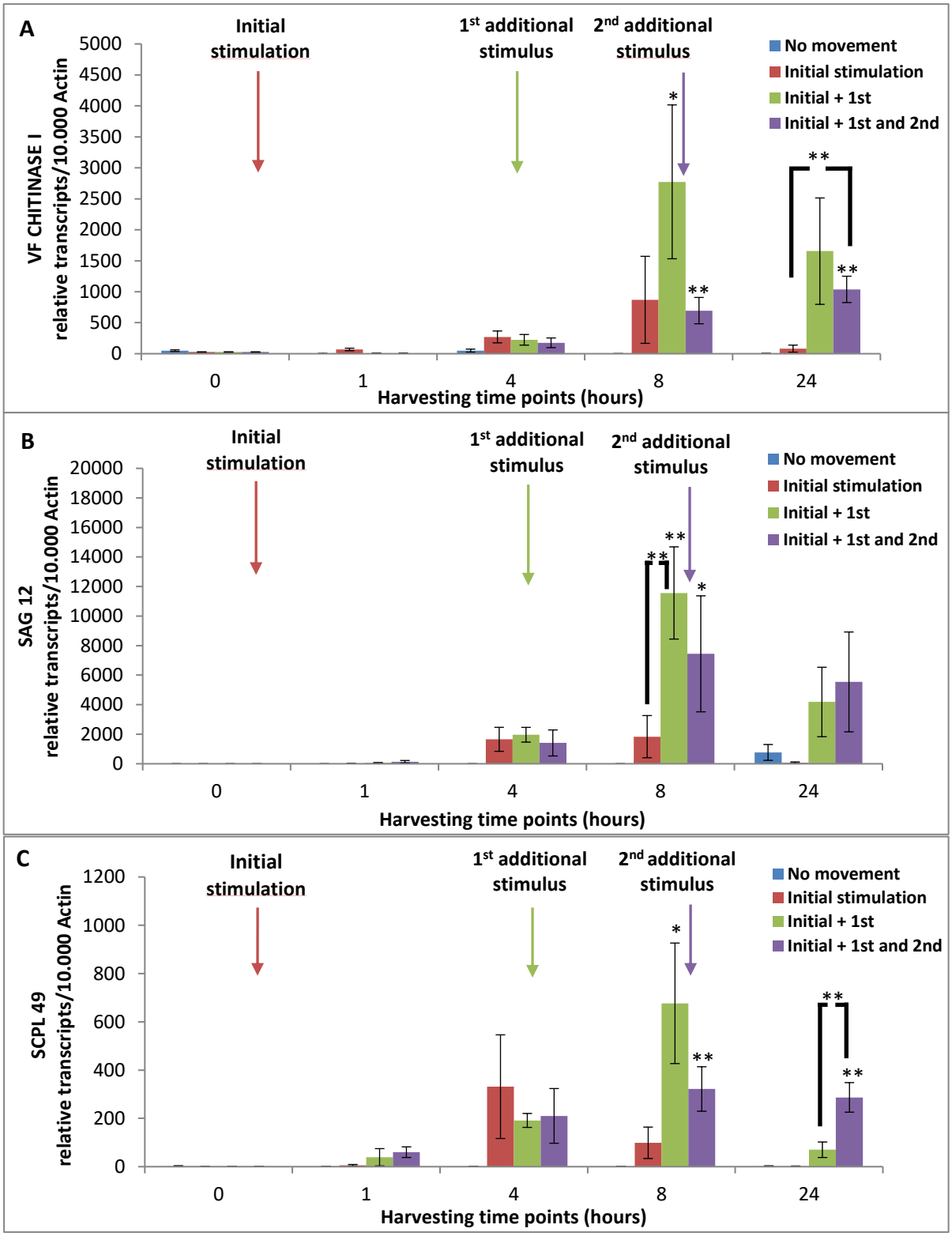


Fig. 3.1.1: Expression levels of digestion marker transcripts after different numbers of mechanostimulation
Using qPCR, the expression rates of the marker transcripts VF CHITINASE I, SAG 12 and SCPL 49 after different treatments and time points were determined. After "initial + 1st" and "initial + 1st and 2nd" stimulus the expression remained elevated after 24 h. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks above error bars indicate statistically significant difference to respective treatment at 0 h; asterisks over black bars indicate statistically significant difference between treatment conditions: *p ≤ 0.05; **p ≤ 0.01 by Student´s T-test, n=5, mean ± SE.

In Fig. **3.1.1**, it is shown that the transcript levels of VF CHITINASE I, SAG 12 and SCPL 49 start to rise at 4 h after all treatments (Initial stimulation, Initial + 1st additional stimulus and Initial + 1st and 2nd additional stimulus). The three transcripts show an expression peak at 8 h, with a statistically significant difference between time point 0 h and time point 8 h in condition three and four (Initial + 1st additional stimulus and Initial + 1st and 2nd additional stimulus). The abundance of all three transcripts is reduced to the initial level after 24 h in condition two (if only the initial mechanostimulation is applied). Their expression remained elevated after 24 h in condition three (Initial + 1st additional stimulus) and condition four (initial + 1st and 2nd additional stimulus), which represents the state of a trapped insect (the plant senses mechanostimulation for a prolonged period of time).

3.1.2 Effects of ABA on hydrolase expression in truncated plants

As is has been demonstrated by Escalante-Pérez et al., (2011), water stress as well as the exogenous application of ABA had an inhibiting effect on trap closure. The application of ABA 48 h prior to attempted trap closure desensitized the traps in a way that in 50 % of the plants tested, three and more (up to 10) trigger hair displacements were required for trap closure. A similar effect could be observed when the water content of the soil was reduced to levels less than 50 %.

The effect of ABA on the expression of the digestion marker transcripts was examined by cutting traps at the base of the leaf and by placing them either in pure ddH_20 or in ddH_20 supplemented with 50 µM ABA. Results are shown in **Fig. 3.1.2**. The plants were separated in two groups, one was the control group and the others were mechanostimulated. The plants were preincubated with water or ABA for 24 h prior to the onset of the experiment. After the preincubation, half of the plants were mechanostimulated 60x with a frequency of 1/min by gently squeezing the closed trap between two fingers as described in Böhm et al., (2016a) **(see also 2.7.16)**. The experiment lasted for 24 hours, 4 h and 24 h after initial trap closure samples were taken and hydrolase expression was determined. The expression levels of the three marker hydrolases are similar at time point 4 h as well as at time point 24 h after mechanostimulation in truncated plants with and without ABA pretreatment. No significant difference could be found between the transcript levels of plants with and without ABA, so apparently the ABA treatment had no effect in these particular conditions.

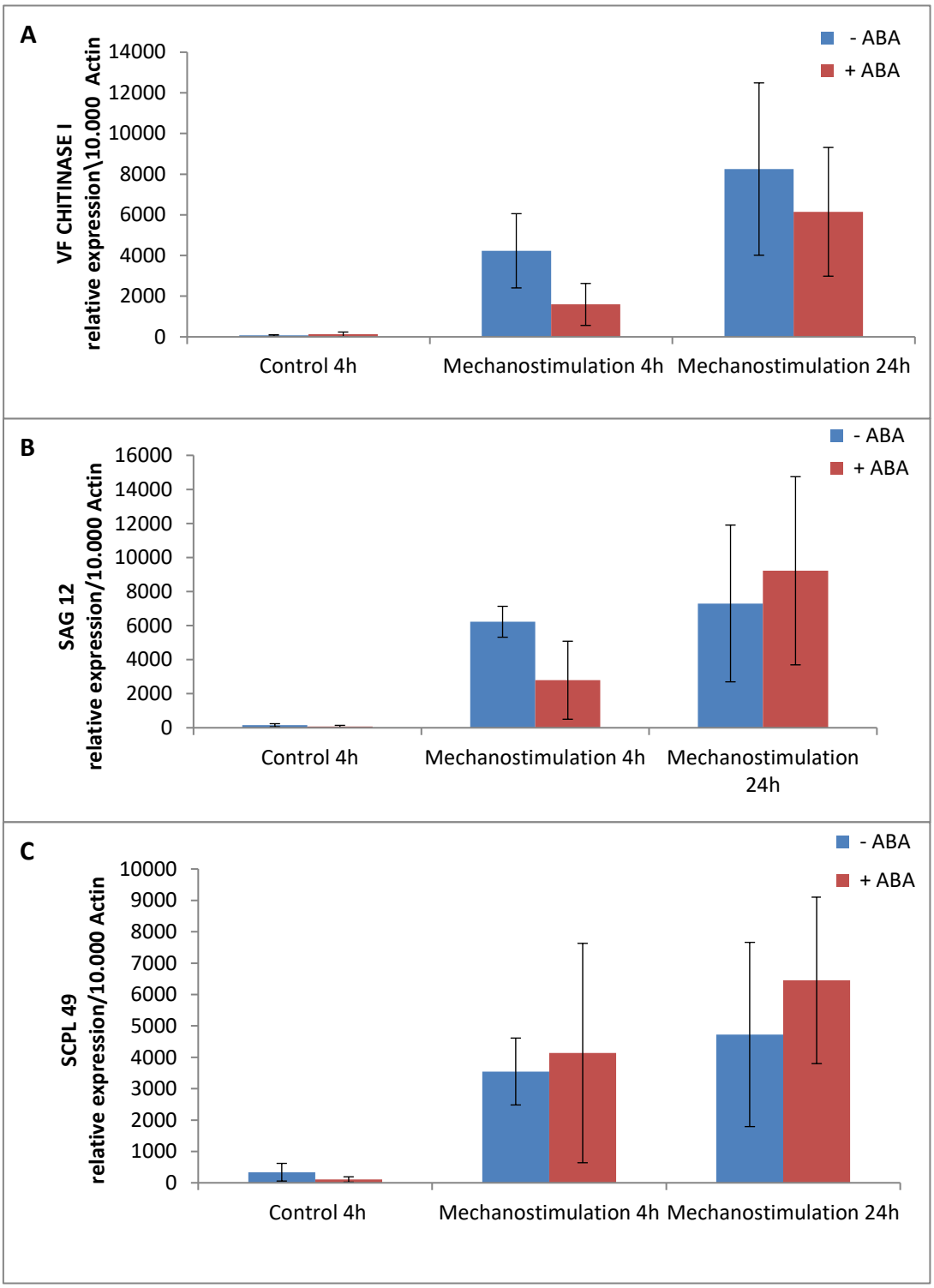


Fig. 3.1.2: Expression levels of digestion marker transcripts in truncated plants after ABA pretreatment followed by mechanostimulation

The effect of the mechanostimulation on marker transcript levels in plants with and without ABA-pretreatment was analyzed in truncated plants. Truncated plants were incubated for 24 h with either 50 µM ABA or ddH_20, after this incubation plants were subjected to mechanostimulation (60x, frequency 1/min). 4 h as well as 24 h after mechanostimulation, the expression levels of the marker transcripts in plants with and without ABA are similar. Transcript numbers are given relative to 10.000 molecules of DmACT, n=5, mean ± SE.

3.1.3 Effect of ABA, truncation and wounding on jasmonate response

It is well known that plants react with the release of jasmonates to a wounding event (Reviewed by Farmer et al., 2014 and Koo and Howe, 2009). To assess the effect of various stimuli on different phytohormone levels in *Dionaea muscipula* traps, a sequence of experiments was performed. With them the effect of truncation of traps (with the leaf still attached, truncation occurred at the base of the petiole) from the plant, mechanostimulation of the traps, and the application of ABA onto the traps on the characteristics of phytohormone levels should be analysed.

The first experiment was performed by Brigitte Neumann and Sebastian Filbeck. This experiment was performed to establish the baselines of phytohormone levels in *Dionaea muscipula* traps.

- Intact traps (still attached to the plant) were either
    a. Wounded using forceps (in **Fig. 3.1.3** referred to as “wounded”)
    b. Gently squeezed between the finger tips to elicit action potentials (1/min, 10 APs) (referred to as “Mechanostimulation intact”)
    c. Left untreated and attached to the plant, (referred to as “control”)
- In addition, some traps were severed from the plant at the base of the petiole (in **Fig. 3.1.3** referred to as “cut”) with the cutting area submerged in water. Those traps were not further wounded or stimulated, therefore, no extra control group for the detached traps was necessary.

The plants were prepared for the experiment, then the experiment commenced without further waiting time to immediately register the physiological responses. The traps were harvested at the beginning of the experiment (0 minutes) and also after 15 minutes, 30 minutes, 1 hour, 3 hours, and at last after 24 hours, which marked the end of the experimental phase. Afterwards the traps were processed for UPLC-measurement (See **2.7.18**). All levels of Jasmonic acid (JA) and Jasmonic acid-Isoleucine (JA-Ile) were determined using UPLC measurements by Dr. Markus Krischke (Julius-von-Sachs-Institute, Department of Pharmaceutical Biology, University of Würzburg).

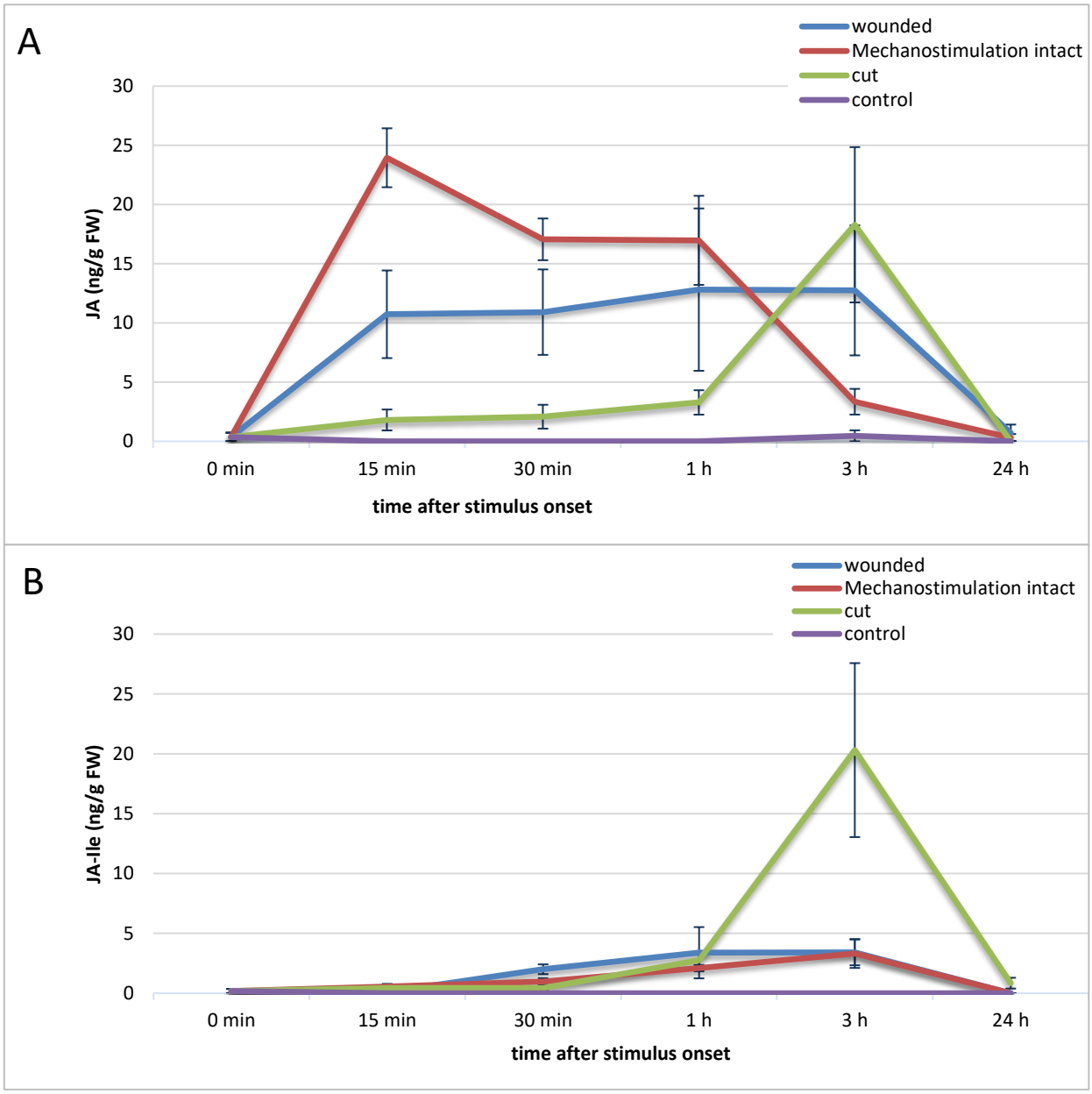


Fig. 3.1.3 JA and JA-Ile levels in trap tissue after different stimuli
Different stimuli were applied to *Dionaea muscipula* traps. In **blue** the traps of intact plants were wounded with a forceps, in **red** traps of intact plants were subjected to mechanostimulation (10x, 1/min), **green** shows the results after the trap with the attached leaf was cut at the base of the petiole and then submerged in ddH_20 without further manipulation. **Violet** shows the control plants which were untreated. Sampling started immediately after treatment. The amount of the phytohormones (JA in 3.1.3 A, JA-Ile in 3.1.3 B) is given in ng/g FW, n=5, mean ± SE.

Fig. 3.1.3 shows the results of these experiments. **Fig. 3.1.3 A** represents the JA levels and shows that, after the **wounding stimulus (blue)**, the JA levels were already elevated after 15 minutes to approx. 10 ng/g fresh weight (FW) and remained at this level until 3 h. **Mechanostimulation (red)** elevated JA levels to approximately 23 ng/g FW at 15 minutes, with levels slowly decreasing to about 17 ng/g FW at 30 minutes and 1 h, and at 3 h they were almost back to base levels. **Cutting (green)** resulted in a slow increase of JA-levels, with approximately 3 ng/g FW at 1 h and a peak at 3 h with approximately 18 ng/g FW. After 24 h, the JA levels of all treatments were back to base levels.

Compared to JA-levels, **Fig. 3.1.3 B** shows that JA-Ile-levels rose slower after wounding and mechanostimulation, with much lower final concentrations. After **wounding (blue)**, JA-levels already reached their maximum after 15 minutes, whereas JA-Ile-levels only started to increase measurably 30 minutes after beginning of the experiment and peaked at 1 h after the wounding event.

Mechanostimulation (red) resulted in maximum JA-levels after 15 minutes of approximately 23 ng/g FW and started to decline afterwards, but the corresponding JA-Ile-levels were increasing slowly and steadily from 15 minutes to 3 h to a level of approximately 3 ng/g FW.

Different results were obtained after **cutting (green)**. JA-Ile levels rose steadily from 15 minutes to 1 h, followed by a peak maximum at 3 h with approximately 20 ng/g FW. The corresponding JA-levels behaved similarly. After 24 h, all JA-Ile levels of all treatments except for **cutting** were back to base levels.

The ABA concentration in untreated truncated traps 24 h after truncation was approximately 30 ng/g FW, the ABA concentration in intact trap tissue 24 h after spray application of 50 µM ABA was found to be approximately 4000 ng/g FW, whereas the ABA concentration in truncated traps submerged in water with 50 µM ABA was approximately 30.000 ng/g FW (data not shown).

A follow-up-experiment was performed to analyse the effects of ABA on the JA- and JA-Ile phytohormone levels. Due to the results from the first experiment (**Fig. 3.1.3**), plants were truncated 24 h prior to the actual experiment onset, so the plants reactions to the cutting had faded away again. Additionally, ABA-preincubation was performed for 24 hours (truncated plants were submerged with the cutting area in 50 µM active ABA solution, intact traps were sprayed with ABA solution). In **Fig. 3.1.4.1** the effect of ABA on JA levels after mechanostimulation is shown.

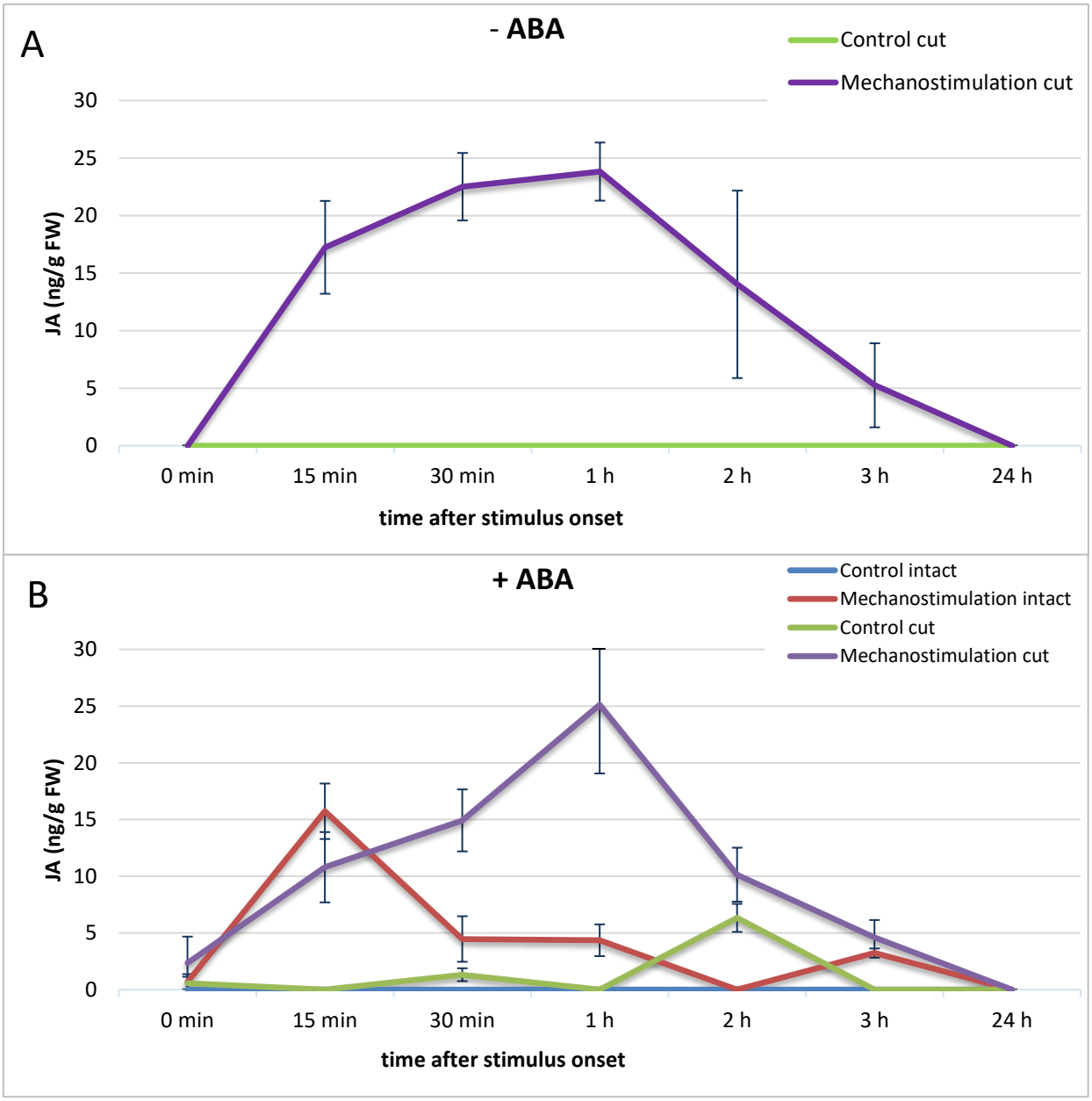


Fig. 3.1.4.1 Effect of ABA-application on JA levels after different stimuli in truncated and intact plants

A In addition to **3.1.3 A**, the effect of truncation on JA-levels in mechanostimulated traps without ABA (- ABA) was examined. Leaves were truncated at the base and submerged in ddH_20 for 24h prior to mechanostimulation (10x, 1/min, violet).

B Subsequently, the effect of ABA (+ ABA) on JA levels was examined. Truncated traps were submerged in ddH_20 + 50 µM active ABA 24 h prior to treatment, and intact plants were sprayed once with 100 µL 50 µM active ABA. Control plants were either truncated (green) or left intact (blue). Mechanostimulation was performed on intact (red) or truncated traps (violet). The amount of JA is given in ng/g FW. n=5, mean ± SE.

Compared with the control treatment in **Fig. 3.1.3 A** and **Fig. 3.1.4.1 A**, the effect of ABA on JA levels after mechanostimulation is evident in intact as well as in truncated plants in **Fig. 3.1.4.1 B**. It is apparent that the rise of JA levels in truncated mechanostimulated traps to the maximum after 1 h happens at approximately the same rate (within the margin of the error bars), regardless of whether the plants were treated with ABA or without ABA, but the peak value after 1 h is similar with approx. 24.5 ng/g FW JA. The decrease of JA level within 2 h after its peak (from 1 h up to 3h) is also characterized by approximately the same rate (within the margin of the error bars).

In **Fig 3.1.3 A** JA levels in mechanostimulated, intact plants rose to maximum levels after 15 min (average of 18 ng/g FW) and remained like this until 1 h, then decreased until 3 h. In **Fig 3.1.4.1 B** JA levels in mechanostimulated, intact traps with ABA rose to approx. the same level after 15 min, but then dropped sharply after 30 min to approx. 5 ng/g FW and stay at this level until 3 h.

To summarize the different results, they were combined in **Fig. 3.1.4.2**. It clearly shows that truncation of the trap vastly alters the perception of ABA, followed by an altered JA-response after mechanostimulation. The intact traps without ABA (**light blue**) show a rapid increase of JA after mechanostimulation, followed by a steady decline on a high level within the first hour. In intact traps with ABA (**orange**), there is also a rapid increase in JA levels, but the peak is notably lower compared to those without ABA, and the decline is also more rapid and on a much lower level. The differences between JA-levels in intact plants with or without ABA are statistically significant (*p ≤ 0.05 for 15 minutes, and **p ≤ 0.01 for 30 minutes and 1 h).

In contrast, truncated traps show a very different behavior. Without ABA (**violet**), the JA increase reaches the same level as in intact traps, but it is reached much later, after 1 h, not after 15 minutes. With ABA (**dark blue**), the JA level increase rate is slightly slower than without ABA, but the peak value after 1 h is similar, and the decline rate afterwards is almost identical with or without ABA.

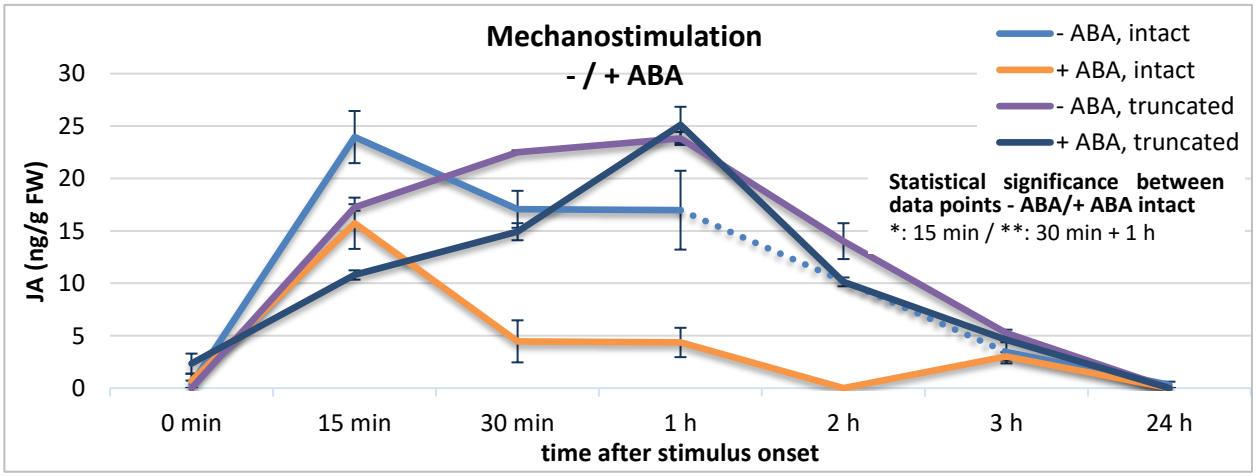


Fig. 3.1.4.2 Summary of the effect of truncation and ABA on JA levels

To summarize the impact of ABA treatment and truncation, the relevant results from Fig. 3.1.3 A, 3.1.4.1 A and 3.1.4.1 B were combined. The amount of JA is given in ng/g FW. n=between 3 and 5, mean ± SE. The line between 1 h and 3 h for- ABA intact (light blue) is dotted because in this experiment (Fig. 3.1.3) no samples were taken at 2 h. Statistically significant differences were found for intact traps without or with ABA (light blue/orange) by Student´s T-test: *p ≤ 0.05 for 15 minutes, **p ≤ 0.01 for 30 min and 1 h.

In **Fig. 3.1.5.1** the effect of ABA on JA-Ile levels after mechanostimulation was analysed. Compared with the treatment in **3.1.3 B** and **3.1.4.1 B**, the effect of ABA on JA-Ile levels after mechanostimulation is evident in intact as well as in truncated plants in **3.1.5.1 B**.

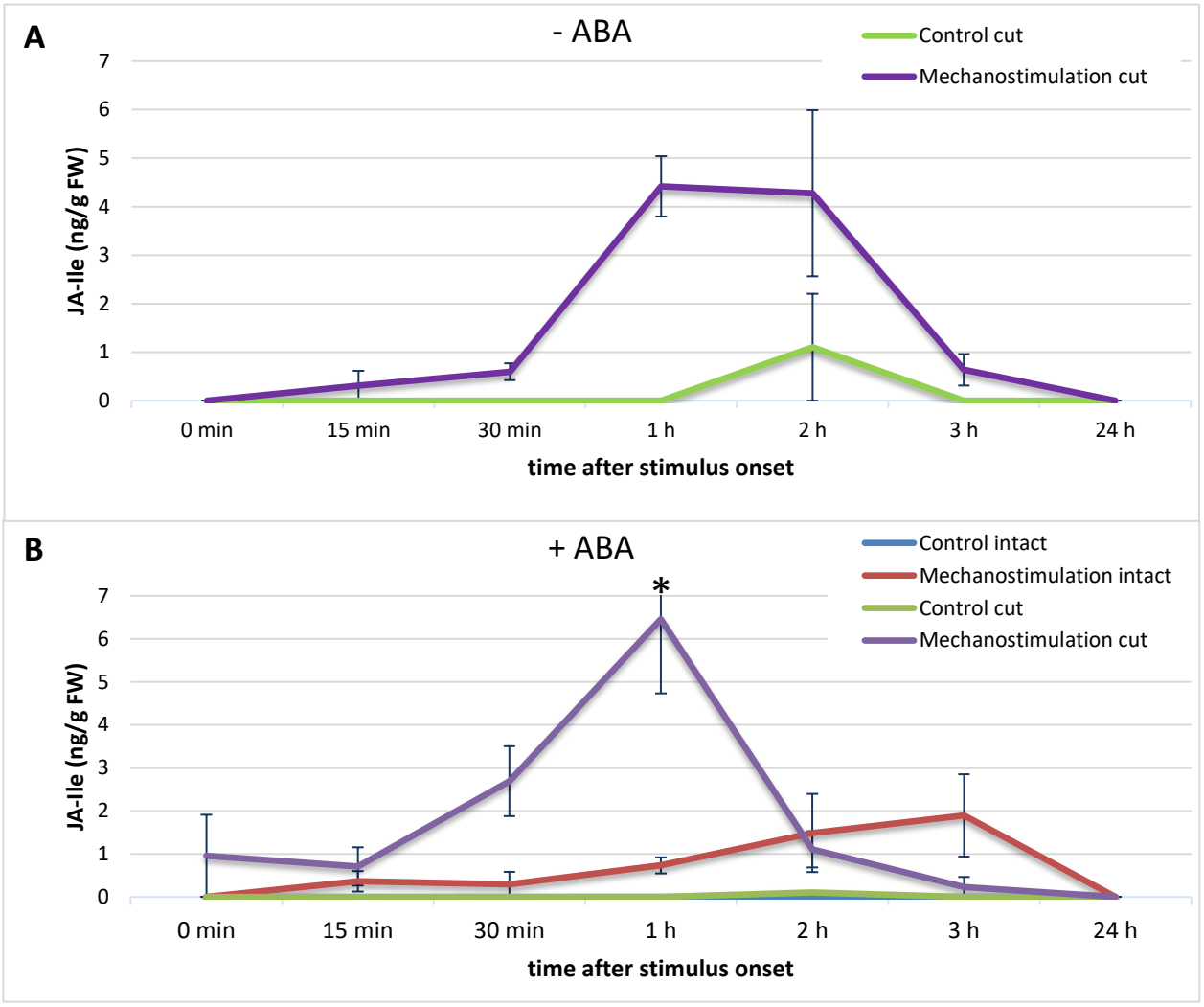


Fig 3.1.5.1 Effect of ABA-application on JA-Ile levels after different stimuli in truncated and intact plants
A In addition to **3.1.3 B**, the effect of truncation on JA-Ile-levels in mechanostimulated traps without ABA (- ABA) was examined. Leaves were truncated at the base and submerged in ddH_20 for 24 h prior to mechanostimulation (10x, 1/min).
B Subsequently, the effect of ABA (+ ABA) on JA-Ile levels was examined. Truncated traps were submerged in ddH_20 + 50 µM active ABA 24 h prior to treatment, and intact plants were sprayed once with 100 µL 50 µM active ABA. Control plants were either truncated (green) or left intact (blue). Mechanostimulation was performed on intact (red) or truncated traps (violet) The amount of JA-Ile is given in ng/g FW. n=5, mean ± SE. (*: In 3.1.5.1 B, Mechanostimulation cut (violet), 1 h, the error bar ended at 9 ng/g FW, but to have the same scale as in 3.1.5.1 A, the error bar was cut at 7 ng/g FW).

In truncated plants with the addition of ABA, JA-Ile-levels rose to their maximum value of an average of 6.5 ng/g FW after 1 h, then declined again. Without ABA, peak-JA-Ile-levels were also reached after 1 h, but the increase was slower, and maximum level was with 4.5 ng/g FW approx. 2/3 of the value with ABA treatment. In intact plants, there was a steady increase of JA-Ile-levels up to 3 hours, but it was small compared to final JA-Ile-levels in truncated plants (Approx. 2 ng/g FW in intact plants and approx. 6.5 ng /g FW in truncated plants).

In **Fig. 3.1.5.2** the different JA-Ile-measurements are combined. Like with JA-levels, a clear impact of truncation can be observed on ABA perception.

In intact plants without ABA (**light blue**), the JA-Ile levels rose slowly and steady after mechanostimulation until their peak at 3 h and declined afterwards. With ABA (**orange**), intact plants showed the same increase pattern and also a peak at 3 h, but the final values were lower (within the margin of the error bars).

Truncated plants showed a very different response. Either with or without ABA, JA-Ile levels strongly started to rise early on, already after 15 minutes (**with ABA, dark blue**) or after 30 minutes (**without ABA, violet**), respectively. Truncated plants reached their peak JA-Ile level at 1 h, and either with or without ABA, the values are very similar (within the error bars, as the difference between the peak with or without ABA is not significant). Until 3 h, the JA-Ile levels of the truncated plants treated with and without ABA have fallen almost to basic levels.

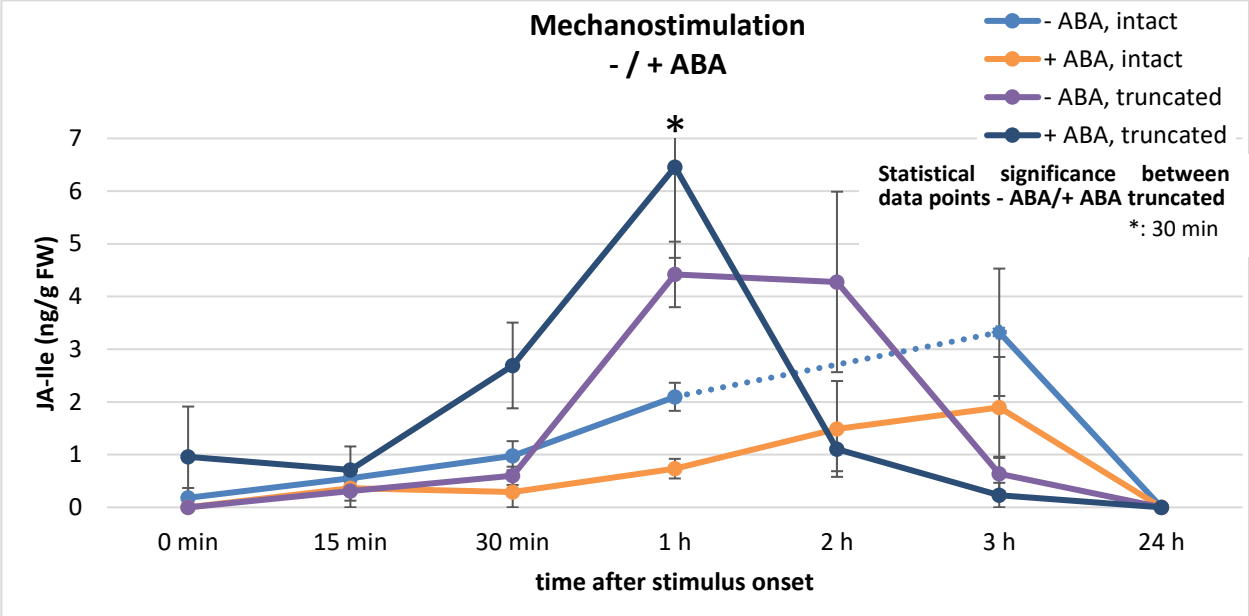


Fig 3.1.5.2 Summary of the effect of truncation and ABA on JA-Ile levels

To summarize the impact of ABA treatment and truncation, the relevant results from Fig. 3.1.3 A, 3.1.4.1 A and 3.1.4.1 B were combined. The amount of JA-Ile is given in ng/g FW. n=between 3 and 5, mean ± SE. The line between 1 h and 3 h for -ABA intact (light blue) is dotted because in this experiment (Fig. 3.1.3) no samples were taken at 2 h. Statistically significant differences were found for truncated traps without or with ABA (violet / dark blue) by Student´s T-test: *$p \leq 0.05$ for 30 minutes. (*: In + ABA truncated, 1 h, the error bar ended at 9 ng/g FW, but to have the same scale as in 3.1.5.1 A, the error bar was cut at 7 ng/g FW.)

To summarize the results, it can be said that the effect of ABA in intact plants on the course of JA levels is clearly visible. Compared to untreated plants, with ABA the JA-levels after mechanostimulation are lower at their peak after 15 minutes and also drop faster again within the first hour after (See Fig. 3.1.4.2). After 3 h, the JA-levels are similar again. The same is true for JA-Ile-levels (Fig. 3.1.5.2).

In truncated plants, the situation is much different. Truncated plants reach the JA-peak after mechanostimulation after 1 h, not after 15 minutes, and the peak level is similar with or without

ABA, although the JA-increase rate is slightly slower with ABA. It is therefore conceivable that the ABA perception is impaired in truncated plants and JA production followed by mechanostimulation is similar. With regards to the JA-Ile-levels it can be stated that the difference between plants with or without ABA is only statistically significant at 30 min ($*p \leq 0.05$). Therefore, the current data suggest an early increasing effect of ABA on JA-Ile-production in truncated *Dionaea muscipula* traps after mechanostimulation, but in later stages of the measurement this effect is not visible anymore.

3.1.4 Gas exchange measurements to determine the effect of phytohormones on secretion

It is well known that the digestion cycle of *Dionaea muscipula* can be externally activated by the application of the phytotoxin coronatine without mechanical stimulation (Escalante-Pérez et al., 2011; Böhm et al., 2016a, Bemm et al., 2016). It is also known that the application of ABA can inhibit trap performance (Escalante-Pérez et al., 2011). The interplay of those two phytohormones in *Dionaea*, however, was not known at all. A fast and, at that time, reliable way to directly observe the effect of phytohormones on the plant´s physiology was the use of gas exchange measurements. Mainly, this method measures variations in water vapor concentration in the air stream caused by opening or closing of stomata (See. **2.7.15**, also described in Bauer et al., 2013). In *Dionaea*, however, water vapor concentration can also be influenced by the onset of secretion of the digestive fluid. It has been shown previously (by Hubert Bauer, published in Scherzer et al., 2017) that spray application of coronatine on the inside of the open trap leads to secretion of digestive fluid which can be picked up by gas exchange measurements if the traps are kept open, as the inside of the trap would otherwise be hermetically sealed from the outside world after trap closure. Hence the influence of coronatine and ABA on the onset and amount of secretion of digestive fluid was to be analysed using truncated leaves with the leaf well-water supplied and the trap kept open using a bent paper clip.

As a precursor experiment the strength of the transpiration pull was analysed to assess after which time ABA could, if supplied in the water via the leaf, reach the trap tissue. Therefore, Fluorescein-diacetate (FDA), a live cell marker (Widholm, 1972), was used.

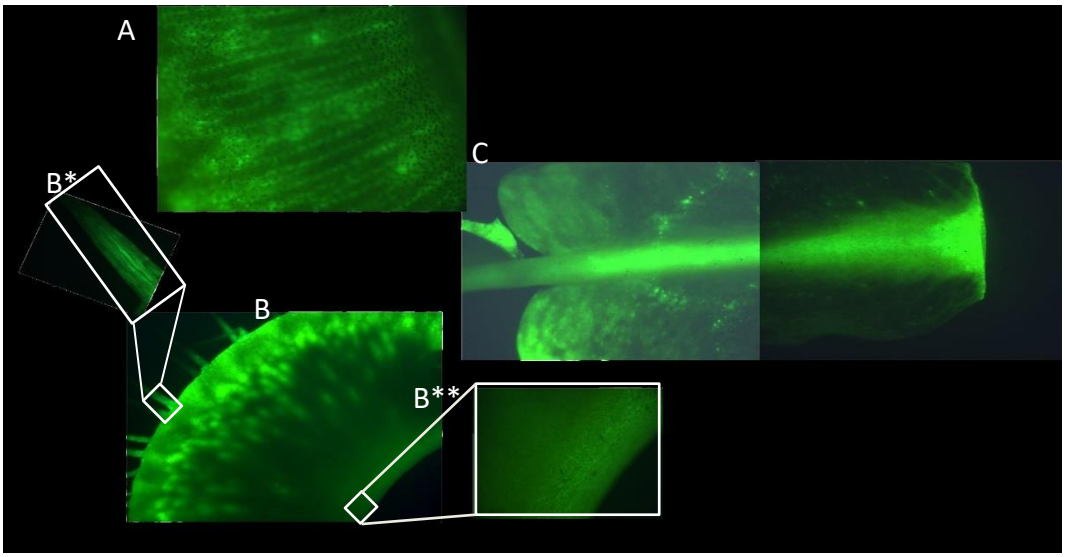


Fig 3.1.6 Distribution of Fluorescein-diacetate (FDA) in the *Dionaea* leaf by transpirational pull
An entire truncated *Dionaea* leaf was, with the cutting site well submerged in ddH_20 containing 0.04 % FDA, incubated for 1 h. Afterwards, the distribution of FDA mediated through the transpirational pull was observed. Fluorescence was found in every part of the leaf. **A** shows the inside of the trap, **B** the outside, with the inset **B*** showing an enlarged marginal spike, and the inset **B**** showing the enlarged area of the midrib. **C** shows the petiole. Control leaves submerged in ddH_20 alone did not exhibit any fluorescence (data not shown).

A *Dionaea* leaf was cut and placed in a reaction vessel containing a solution of 0.04 % FDA (stock 5 mg/ml) in ddH_20. After 1 h the fluorescence was observed using a fluorescence binocular (Leica MZFLIII + DFC500) to determine the strength of the transpirational pull. Fluorescence could be observed in the entire leaf, from petiole tissue (**Fig. 3.1.6, C**) to the inside (**Fig. 3.1.6, A**) as well as the outside (**Fig. 3.1.6, B)** and the midrib (**Fig. 3.1.6, B**)** of the trap tissue, even in the marginal spikes (**Fig. 3.1.6 B***). Control leaves that had been submerged in pure ddH_20 did not show any sign of fluorescence at the same binocular settings (data not shown). Hence, it was shown that the transpirational pull of a truncated *Dionaea* leaf with attached trap is strong enough to distribute ABA throughout the entire leaf within one hour.

Based on these results, truncated plants were subjected to gas exchange measurements. As it was known that cutting of the leaf resulted in an almost immediate release of jasmonates (See **Fig. 3.1.3**), it was to be tested if this release would interfere with the impact of ABA. A direct way to analyse this was to put two truncated leaves of plants that had been in the dark overnight, well water-supplied in two separate measuring chambers of the gas exchange machine and let them adapt to the conditions in the dark. The traps were kept open using a bent paper clip. While the paper clip was inserted into the trap, trap closure was elicited. After the measured water vapor levels reached a steady state, opening of the stomata was induced by switching on the light (using a light source with 600 µE). An hour later 50 µM ABA was administered to only one of the leaves via the xylem, it was administered via a small tube with one end on the outside of the measuring chamber and the other end in the water where the truncated leaf was immersed. In **Fig. 3.1.7**, it could be seen that the water vapor level from the respective measuring chamber started to decline approx. 20 min after ABA had been given. After decreasing for approx. 25 min, the water vapor level from this specific measuring chamber again reached a steady state. After reaching the steady state, lights were switched off, resulting in the complete closure of the stomata and was therefore accompanied by a sharp decrease in water vapor levels in both measuring chambers. The decrease in water vapor content that followed the administration of ABA was interpreted as ABA-mediated closure of the stomata. Therefore, it was concluded that ABA had an impact on the plant´s physiology, even after truncation.

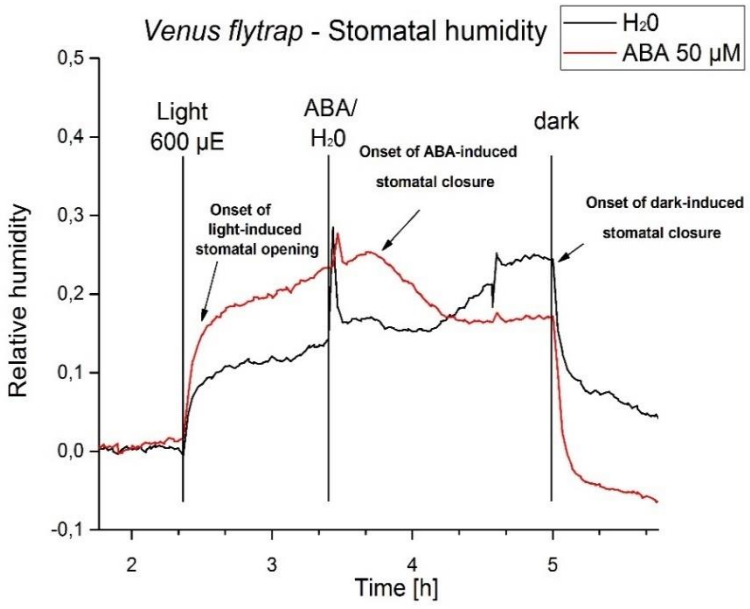


Fig. 3.1.7 Influence of ABA on stomatal aperture in truncated leaves

Leaves from *Dionaea* plants that have been in the dark overnight were truncated and, well water-supplied, placed in separate measuring chambers of the gas exchange machine. At the beginning of the measurement plants were kept in dark to adapt to the conditions. After a steady state in water vapor content was reached, lights were switched on (**Light 600 µE**). One hour after that 50 µM active ABA was given to only one leaf (red trace), the other leaf received H_20 (black trace) (**ABA/H_20**). After approx. 90 minutes, lights were switched off again (**dark**), resulting in a decrease in water vapor from both leaves.

Based on the findings above, the interplay of COR and ABA was assessed. First, light-adapted traps were subjected to either ddH_20 or COR, with the traps kept open using a paper clip. Measuring conditions for all the experiments shown below were 50 % rh, 20 °C, 400 PPM CO_2 and the plants were kept in the dark. In plants that were sprayed with COR, approximately 3 hours after spraying water vapor levels increased (**Fig. 3.1.8 A**, red trace), whereas traps that have been sprayed with water did not show any such increase (**Fig 3.1.8 A,** black trace).

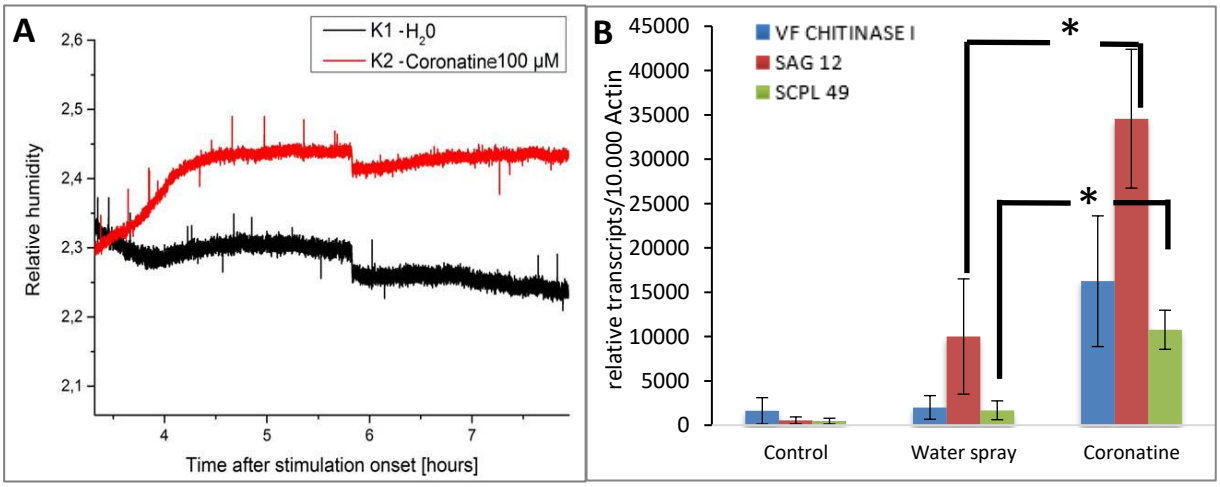


Fig. 3.1.8 Traps response to COR spray

A) Exemplary graph of truncated *Dionaea* leaves in separate dark measuring chambers, one trap has been sprayed with ddH_20 (black trace), the other with 100 µl 100 µM COR (red trace). Using a paper clip, the traps were prevented from closing. Elevated water vapor levels could be observed emerging only from the trap that had been treated with COR, the elevation is visible from approx. 3 h to approx. 4.5 h after spraying. **B)** qPCR data of traps that have been sprayed with either water or COR and been subjected to gas exchange measurements. COR spray highly induced transcription of marker hydrolases. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks indicate statistically significant difference: *p ≤ 0.05 by Student´s T-test n=4, mean ± SE.

After the measurement was finished the traps were immediately frozen and RNA was isolated for further gene expression studies. In **Fig**. **3.1.8 B** the qPCR results show a strong increase of marker hydrolase expression in the COR-sprayed traps after 7 ± 1 h of incubation compared to the water-treated traps. The water-treated traps showed only moderately elevated digestion marker transcript levels, which can probably be explained by the mechanostimulation while applying the paperclip to keep the traps open. Therefore, it could be concluded that the elevated water vapor content level caused by COR application seen in **Fig. 3.1.8 A** can be correlated to increased marker hydrolase expression in **Fig. 3.1.8 B**.

Consequentially, the effect of ABA administration after COR-spray was analyzed. **Fig. 3.1.9** shows measurements of each a single trap with only COR-spray (**Fig 3.1.9 A**) or additionally with ABA administered after 5 h (**Fig 3.1.9 B**). **Fig. 3.1.9 A** shows that COR leads to an increase in water vapor levels in the first 2 to 4 h, **Fig. 3.1.9 B** shows that the administration of ABA stops or reverses this increase approx. 30 min after administration. After the experiment was finished, the traps were frozen in liquid nitrogen.

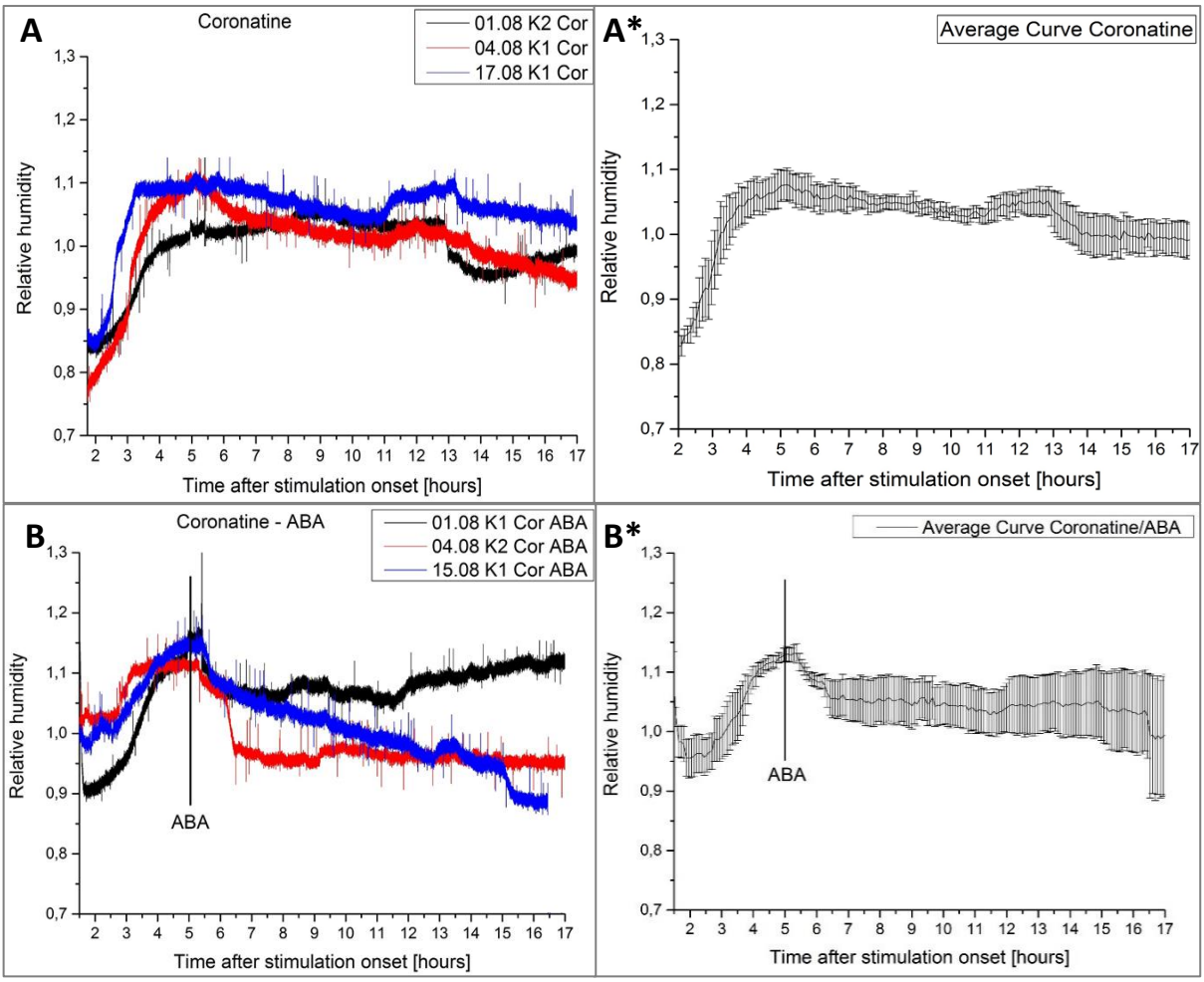


Fig. 3.1.9 Traps response to the application of only coronatine and coronatine followed by ABA

Response of single truncated traps to either being treated with only COR (**A, A***) in comparison to coronatine followed by ABA **(B, B***). **A** and **B** show the raw data of three separate measurements (the number indicates the day of the measurement in the form dd.mm), **A*** and **B*** show the statistic average curves, assembled from the raw data. COR treatment leads to elevated, administration of ABA (the time point when ABA was administered is indicated in **B** and **B*** by the black line) after COR treatment leads to a slight decrease in water vapor levels after approx. 30 min (n=3, mean ± SE)

This reduction of the water vapor levels is not represented on the mRNA level, as the marker hydrolases are expressed at high levels in the traps treated with COR and with COR/ABA, no significant difference could be detected (see **Fig. 3.1.10**).

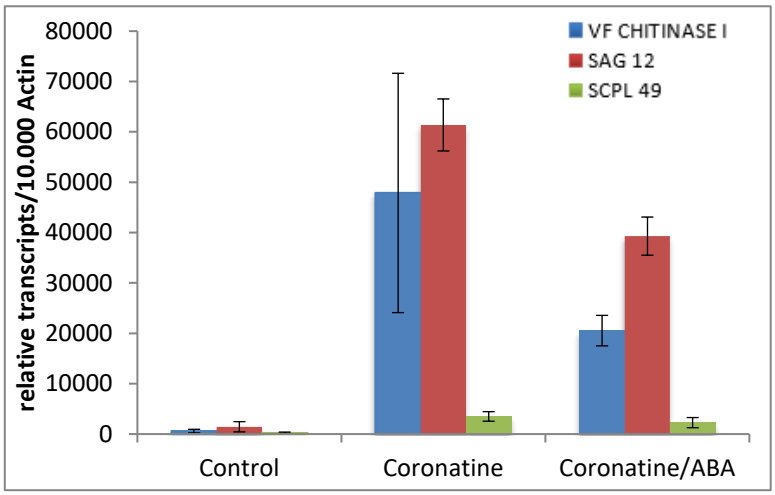


Fig 3.1.10 Transcription levels of digestion marker hydrolase in COR and COR/ABA treated traps are similar
Transcript levels of the marker hydrolases from traps in **Fig 3.1.9** were analysed. There was no significant difference between only COR- and COR/ABA treated traps. Transcript numbers are given relative to 10.000 molecules of DmACT; n=3, mean ± SE.

3.1.5 Influence of coronatine (COR) and abscisic acid (ABA) on marker transcript expression in intact plants

In the previous experiments the expression rate of the marker hydrolases could not be reduced by the subsequent application of ABA after the plants were treated with COR. As the tests have been performed on truncated plants, where truncation leads to the demonstrated alterations in phytohormone levels (see **Figs. 3.1.3 to 3.1.5**), the experiment was repeated with intact plants.

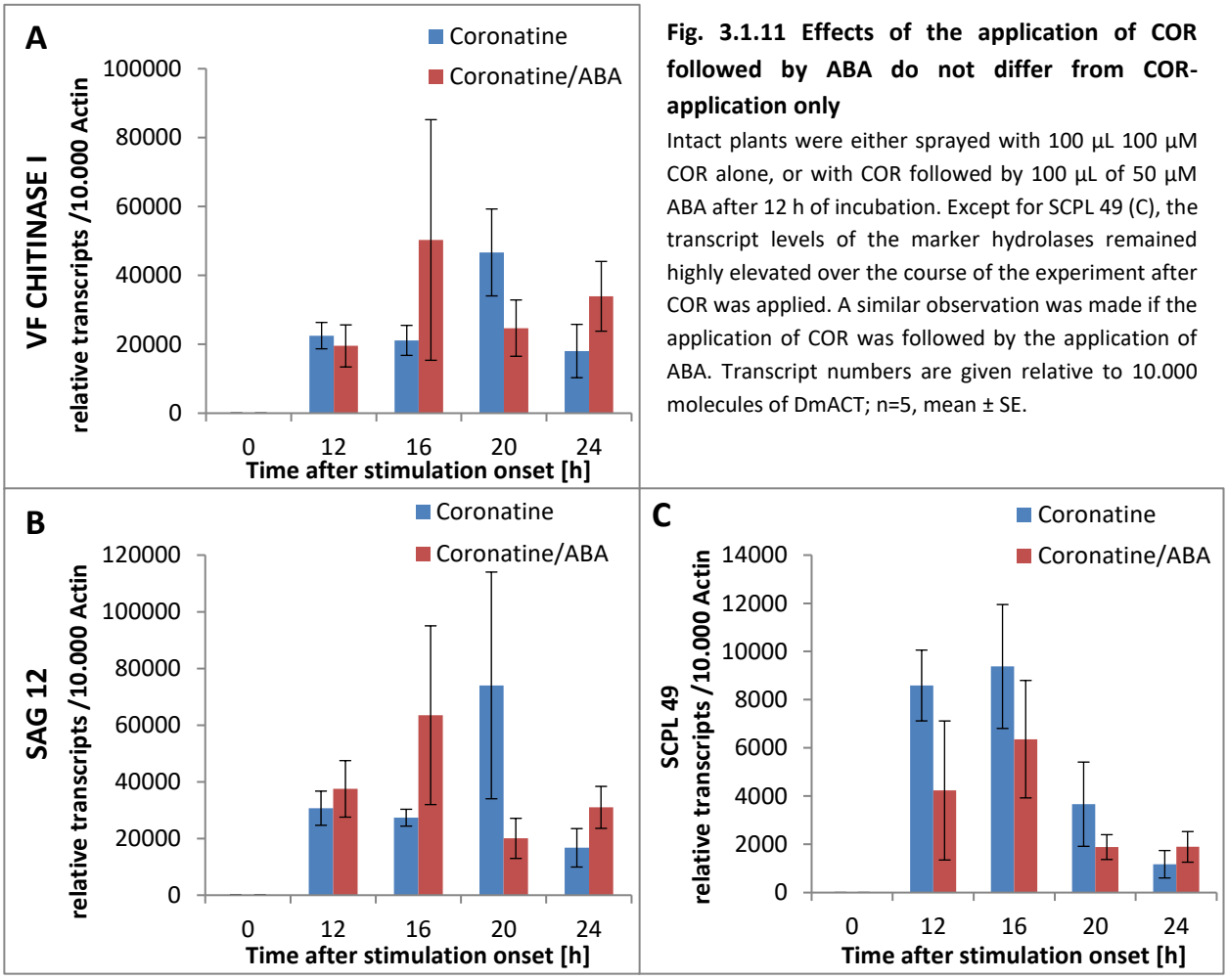


Fig. 3.1.11 Effects of the application of COR followed by ABA do not differ from COR-application only

Intact plants were either sprayed with 100 µL 100 µM COR alone, or with COR followed by 100 µL of 50 µM ABA after 12 h of incubation. Except for SCPL 49 (C), the transcript levels of the marker hydrolases remained highly elevated over the course of the experiment after COR was applied. A similar observation was made if the application of COR was followed by the application of ABA. Transcript numbers are given relative to 10.000 molecules of DmACT; n=5, mean ± SE.

In **Fig. 3.1.11**, intact plants were either sprayed with only 100 µL 100 µM COR, or with COR followed by the spray application of 100 µL of 50 µM ABA after 12 h, and the expression of the digestion marker hydrolases was assessed. The spray application of COR alone elevated the transcripts drastically, and except for SCPL 49 these transcript levels were maintained for 24 hours. A similar observation could be made in the plants that received the ABA spray treatment. Based on the presented results it was concluded that the spray application of ABA 12 h after the application of COR was not adequate to repress the effect of COR.

3.1.6 Effects of ABA on hydrolase expression in intact mechanostimulated plants

It was described in Escalante-Pérez et al., (2011) that the treatment of *Dionaea* traps with ABA 48 h prior to mechanostimulation drastically changed trap performance in terms of trigger hair displacements required to elicit trap closure. The following experiment was aimed to reveal the effects of ABA pretreatment followed by mechanostimulation on mRNA transcript level. On these grounds, intact traps were pretreated with 50 µM ABA or ddH_20 as control via spray application 24 h prior to mechanostimulation. After the incubation time, plants were mechanostimulated 60 x with a frequency of 1/min.

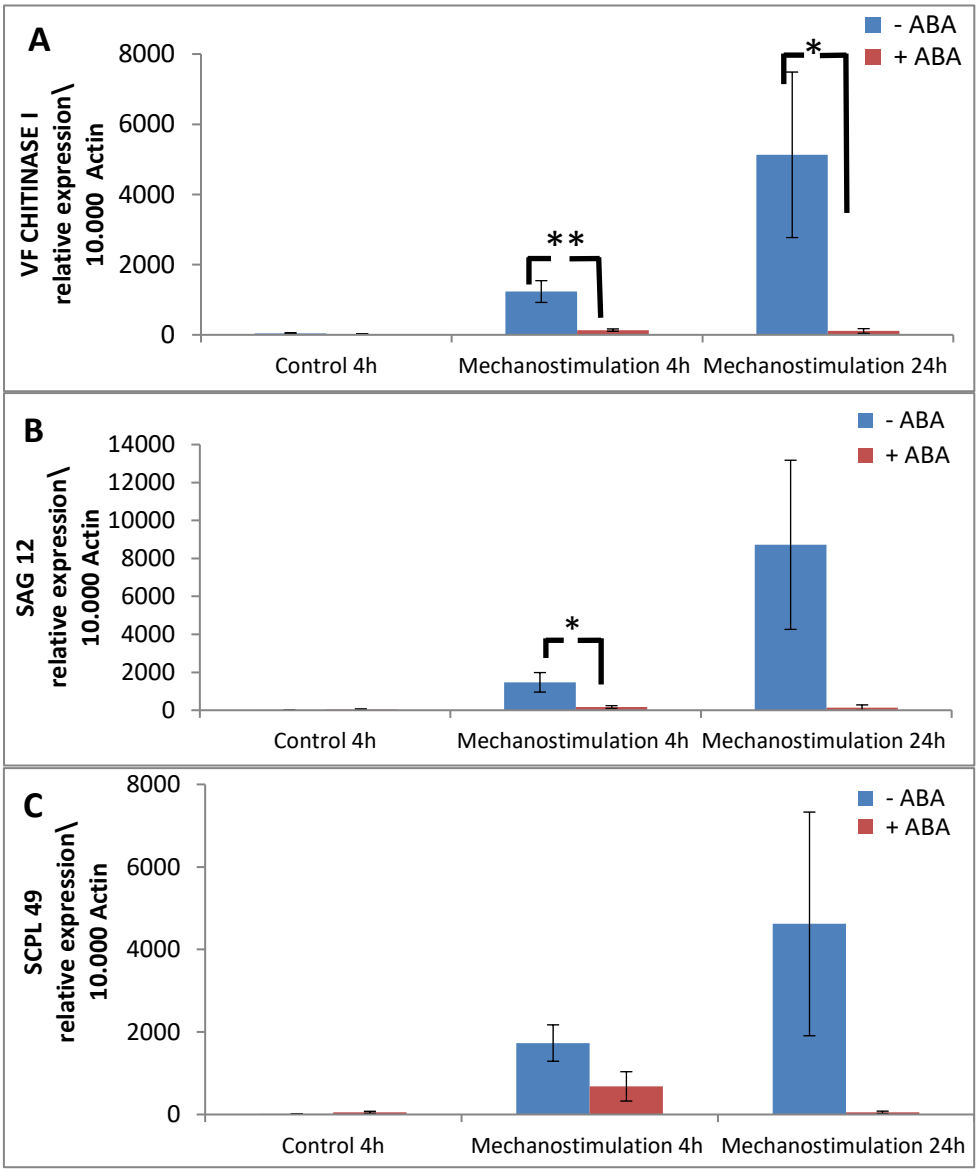


Fig. 3.1.12 ABA-preincubation prevented mechanostimulation-induced marker transcript expression in intact plants

Intact traps were sprayed with 100 µL 50 µM ABA or ddH_20 and incubated for 24 h. After the incubation period traps were mechanostimulated 60 x with a frequency of 1/min. Plants were harvested either 4 h or 24 h after mechanostimulation. The water-sprayed traps show the rise in marker transcripts already known to be associated with mechanostimulation. The ABA-sprayed traps, however, only show negligible transcript numbers. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks indicate statistically significant difference: *$p \leq 0.05$; **$p \leq 0.01$ by Student´s T-test; n=5, mean ± SE.

The transcript analysis depicted in **Fig. 3.1.12** shows that water-sprayed traps reacted, as it was known from previous experiments, with a rise in marker transcript levels after mechanostimulation. ABA-sprayed traps, however, showed negligible transcript numbers. Consequently, ABA inhibits mechanostimulation-induced transcript levels in intact plants.

3.1.7 Influence of an inhibitor on transcript expression

As ABA has a variety of effects on the physiology of plants (reviewed by Finkelstein, 2013), the need for a specific inhibitor of the jasmonate pathway arose to study the connection and fine-tuned regulation between mechanical stimulation of the trap and expression of hydrolase transcripts further. Monte et al., (2014) described the development of a ligand-based antagonist of jasmonate perception, namely coronatine-0-methyloxime (COR-MO), a derivative of COR. In *A. thaliana*, COR-MO binds to the COI1-receptor, as well as JA-Ile or COR, but instead of activating, COR-MO is preventing COI1-JAZ – interaction, JAZ-degradation and the effects of JA-Ile or COR on several JA-mediated responses. COR-MO was therefore identified as a potential candidate to interfere with the jasmonate pathway in *Dionaea*.

Intact plants were sprayed with either 100 µL ddH_20 or 100 µM COR-MO, and after 4 h those plants were mechanostimulated 0x as control, 10x or 60x (frequency 1/min) and hydrolase expression was assessed 4 h after mechanostimulation. Trap-closure was elicited mechanically.

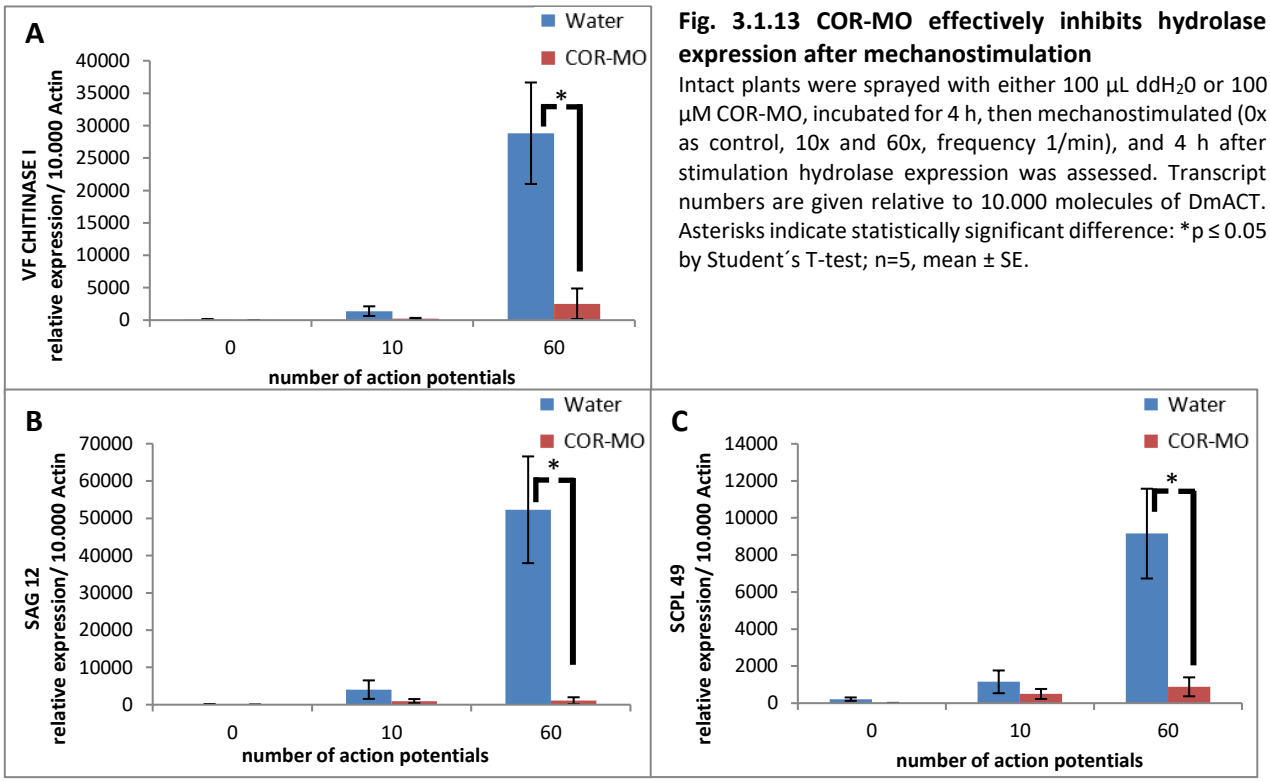


Fig. 3.1.13 COR-MO effectively inhibits hydrolase expression after mechanostimulation
Intact plants were sprayed with either 100 µL ddH_20 or 100 µM COR-MO, incubated for 4 h, then mechanostimulated (0x as control, 10x and 60x, frequency 1/min), and 4 h after stimulation hydrolase expression was assessed. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks indicate statistically significant difference: *p ≤ 0.05 by Student´s T-test; n=5, mean ± SE.

The data in **Fig. 3.1.13** proves that, after 60 stimulations, JA-responsive genes are highly expressed in plants sprayed with ddH_20, shown here by example of the digestion marker transcripts VF CHITINASE I, SAG 12 and SCPL 49. However, expression is almost negligible in plants treated with the inhibitor.

This proves on the one hand the ability of COR-MO to act as a potent jasmonate-pathway inhibitor in *Dionaea muscipula* (this has also been proven in Böhm et al., (2016a)), and on the other hand shows that, with the jasmonate pathway blocked, the mechanostimulation signal is not perceived by the trap, and the molecular response of hydrolase expression is not triggered.

In **Fig. 3.1.14** the effects of the inhibitor COR-MO on COI1- and JAZ1 transcript expression are shown. Most strikingly is the fact that the application of COR-MO without mechanostimulation is already enough to significantly elevate COI1-levels compared to water treatment, as also seen in Böhm et al., (2016a).

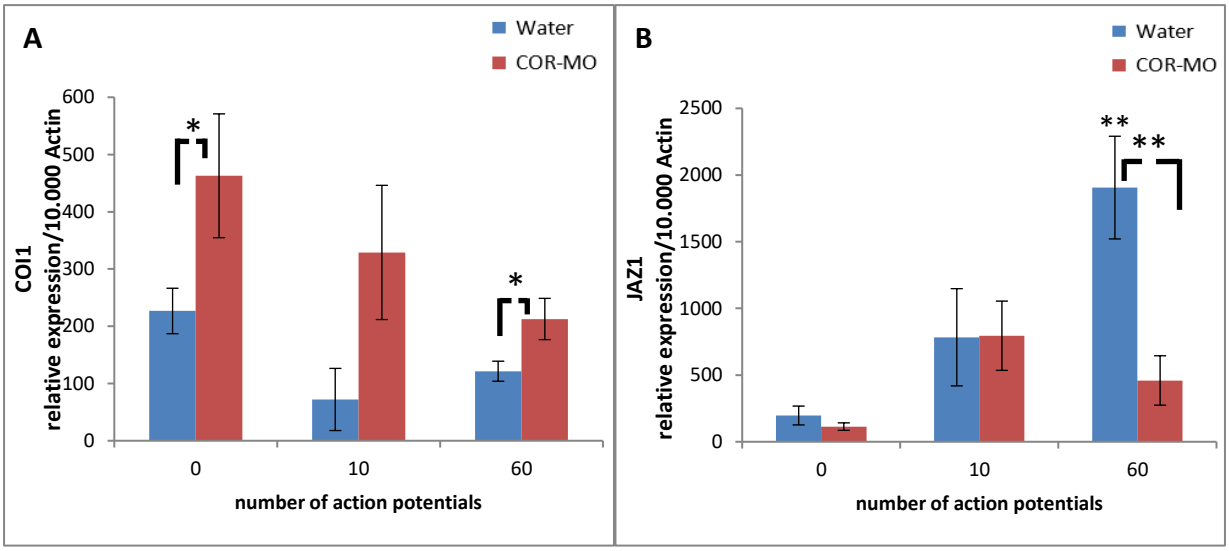


Fig 3.1.14 Jasmonate pathway inhibitor COR-MO influences JAZ1/COI1-mRNA-expression levels
The jasmonate pathway inhibitor coronatine-O-methyloxime (COR-MO) binds to COI1 and prevents COI1/JAZ1 interaction. Intact plants were sprayed with either 100 µL ddH20 or 100 µM COR-MO, and after 4 h those plants were mechanostimulated 0 x, 10 x or 60 x (frequency 1/min) and expression of transcripts for COI1 and JAZ1 was assessed 4 h after mechanostimulation. On the mRNA level, COR-MO significantly inhibits COI1 transcript repression and JAZ1 transcript elevation. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks with bars indicate statistically significant difference between indicated columns, singles asterisks indicate statistically significant difference between indicated column and value at 0 AP: *p ≤ 0.05; ** *p ≤ 0.01 by Student´s T-test; n=≥4, mean ± SE)

The inhibiting effect of COR-MO becomes obvious when analyzing JAZ1 transcripts. The application of COR-MO significantly inhibits JAZ1 transcript elevation after 60 mechanostimulations, therefore proving its ability to act as a potent jasmonate pathway inhibitor in *Dionaea muscipula* and also showing its ability to block the molecular transmission of the mechanic stimulus.

In addition to COR-MO, the inhibitors Phenidone, which inhibits lipoxygenase (LOX), thereby preventing conversion of linolenic acid to 13(S)-HPOT, a precursor of MeJA (Cucurou et al. 1991), and Neomycin, which inhibits the accumulation of JA-Ile by increasing the turnover to 12-hydroxy-JA-Ile and thereby inhibiting downstream expression of JA-Ile-responsive genes (Vadassery et al. 2014), were also used in this experiment. Phenidone was able to repress the expression of marker transcripts after up to 10 mechanostimulations, but after 20 and more stimulations this effect was gone. Neomycine was only able to repress the expression of VF CHITINASE I until up to 10 stimulations, afterwards the effect was gone (data not shown).

3.1.8 ABA-pretreatment experiments as preparation for RNAseq-experiment

Summarizing the work so far, it could be shown that intact plants respond differently to mechanostimulation paired with the ABA-treatment as compared to truncated plants. When ABA was administered 24 h prior to mechanostimulation, hydrolase expression levels in intact plants were minimal, whereas in truncated plants levels were similar compared to control plants. The incubation of intact plants with COR-MO for 4 h was sufficient to abolish any JA-induced gene expression. Intact plants that were incubated with COR for 12 h prior to receiving an ABA-dose did not reduce hydrolase expression compared to plants that have only been incubated with COR.

Over the course of this thesis, it became clear that the impact of COR as an artificial stimulus is excessive and artificial and therefore poorly suited to analyse the effects and the mechanism of mechanostimulation or the impact of jasmonates (personal communication Dr. Kreuzer).

To gain a better insight in the interplay of ABA and jasmonates, a different approach was chosen. In order to find ABA-responsive genes as well as genes that respond to mechanostimulation and were affected by the application of ABA, a nested experimental design was developed: *Dionaea* traps were either sprayed with water or with 50 µM active ABA solution, and were either incubated for 4 h or for 24 h prior to 10 mechanostimulations with a frequency of 1/min. Afterwards, the such-treated plants were either harvested 4 h or 24 h after mechanostimulation (see also **Fig. 3.1.15**)

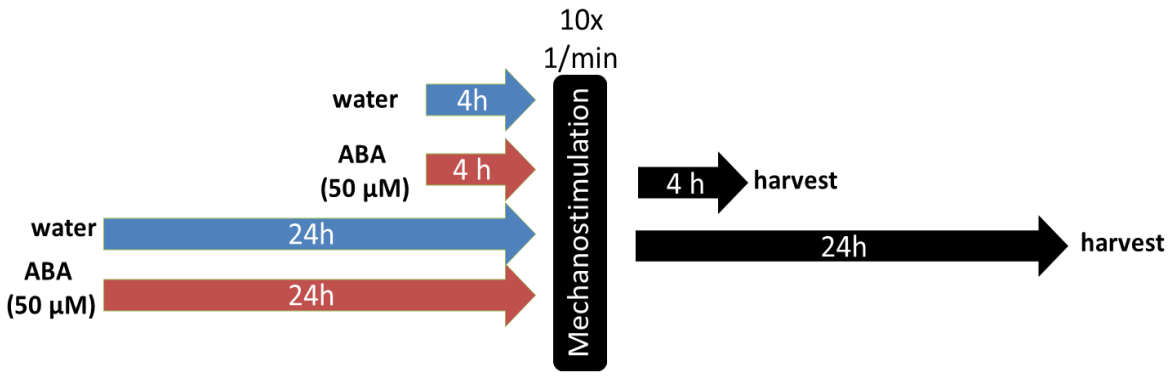


Fig 3.1.15 Experimental design for RNAseq-approach

Experimental design of the RNAseq-approach to identify genes that are affected either by the administration of ABA or via mechanostimulation. Intact traps were either incubated with 100 µL ddH_20 or 100 µL ABA (50 µM active) for either 4 h or 24 h. After each incubation time, the plants received mechanostimulation, 10 x with a frequency of 1/min. After that they were further incubated for either 4 h or 24 h prior to harvest.

Fig. 3.1.16 shows that ABA effectively reduces hydrolase expression after incubation times as short as 4 h prior to mechanostimulation. The expression of VF CHITINASE I and SAG 12 is reduced by the effects of 4 h as well as 24 h of ABA incubation by a range of approx. 1/3 to 1/7 compared to the expression in water-treated control plants 4 h after mechanostimulation.

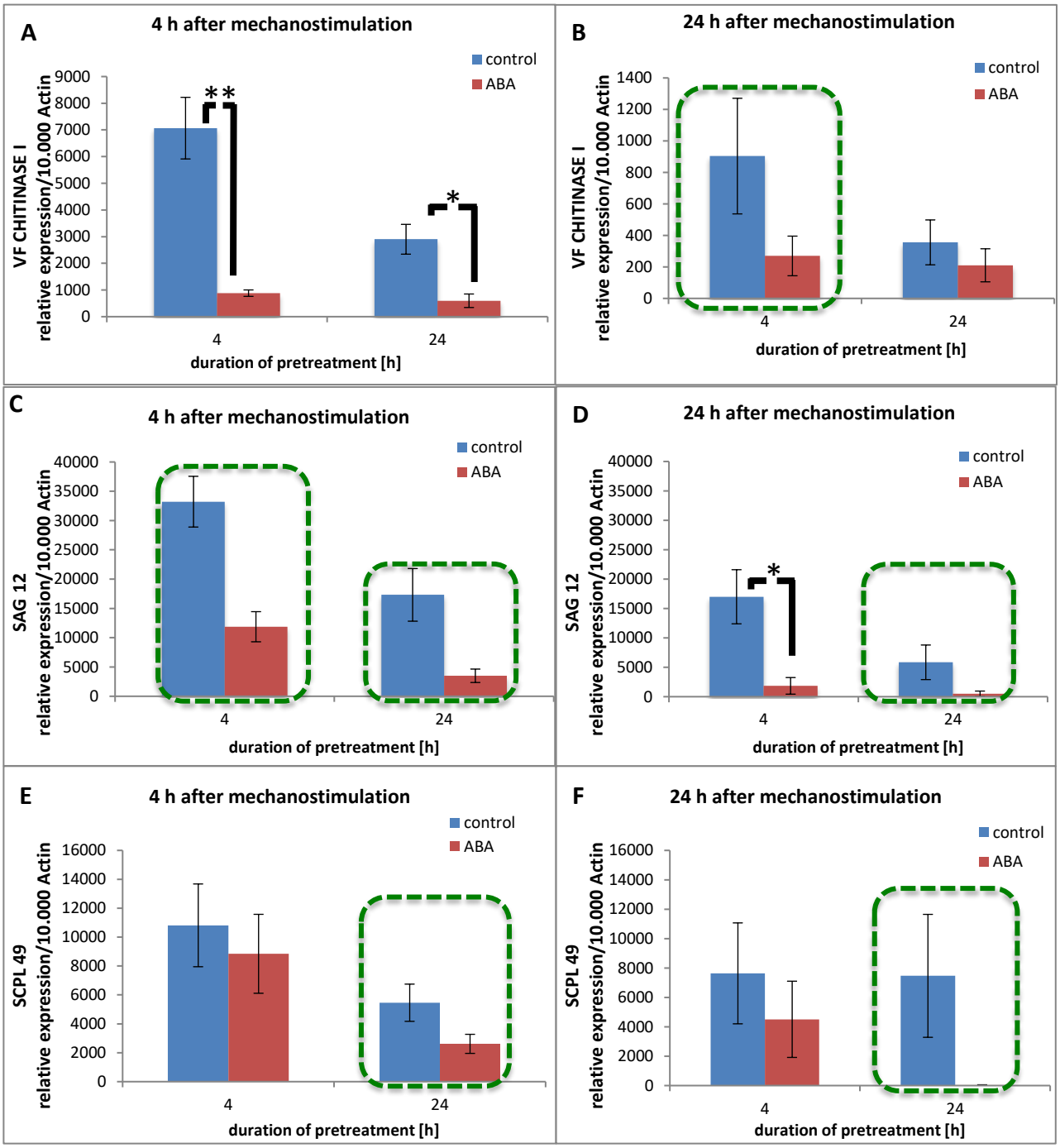


Fig 3.1.16 ABA effectively reduces hydrolase expression after short as well as long incubation times

Intact traps were sprayed with 100 µL of ddH_20 (control) or 50 µM ABA 4 h or 24 h prior to mechanostimulation (10 x, frequency 1/min). Duration of pretreatment indicates if the pretreatment with water or ABA has lasted 4 h or 24 h prior to mechanostimulation. qPCR data of hydrolase expression in the traps were obtained 4 h or 24 h after mechanostimulation. For VF CHITINASE I and SAG 12, the expression levels after 4 h incubation period with ABA are strongly reduced compared to water treatment. For 24 h pretreatment, this reduction is not so distinct. For SCPL 49, no significant difference can be observed between different treatments. Transcript numbers are given relative to 10.000 molecules of DmACT. Bars with asterisks indicate statistically significant difference: *p ≤ 0.05; **p ≤ 0.01 by Student´s T-test; n=3-5, mean ± SE. Green boxes indicate noteworthy results which should be repeated with a larger sample size to evaluate if there really is no statistical significant difference.

At 24 h after mechanostimulation, a notable difference between water- and ABA-treated plants can only be observed after 4 h of ABA incubation. In water-treated plants the transcript levels of VF CHITINASE I and SAG12 are elevated (but visibly less compared to 4 h after mechanostimulation), in ABA-treated plants the levels are negligible. The differences in expression after 24 h of ABA-incubation are not that pronounced.

The expression pattern of the hydrolase SCPL 49 is a noteworthy exception, as it does not show a difference between ddH_20- and ABA-treatment after 4 h of incubation and 4 h after mechanostimulation. The measurement after 24 h of ABA incubation and 24 h after mechanostimulation showed almost complete SCPL 49 expression inhibition, but due to the large standard error for the water treatment the result is not significant.

As statistical testing did not result in statistical differences for every data point, results which suggest a trend are marked with a green box to highlight them for further testing with a larger sample size. In control plants without mechanostimulation, no hydrolase expression was detected at any time point (data not shown).

The effect of ABA-treatment and mechanostimulation on JAZ1 and COI1 mRNA expression was analysed as well (**Fig. 3.1.17**).

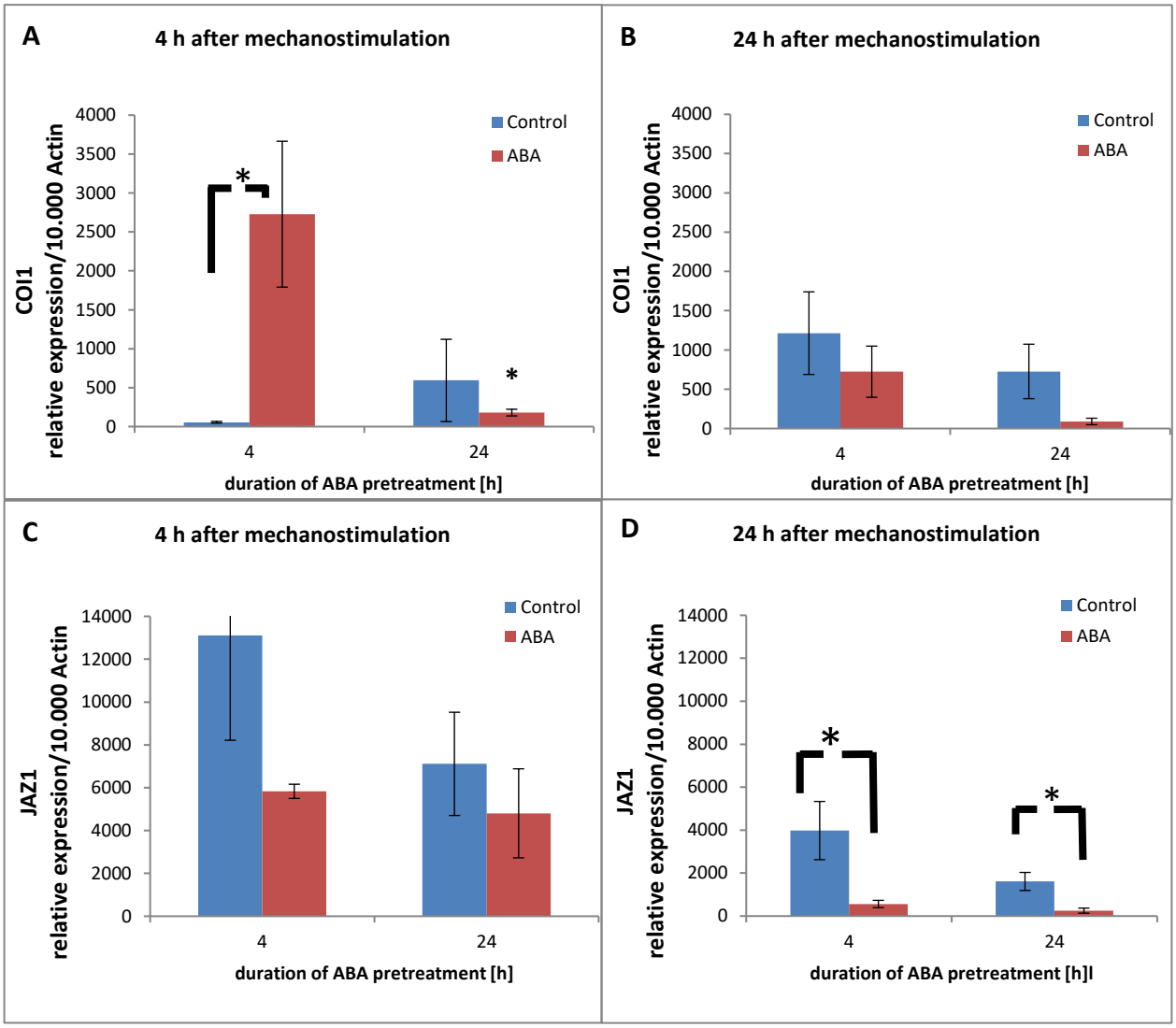


Fig 3.1.17 Effect of mechanostimulation and ABA-treatment on JAZ1 and COI1 mRNA

Intact traps were sprayed with 100 µL of ddH_20 (control) or 50 µM active ABA 4 h or 24 h prior to mechanostimulation (10 x, frequency 1/min). qPCR data of JAZ1 and COI1 mRNA expression in the traps were obtained 4 h or 24 h after mechanostimulation. Transcript numbers are given relative to 10.000 molecules of DmACT. Asterisks over columns indicate statistically significant difference to the same treatment at the other time point. Asterisks with bars indicate statistically significant difference: *p ≤ 0.05; by Student´s T-test; n=3-5, mean ± SE. In C, the axis was cut at 14000 for better visibility, the error bar for JAZ1 after 4 h of ABA pretreatment ranges from 8000 to 18000 transcripts.

It is known from Böhm et al., (2016a) that DmJAZ1 is expressed in resting traps at base levels (341 ± 131 transcripts/10,000 DmACT), and expression is elevated after mechanostimulation. After the first two applied APs, which close the trap, the DmJAZ1 expression levels were elevated 5.5-fold compared to unstimulated traps. Böhm et al. found that, with more than five APs elicited, JA gene-associated transcripts tended toward maximal levels, approx. 3000 ± 1000 transcripts. Traps were harvested by Böhm et al. 4h after the first mechanostimulation signal was given.

They found that COI1 was already expressed in resting traps at about 1250 ± 500 transcripts, but after 2 mechanostimulations expression was significantly reduced to about 250 transcripts.

In **Fig. 3.1.17 A**, it is shown that COI1 expression was almost completely repressed 4 h after mechanostimulation in 4 h-incubated, water-treated control plants, whereas in 4 h-incubated ABA-treated plants expression was almost unchanged compared to non-mechanostimulated plants. This was also shown in Böhm et al., (2016a).
In plants that were incubated with ABA for 24 h , COI1 levels were also drastically reduced, but the same was true for the water-control, no difference between water- and ABA-treatment could be detected in plants 4 h after mechanostimulation. Analysis of the expression values by Students T-Test did not result in any significant differences. In plants that were harvested 24 h after mechanostimulation (**Fig. 3.1.17 B**), no significant difference could be observed between the COI1-levels at the different time points between water- and ABA-treatment.

For JAZ1, no significant difference in transcript levels could be observed 4 h after mechanostimulation between water- and ABA-treated plants (**Fig. 3.1.17, C**), independent of the incubation time, but 24 hours after mechanostimulation, the difference in JAZ1 transcript levels between water- and ABA-treatment was significant for both 4 h and 24 h incubation time (**Fig. 3.1.17 D**). This is discussed in **4.3.3.3**.

3.1.9 Effect of exogenous ABA application on trap closure in intact plants

It was already described by Escalante-Pérez et al. in 2011 that the spray application of ABA on the traps of intact plants has an effect on trap closure after 48 h. Supplementary to the experiments presented above where it was shown that ABA has an effect on transcription levels already after 24 h, the effect of ABA spray application on trap closure and reopening in intact plants was examined. Intact plants were either sprayed once with 100 µl ddh_20 as a control or with 100 µL 50 µM active ABA-solution. Trap closure behavior was assessed 6 h and 24 h after spray treatment, and trap reopening behavior was assessed 24 h after trap closure.

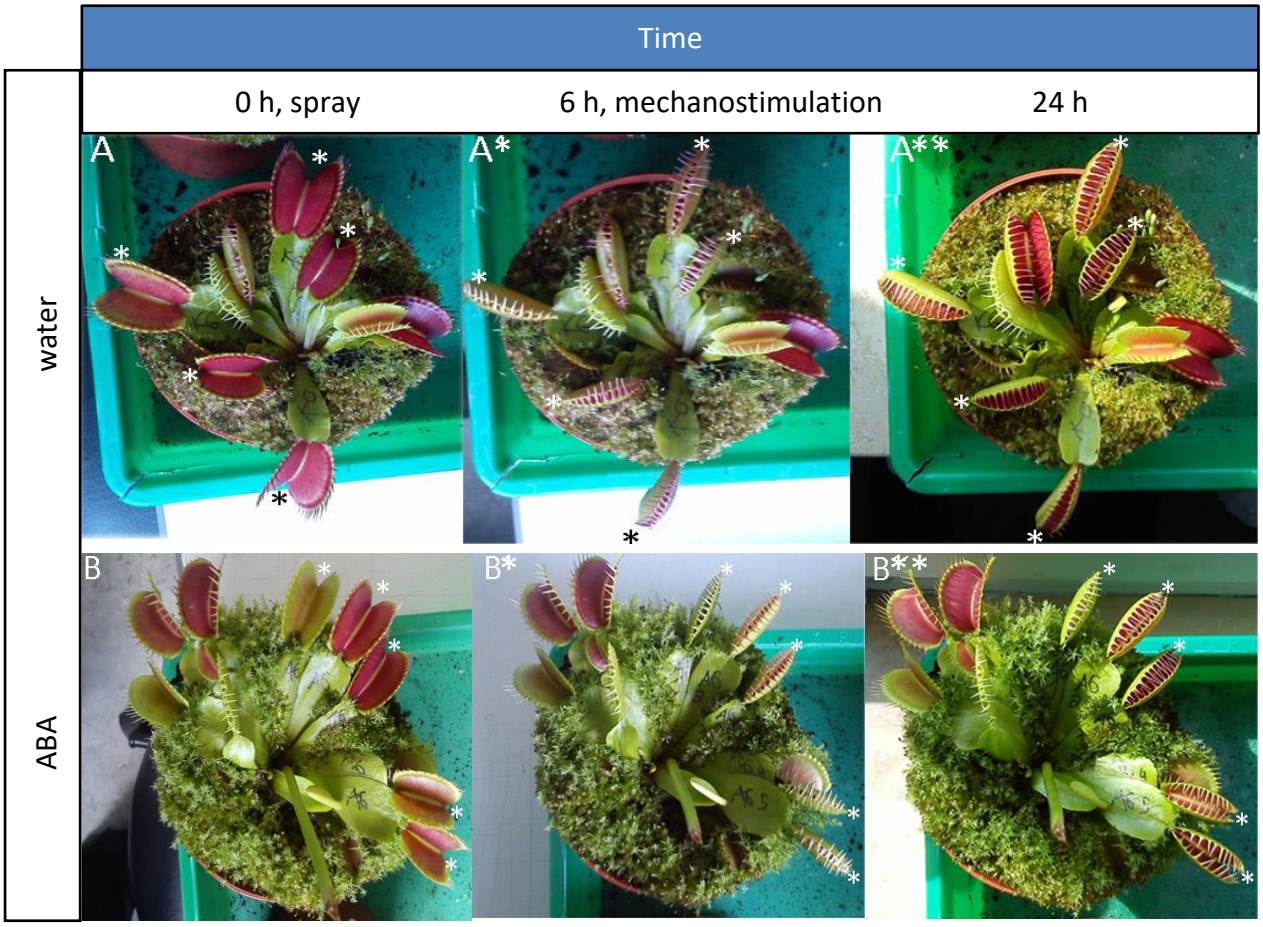


Fig 3.1.18 Effect of ABA spray-application after 6 h incubation
The effects of ABA (50 µM active, 100 µL) or water treatment after 6 h of incubation time are depicted. A and B show the respective traps (indicated by asterisks) directly after either water (A) or ABA (B) treatment. A* and B* show the same traps after 6 h incubation time and successfully elicited trap closure by two mechanostimulations. A** and B** show the same traps 24 h after mechanostimulations. In A** and B**, all traps are slowly reopening.

In **Fig. 3.1.18** the effects of water and ABA spray are shown after an incubation time of 6 h. There is no difference in closing and reopening behavior between the water control and the ABA sprayed plants.

Fig 3.1.19 Effect of ABA spray-application after 24 h incubation
In this figure the effects of ABA (50 µM active, 100 µL) or water treatment after 24 h of incubation time are depicted. A and B show the respective traps (indicated by asterisks) directly after either water (A) or ABA (B) treatment. A* and B* show the same traps after 24 h incubation time and successfully elicited trap closure by two mechanostimulations. A** and B** show the same traps 24 h after mechanostimulations. In A** and B**, all traps are reopening.

In **Fig. 3.1.19** the effects of water (control, A) or ABA (B) are shown after an incubation period of 24 h. No difference in closing and reopening behavior between the water control and the ABA sprayed plants was observed.

This is in agreement with previous experiments (data not shown) performed with truncated plants which have been submerged with the cutting area in ABA (50 µM active) for 15 min, 30 min, 1 h, 2 h, 3 h and 4 h. The traps closed after 2 mechanostimulations after all the different incubation periods, ABA could not alter the closing behavior.

3.1.10 Summary of molecular biological results

In 3.1.1 it could be shown that the height and duration of the digestion marker transcript expression (VF CHITINASE I, SAG 12 and SCPL 49) is positively correlated with the number of mechanostimulations the plant senses. The more stimulations, the higher the expression levels and the longer these levels remain elevated.

In 3.1.2 it was observed that a 24 h pretreatment of truncated *Dionaea* traps with ABA, followed by mechanostimulation, had no visible effect on the expression of the digestion marker transcript expression levels, as compared to the water-treated control group. The expression levels were comparable after both treatments in plants harvested 4 h or 24 h after the mechanostimulation occurred.

In 3.1.3 the effects of wounding, mechanostimulation of intact traps and severing the trap from the plant (cutting) on the production of the phytohormones jasmonic acid (JA) and JA-isoleucine (JA-Ile) was analyzed. The results showed that for JA, wounding and mechanostimulation had a similar effect at the beginning, JA levels were strongly elevated already after 15 minutes. In mechanostimulated traps, JA levels were reduced again after 3 h, whereas in wounded plants they were still elevated. The case was different for truncated plants, JA levels were slightly elevated within the first hour, much less than in wounded and mechanostimulated traps, but strongly elevated after 3 h. The maximum JA level was comparable in all treatments. In all cases, JA levels were back to base level after 24 h.

As JA is converted to JA-Ile, the change in JA-Ile-levels were compared to JA-levels. The JA-Ile-levels in wounded, mechanostimulated and truncated plants were rising over the course of the first hour in a comparable way. But after three hours, the JA-Ile-levels in truncated plants were approximately three times as high as in wounded or mechanostimulated traps. After 24 hours, only in truncated plants residual levels of JA-Ile were detectable.

In 3.1.4, as a preparation for gas exchange measurements, it was assessed if the transpirational pull in a truncated trap was strong enough to distribute ABA from the surrounding water to all parts of the trap. This was proven, after 1 h the used live-cell marker was found in every compartment of the trap. Later on, it was shown that the spray application of coronatine (COR) is able to elicit secretion of digestive fluid, which can be picked up by gas exchange measurements, and that the administration of ABA after the onset of secretion can diminish this secretion again. The spray application of COR strongly induced the transcription of the digestion marker hydrolases, but the later application of ABA does not reduce this transcriptional activation.

In 3.1.5, intact plants were either sprayed with COR, or COR first and ABA 12 h later. The digestion marker transcript levels were comparable in both treatments, which shows that ABA is not sufficient to counteract the effects of COR after 12 h.

In 3.1.6, intact plants were preincubated with either water or ABA for 24 hours, afterwards they were mechanostimulated. It could be shown that ABA preincubation strongly inhibits the digestion marker transcript expression in intact plants.

In 3.1.7, intact plants were sprayed with COR-MO, which is an inhibitor of the jasmonate pathway. It was shown that intact plants which were treated which COR-MO did no longer react to mechanostimulation with digestion marker transcript expression.

In 3.1.8, intact traps were either sprayed with water or ABA and incubated for a duration of 4 h or 24 h. After this incubation time, they received mechanostimulation of 1/min for 10 times. 4 h or 24 h after the mechanostimulation, the different groups were harvested. Transcript analysis showed that for the transcripts VF CHITINASE I and SAG 12, the transcription levels were strongly reduced after the plants have been incubated with ABA. This shows that the experimental design is suitable to identify ABA-responsive or mechanostimulation-responsive genes and analyse the interplay of ABA and mechanostimulation.

In 3.1.9, intact traps were sprayed and incubated with ABA for 6 h or 24 h, followed by mechanostimulation. All traps closed after two triggers were bent twice. 24 h or 48 h after the spraying of ABA, which is 18 h and 24 h after mechanostimulation, all traps were slowly reopening.

3.2 Plant micropropagation and protoplast transformation

3.2.1 Tissue culture experiments

Generation of plants from sterilized seeds

As *Dionaea muscipula* is a slow-growing plant that can take up to 5 years from seed to develop into a mature plant in soil, other ways of propagating the plants were explored. As is has been described before (Beebe 1980; Bartsch et al., 2014), accelerated growth could be achieved in sterile conditions using tissue culture techniques and nutrient medium as a surrogate for soil.

Dionaea seeds that were harvested in the greenhouse were surface – sterilized (**see 2.7.12**) and placed on ½ strength MS – medium. Seedlings developed after 6 to 10 weeks, and little plantlets could be obtained after 3 months (See **Fig. 3.2.1, A-C**). Plant growth was vivid and healthy adult plants were obtained after 7 to 9 months. They could be transferred safely to the greenhouse by placing them in soil and maintaining high humidity conditions (e.g. by wrapping the entire pot in cling wrap) for 2 weeks.

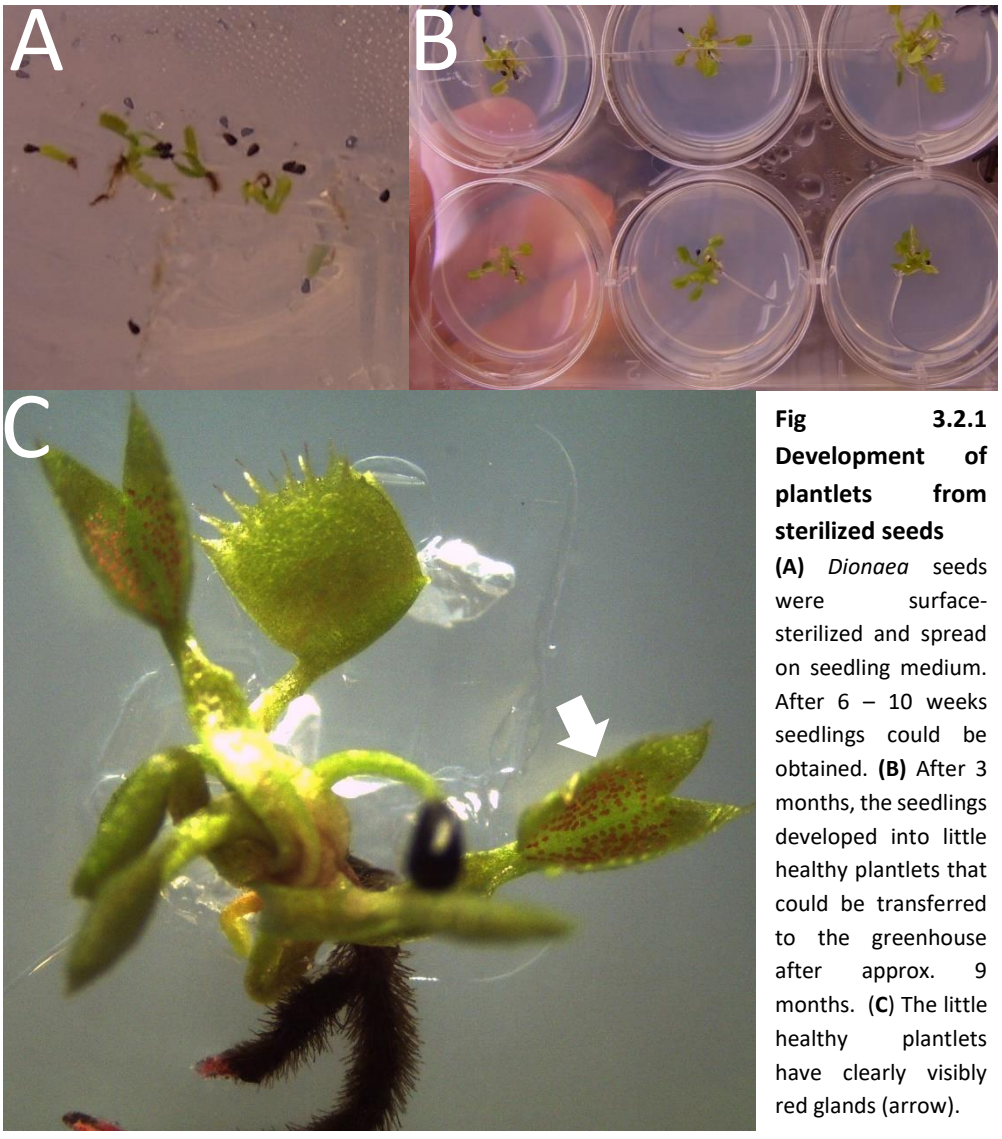


Fig 3.2.1 Development of plantlets from sterilized seeds
(A) *Dionaea* seeds were surface-sterilized and spread on seedling medium. After 6 – 10 weeks seedlings could be obtained. **(B)** After 3 months, the seedlings developed into little healthy plantlets that could be transferred to the greenhouse after approx. 9 months. (**C**) The little healthy plantlets have clearly visibly red glands (arrow).

Micropropagation of *Dionaea* by using sterile green plant parts as nurse tissue

Entire sterile culture plants could be separated into single parts (e.g. a single leaf with attached trap, or a single trap) and placed on ½ MS – medium (**see 2.7.12**). Without the addition of growth hormones, approx. 20 % of plant parts developed adventitious buds (**Fig. 3.2.2 A**) after a time period of 8 – 12 weeks, even after the tissue apparently turned necrotic (indicated by a color-change from green to black). After approx. 16 weeks the plant part that acted as nurse tissue was covered in about 20 to 30 small plantlets complete with root and traps (Fig. 3.2.2 A**).

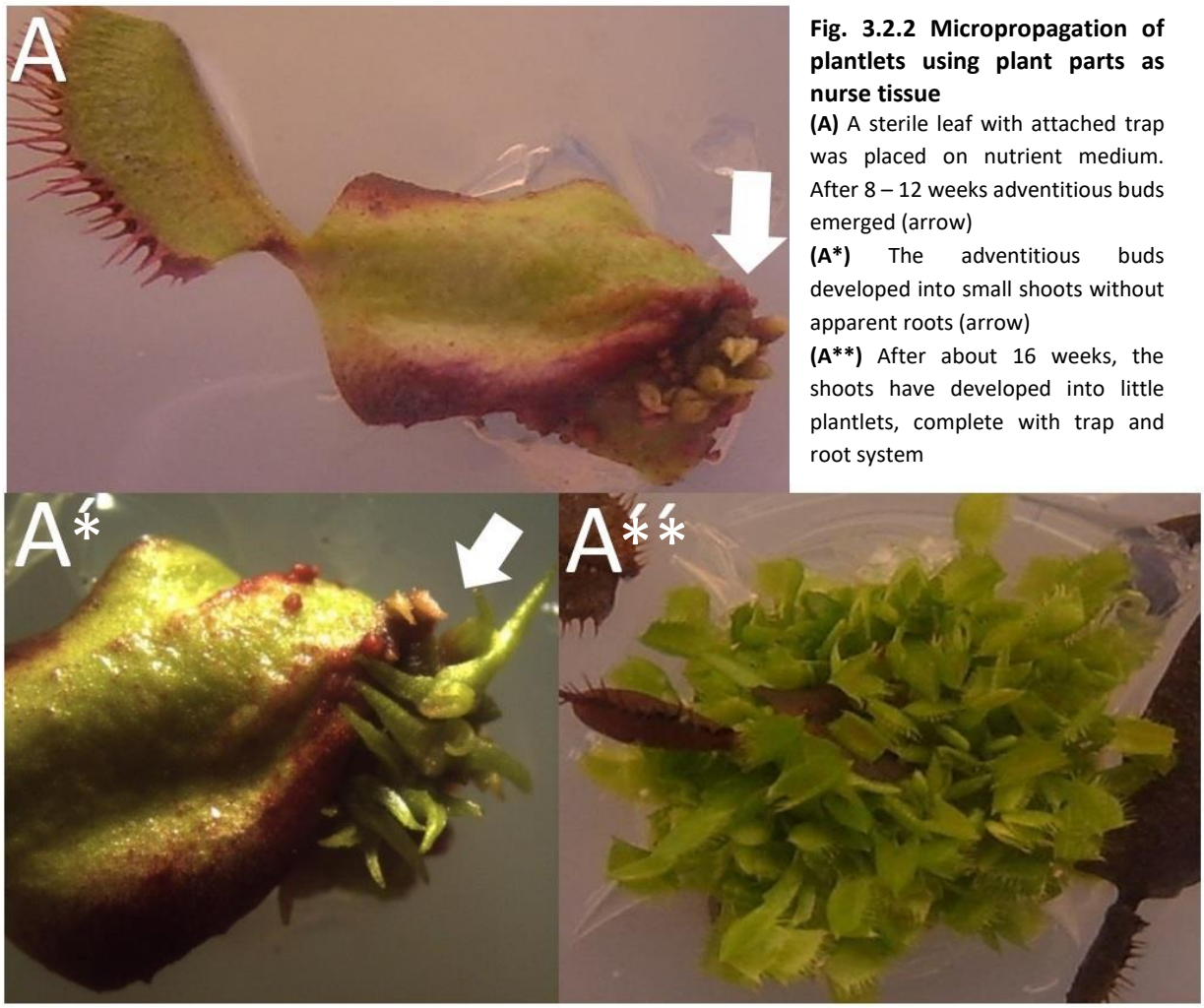


Fig. 3.2.2 Micropropagation of plantlets using plant parts as nurse tissue
(A) A sterile leaf with attached trap was placed on nutrient medium. After 8 – 12 weeks adventitious buds emerged (arrow)
(A*) The adventitious buds developed into small shoots without apparent roots (arrow)
(A)** After about 16 weeks, the shoots have developed into little plantlets, complete with trap and root system

The plantlets from **Fig 3.2.2** A** could be separated carefully and singled out to grow into a healthy, mature plant. Once the plant was transferred from *in vitro* conditions to soil, it was important to keep humidity levels high at the beginning. This was achieved by wrapping the plant in simple commercially available cling wrap. This provides a fast and reliable opportunity to maintain a population of specific, defined plants.

The plant micropropagation technique described above is not only suitable for the multiplication of plants derived from sterile culture, but can also be applied to multiply plants from non-sterile conditions. For that, those plants have to be surface-sterilized prior to be placed on nutrient medium (described in Bartsch et al., 2014). This has proven to be not trivial and requires some experience.

3.2.2 Generation and transformation of protoplasts

Protoplasts were generated as described in **2.7.10** from 1 g of healthy green plant parts (from the *Dionaea* petiole, mostly leaf blade without stem tissue) with the enzyme solution adjusted to 350 mOsmol/kg. For transformation, 30 µg of the vector pSAT-UBQ10prom::VenusYFP::mTurqouise2 (further referred to as "vector", provided by Dr. Kai Konrad, Department of Plant Physiology and Biophysics, University of Würzburg) were used and the PEG-transformation was performed after the protoplasts were incubated at 4 °C overnight (ON). 24 h after transformation they were analysed using a Keyence BioZero inverse fluorescence microscope and 20x magnification. As VenusYFP was detectable using the in-built GFP-filter, the microscope was suited for detection of fluorescence.

The vector was tested on protoplasts derived from 5-7 weeks old *A. thaliana* Col-0 mesophyll tissue to establish the functionality of the vector. To establish the transformation system, untransfected protoplasts generated from *A. th* Col-0 – mesophyll tissue were analyzed with regard to fluorescence and autofluorescence. **Fig. 3.2.3 A** shows the *A. th.* protoplasts in transmission light, **Fig. 3.2.3 B** in fluorescence light using the GFP filter. As Fig. 3.2.3 B shows, they did not exhibit any autofluorescence. Fig. 3.2.3 C shows the chlorophyll autofluorescence after being excited with UV light, detected while using the RFP filter.

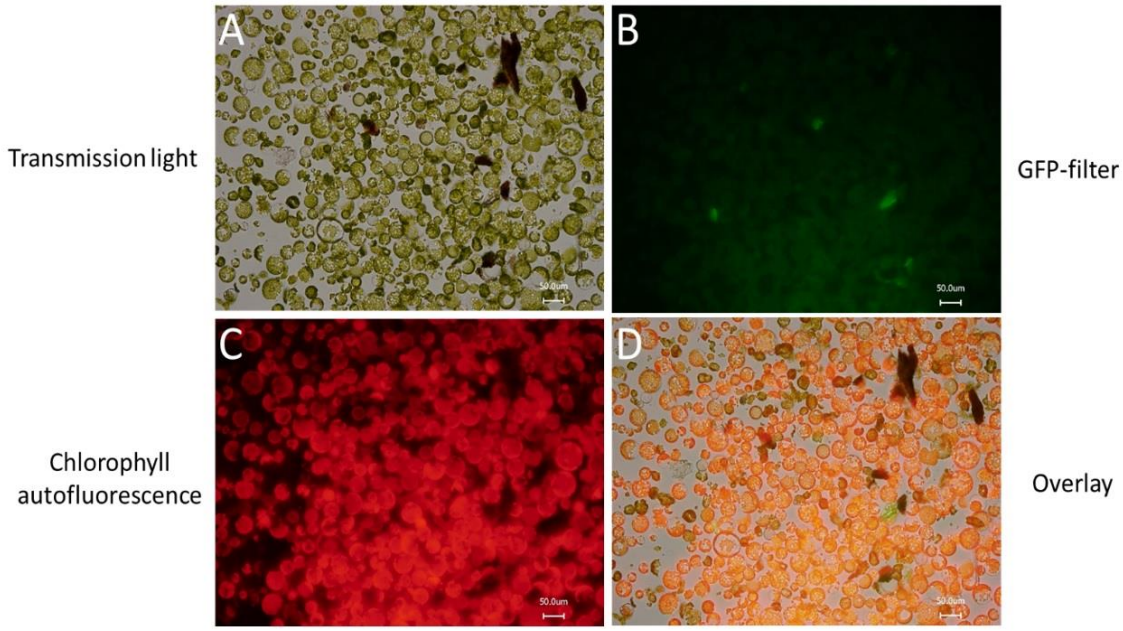


Fig 3.2.3 Transformation control – untransfected *A.th* Col-0 protoplasts
Untransformed *A. th* protoplasts were tested as a control. Transmission light **(A)** was used to assess form and chloroplast distribution of the protoplasts. Fluorescence light **(B)** was used to analyse possible autofluorescence radiating from the protoplasts themselves. Chlorophyll autofluorescence **(C)** was examined to further assess the status of the protoplasts using RFP-filter, and **(D)** is an overlay of pictures A-C. The scale bar represents 50 µM.

To verify the working condition of the vector, it was tested on the *A. th* - protoplasts shown in **Fig. 3.2.4**

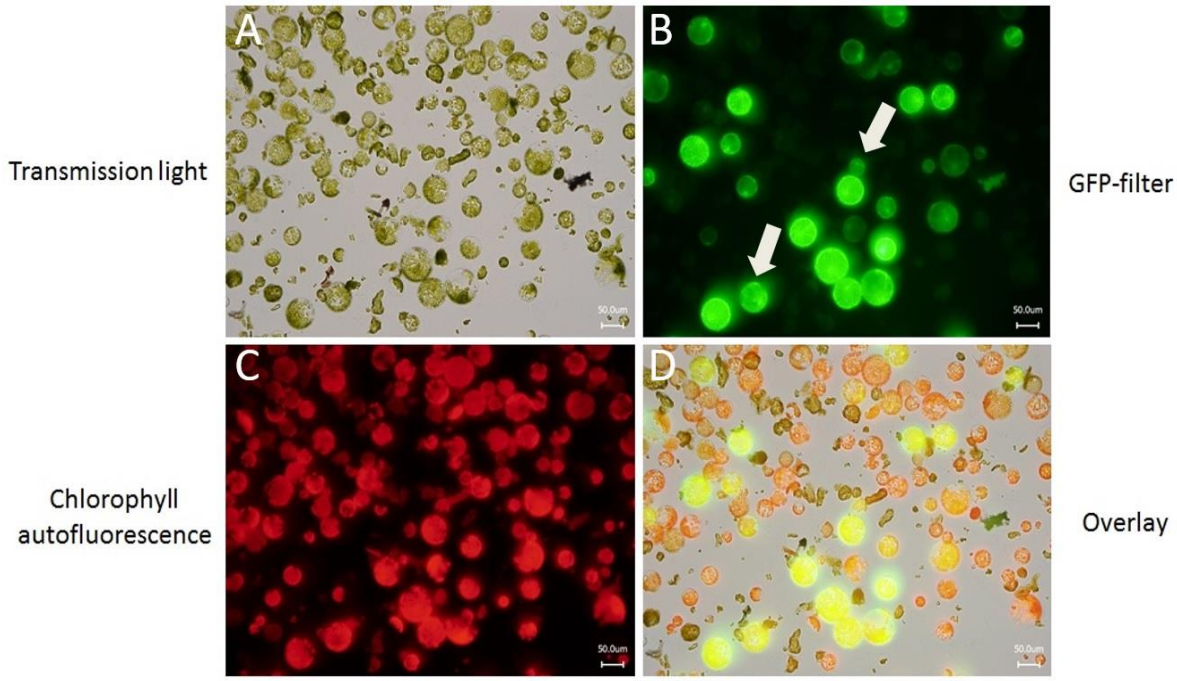


Fig 3.2.4 Transformation test – Vector expression in *A.th* Col-0 - protoplasts

The transformation vector was first tested on protoplasts generated from 1 g of 5-6 weeks old *A. th*. Col-0 – mesophyll tissue. Transmission light **(A)** was used to assess form and chloroplast distribution of the protoplasts. Fluorescence **(B)** was used to analyse the fluorescence distribution in the protoplasts (arrows). Chlorophyll autofluorescence **(C)** was used to further assess the status of the protoplasts, and **(D)** is an overlay of pictures A-C. The scale bar represents 50 µM.

The transformation of *A. th* protoplasts with pSAT-UBQ10prom::VenusYFP::mTurqouise2 resulted in bright visible fluorescence in the protoplasts concentrated to the chloroplasts (**Fig 3.2.4 B**, arrows), proving that the vector is working and fluorescence could be observed in *Dionaea* protoplasts after successful transfection, if the promoter is functional and if the fluorescent protein is correctly produced and folded in the *Dionaea* protoplast system.

Generating protoplasts from *Dionaea* mesophyll tissue is more complicated than from *Arabidopsis*. In *Dionaea* protoplasts (**Fig 3.2.5**), chloroplast distribution after 24 h incubation is not as even (**Fig 3.2.5 A**) as it can be observed in *A. th* – protoplasts (**Fig. 3.2.3 A**), they tend to accumulate to one side of the protoplast. Untransformed *Dionaea* protoplasts were examined with regard to fluorescence, but no autofluorescence could be observed using the GFP-filter (**Fig. 3.2.5 B**).

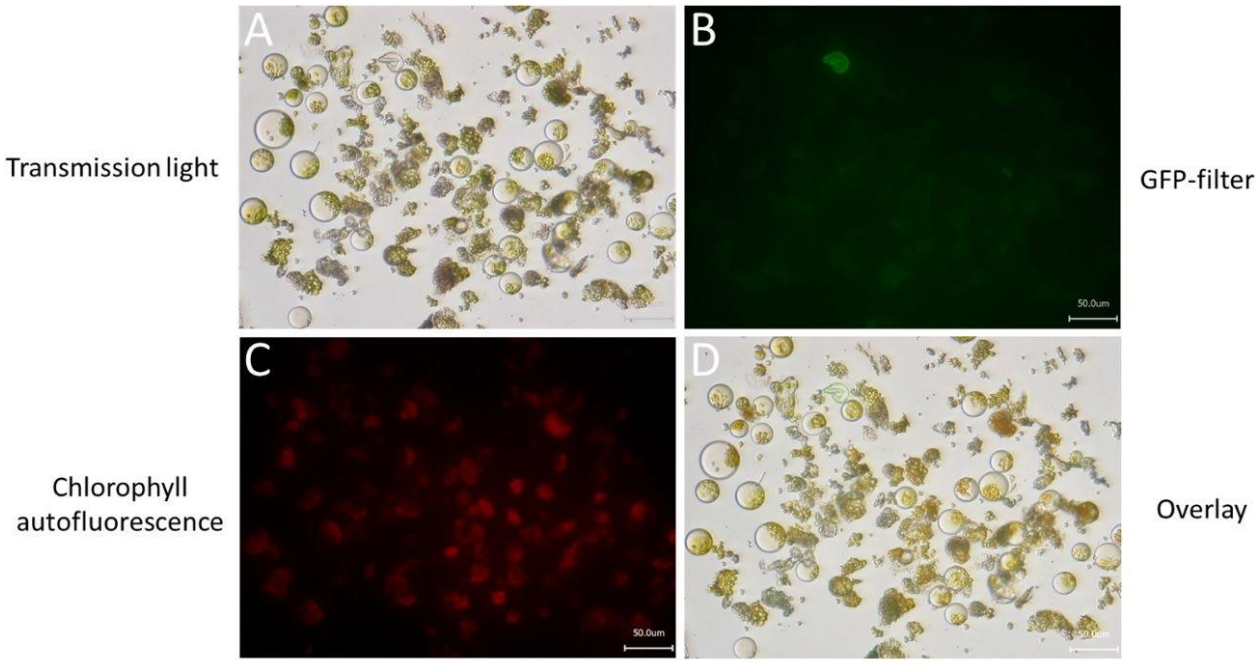


Fig 3.2.5 Transformation control - untransfected *Dionaea* protoplast
Untransfected protoplasts were tested as a control. Transmission light **(A)** was used to assess form and chloroplast distribution of the protoplasts. Fluorescence **(B)** was used to detect possible autofluorescence radiating from the protoplasts themselves. Chlorophyll autofluorescence **(C)** was used to further assess the status of the protoplasts, and **(D)** is an overlay of pictures A-C. The scale bar represents 50 µM.

After the *Dionaea* protoplast system was determined adequate to be tested for fluorescence generated by the vector due to a lack of autofluorescence, *Dionaea* protoplasts that have been incubated at 4 °C ON after generation were transfected with 30 µg of pSAT-UBQ10prom::VenusYFP::mTurqouise2 and fluorescence was examined 24 h after transfection.

Dionaea protoplast transformation was successfully achieved (**Fig. 3.2.6 B**, arrows). Fluorescence could be observed concentrated in the chloroplasts of the protoplasts.

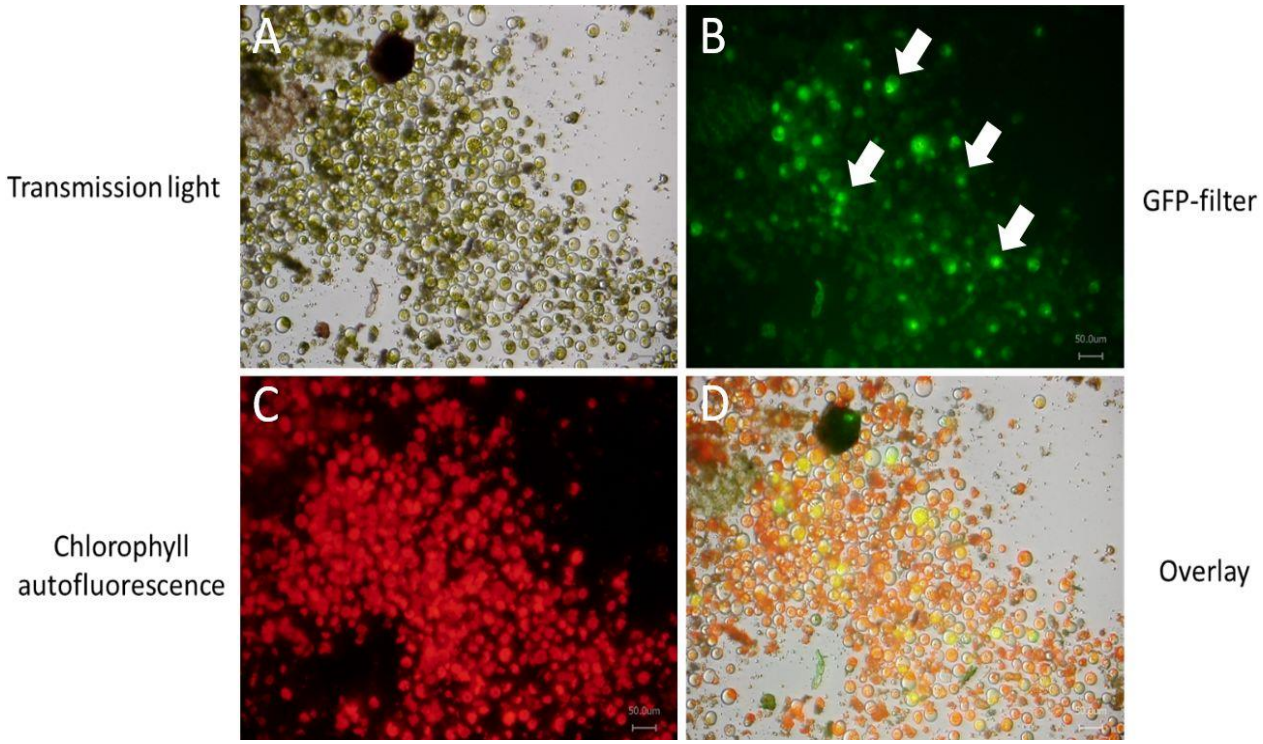


Fig 3.2.6 Transformation test – Expression of fluorescent proteins in *Dionaea* - protoplasts
Dionaea protoplasts were transfected with 30 µg of pSAT-UBQ10prom::VenusYFP::mTurqouise2. Fluorescence was examined 24 h after transfection. Transmission light **(A)** was used to assess form and chloroplast distribution of the protoplasts. Fluorescence **(B)** was used to analyse the fluorescence distribution in the protoplasts (arrows). Chlorophyll autofluorescence **(C)** was used to further assess the status of the protoplasts, and **(D)** is an overlay of pictures A-C. The scale bar represents 50 µM.

3.2.3 Herbicide tolerance test of *Dionaea muscipula*

Herbicides are used in tissue culture experiments to distinguish transformed from untransformed plants by introducing an herbicide resistance gene along with the transformation vector as a genetic marker. As the potency of the herbicide resistance gained by the transformant varies depending on many variables (Park et al., 1998), singling out transformed plants by the use of the herbicide they are supposed to be resistant against is not a trivial task. To establish a baseline, wildtype plants have to be subjected to the herbicide in various concentrations to discover the maximum dose they could tolerate. As there were no working transformation systems for *Dionaea muscipula* at the time of the thesis, but the generation of a transgenic plant via protoplast regeneration was possible, the impact of two herbicides has been tested.

As the antibiotic kanamycin A (further referred to as kanamycin) is frequently used as a selective agent (Nap et al., 1992), a number of kanamycin concentrations have been tested in tissue culture conditions using 3 months – old plantlets derived from sterile seeds. Those plantlets have been transferred to plant nutrient medium containing 0 mg/L, 50 mg/L, 100 mg/L, 200 mg/L, 300 mg/L and 400 mg/L of kanamycin. After four months, survival rate has been determined. All plantlets in 0 mg/L and 50 mg/L survived and thrived, at 100 mg/L only 1 out of 4 plants survived and thrived, 3 out of 4 were degenerated (**Fig. 3.2.7 A**, asterisks), and at 200 mg/L and above all plants were necrotic and presumably dead (**Fig 3.2.7 B**, asterisks).

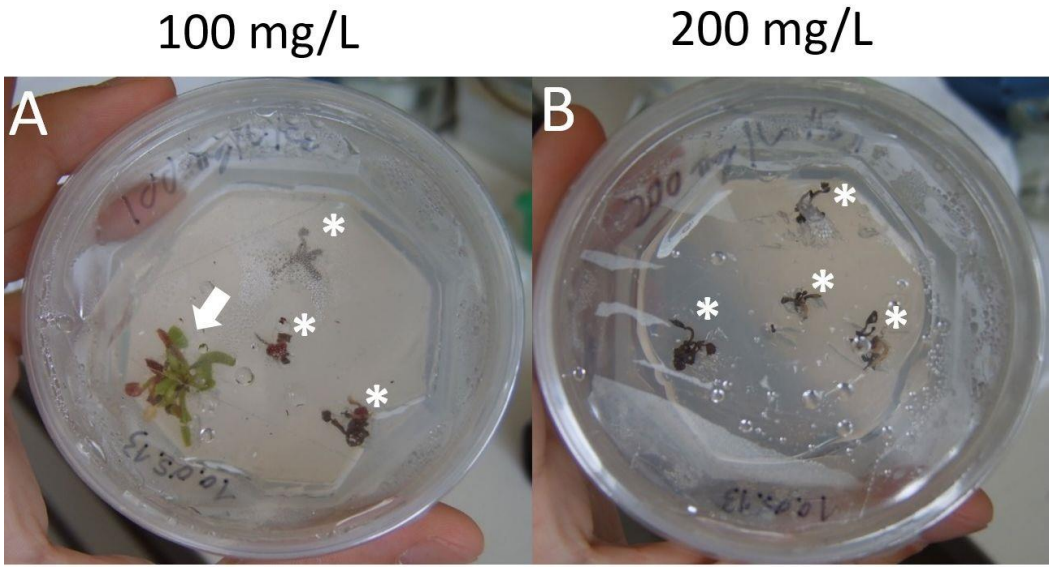


Fig. 3.2.7 Test of kanamycin as herbicide for *Dionaea muscipula* transformation system
Different concentrations of kanamycin (0 mg/L to 400 mg/L) were used on 3 months old *Dionaea* plantlets derived from sterile seeds. After 4 month of incubation survival rate was assessed. At 100 mg/L **(A)** only 1 out of 4 plantlets (arrow) was thriving, and at concentrations of 200 mg/L **(B)** and above all plants were necrotic, of brown/black color and presumably dead (asterisks).

Another very effective herbicide is N-(phosphonomethyl)glycine, also known as Glyphosate (Baird et al., 1971). Because it was not available in an adequate pharmaceutical form, the impact of Glyphosate on *Dionaea muscipula* was tested on adult plants in the greenhouse in the form of the spray application of Celaflor Roundup Alphee, a herbicide containing 7,2 g/L Glyphosate. Healthy green adult *Dionaea* plants (**Fig. 3.2.8** A, B) were sprayed entirely once. After only 11 days of incubation, the plants showed severe signs of necrotic (black) tissue (**Fig 3.2.8**, A*, B*)

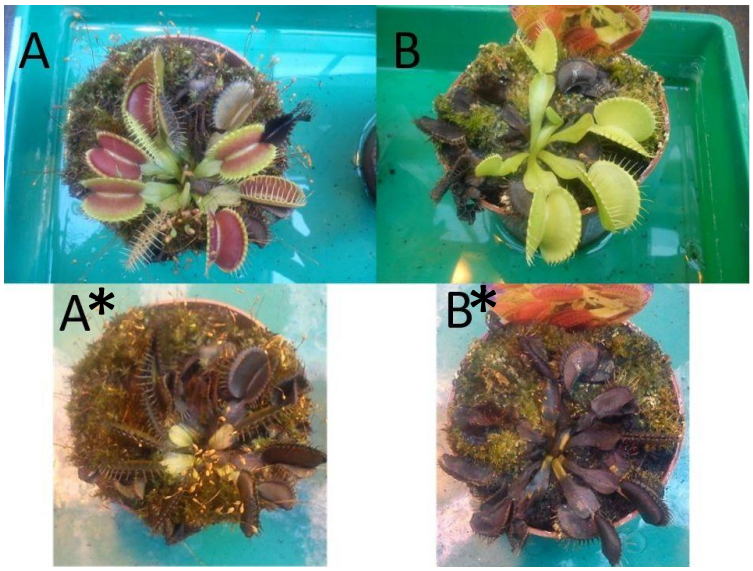


Fig 3.2.8 Impact of Glyphosate on *Dionaea muscipula*
Healthy green adult plants were sprayed entirely with Celaflor Roundup Alphee (A, B). After 11 days (A*, B*), the plants showed severe signs of necrotic tissue

3.2.4 Summary of plant micropropagation and protoplast transformation experiments

In 3.2.1, it could be shown that *Dionaea* can be raised in sterile cultures from sterilized seeds, and can also be multiplied in sterile culture by using parts of a whole plant to generate numerous plantlets.

In 3.2.2, protoplasts were generated from *Dionaea* petiole tissue and transfected with a pSAT-UBQ10prom::VenusYFP::mTurqouise2-vector. The YFP fluorescence could be detected in *Dionaea* protoplasts.

In 3.2.3, kanamycin as a potential selective agent was assessed. Concentrations of 200 mg/L and above proved lethal for developing plantlets in sterile cultures after 4 months. The effects of spraying Glyphosate were also assessed on whole plants in the greenhouse, a one-time spray treatment was sufficient to kill the plant after 11 days.

4. Discussion

Dionaea muscipula, the Darwin plant, has fascinated researchers for more than 200 years (Ellis, 1768; Darwin, 1875; Hedrich and Neher, 2018). Its ability to trap and digest insects and other prey, together with its exceptional catching mechanism, the snap-traps, puts it in an unprecedented position within the plant kingdom.

The initial procession of information is facilitated using electric impulses. Prey entering the snap-traps elicit action potentials by mechanical bending of the trigger hairs, which in turn generates an action potential that travels across the whole trap. If the trap is ready to digest, two action-potentials are enough to trigger trap closure. The trapped insect keeps moving within the trap and is struggling to escape, thus eliciting more action potentials. 3 APs are sufficient to elicit a distinct rise of the gland cell cytoplasmic Ca^{2+} level, which most probably triggers the jasmonate pathway. More than 5 APs trigger the production and secretion of the digestive fluid and the digestive enzymes. They also induce the building of transport proteins in the gland cells which are required for the uptake of nutrients obtained from the digested insect (Volkov et al., 2011; Böhm et al., 2016a; Hedrich and Neher, 2018).

It was known from previous experiments (Escalante-Pérez et al., 2011) that the exogenous application of hormones (or their mimics) can interfere with *Dionaea*'s natural trapping system. The effect of these hormones on the transcription level of the marker hydrolases was not well understood at the beginning of this work.

4.1 DNA-barcoding for species analysis

For further research, and with huge varieties within the cultivars, it is important to distinguish if *Dionaea* really is a single species with polymorph appearance, or if it includes different subspecies. Or can different ecotypes be distinguished, like in *A. th*, which would explain different molecular behaviors? Effects of long *in vitro*-cultivation periods have to be established compared to wildtype plants with a possible effect on the genome. To establish *Dionaea muscipula* as a model organism for biological research, uncertainties like this have to be eliminated in order to be able to compare research done world-wide. The plants used in this thesis were all obtained from one large breeder, Cresco Carnivora V.O.F. from the Netherlands, who apparently used *in vitro* multiplication techniques to generate huge amounts of plants (oral communication Joachim Rothenhöfer, head horticulturalist Department of Plant Physiology and Biophysics, University of Würzburg), so one might assume genetic identity between the plants. But plants were delivered with unknown age and unknown growth conditions prior to being transferred to the institute´s green house. This is due to the long generation time of a plant. These differences in plants age and condition might explain the different expression levels obtained in different experiments in this thesis, and also the huge error bars which showed up in some experiments.

The *maturaseK*-gene (*matK*) from chloroplasts could be used to distinguish different species, as described in Hilu and Liang (1997), or the *rbcL* (RuBisCO large chain)-gene together with *matK* (Hollingsworth et al., 2009). The suitability has to be assessed; other genes might be more suited for *Dionaea muscipula*. It was shown that the nuclear ribosomal internal transcribed spacer *ITS* + *matK* are best suited to discriminate species within the *Nepenthes* genus, whereas *rbcl* + *matK* were not suited to distinguish *Nepenthes* species(Gogoi and Bhau 2018).

If it has been established that all existing 130 *Dionaea* cultivars belong to the same species by comparing certain specific genes, the complete comparison of different transcriptomes or different genomes could be used to further characterize the different cultivars. These findings can then be used to better interpret different results obtained by different researchers.

4.2 Tissue culture experiments can be the basis for a whole new field of *Dionaea muscipula* research

The tissue culture experiments performed in **3.2** returned very promising results, as it was shown that expression of genetic constructs in *Dionaea* protoplasts is possible and fluorescent signals can be observed.

Under the right conditions, from a single leaf of a sterile plant a multiplicity of clones can be generated, which can be utilized for stock-keeping of plant lines (**3.2.1**). Callus tissue is also relatively easy to create and could serve as basis tissue for transgenic plant generation.

This is especially important for maintenance of future transgenic lines, which could be created by creating transgenic protoplasts (**3.2.2**) and using them as a basis for callus generation (Nagy and Maliga, 1976) or by using *Agrobacterium tumefaciens*-mediated gene transfer (De Buck et al., 1998) as has been published recently for the first time (Suda et al., 2020). This is especially important because via transient protoplast expression the functionality of vector systems, fusion proteins and promotor constructs can be tested before transgenic lines are established.

As the *Dionaea* protoplasts did not look particularly healthy, with regard to plastid distribution and cell volume increase, compared to the *Arabidopsis* protoplasts (**Fig. 3.2.4** and **Fig. 3.2.5**), protoplast generation conditions have to be improved, mainly the osmotic conditions.

It has been further demonstrated (**3.2.3**) that *Dionaea* has an intrinsic tolerance to kanamycin up to a certain threshold, which could be used to further enhance in vitro cell culture conditions for *Dionaea* to reduce bacterial growth which occurs occasionally, mainly after surface sterilization of non-in vitro plant tissue (Bartsch et al., 2014) or to distinguish transfected from non-transfected cells by including kanamycin resistance. *Dionaea* has been shown to be very susceptible to Glyphosate, therefore this can also be used as a powerful

selective agent to distinguish transfected from non-transfected plants. The right dosage for successful transfected plant survival has yet to be determined.

In summary, with the protoplast method described here in this thesis, it is possible to test the genetic constructs intended to be used for the generation of the transgenic lines, to verify that they are working in *Dionaea* as intended. With those verified constructs, transgenic lines can be established, either by generating callus-tissue from transgenic protoplasts or by using *A. tumefaciens*. In addition, it has been shown that *Dionaea* is susceptible to higher amounts of kanamycin over a longer period of time, and to Glyphosate after a very short period of time.

4.3 Transcriptional and hormonal regulation

4.3.1 *Dionaea muscipula* can discriminate between accidentally trapped objects and living prey by spatio-temporal signal integration

In accordance with published research (Escalante-Pérez et al., 2011; Böhm et al., 2016a), it was found that *Dionaea* can discriminate between inanimate, trapped objects and a worthwhile prey. Based on a multi-level safeguard system, *Dionaea´*s trapping mechanism is concentrated on maximizing gain of nutrients and minimizing loss of water and energy. As the pre-tensing of the trap requires energy, so a prey can be trapped within a fraction of a second, it is optimal for the plant to develop mechanisms that minimize energy costs. Two action-potentials elicited by mechanostimulation are required to trigger trap closure (Escalante-Pérez et al., 2011); so, if a leaf, which does not possess any nutritious value whatsoever, falls into the trap, this is usually not sufficient to cause trap closure. An insect on the other hand that visits the trap on its search for food (Kreuzwieser et al., 2014) is much more likely to cause two separate action potentials. Once the trap has snap-shut and the insect is trapped, the second layer of safeguard mechanisms sets in. Most of the time the trap does not close all the way immediately, leaving a small opening between the two trap lobes. The interlocking marginal spikes are sufficient to prevent larger prey worth digesting from exiting, but smaller prey with only a minimum amount of nutrients is able to break free (Volkov et al., 2011; Libiaková et al., 2014). After several minutes, the trap-lobes have closed completely. This is the time for the third layer of safeguard mechanism to take action. If by accident an inanimate object like a leaf has triggered complete trap closure (closed state), it will not move while being in the trap, therefore not eliciting any more action potentials. This will lead to trap-reopening in the next 24 to 48 hours (own observation; Volkov et al*.,* 2011; Libiaková et al., 2014). If a prey worth the digesting effort has been caught, it is trapped within the two trap lobes and is trying to escape. In doing so, it struggles to break free, thereby touching the trigger hairs and eliciting more action potentials. Böhm et al., (2016a) have found that five action potentials were required to trigger significant gene expression of the three digestion marker hydrolases VF CHITINASE I, SAG 12 and SPCL 49.

In **3.1.1** it was shown that only trap closure and the initial stimulation during the first hour (3 times each 15 minutes) was not enough to elicit a long-lasting hydrolase expression. With only the initial stimulation present, hydrolase expression was almost non-detectable after 4 hours. With an additional stimulus after 4 hours and a second additional stimulation after 8 hours, the hydrolase expression levels remained elevated even 24 h after trap closure. In light of the findings by Böhm et al., (2016a), this points towards a spatio-temporal signal incorporation by *Dionaea muscipula*. As elevated JA- and JA-Ile-levels have been measured after 10 mechanostimulations and from as early as 15 minutes to 3 hours, (see **3.1.3**), it stands to reason that after 4 hours elevated levels of JA-Ile will also be detectable when the additional first stimulus is applied. Within this primed system, the effect of the additional stimulus is enough to keep marker hydrolase expression elevated. This represents the situation of a trapped insect struggling to escape its cage.

To understand the full complexity of this regulation, future research should combine all three examination foci: After trap closure is elicited, a various amount of trigger hair stimulations should be applied, this should be correlated with different time points of (additional) stimulations. Complementary, marker hydrolase transcript expression as well as COI1- and JAZ-expression should be measured, and from the same samples JA- und JA-Ile-levels should be determined. This can be cross-referenced with the determination of enzymes in the digestive fluid. This would give a wholistic picture of the spatio-temporal regulation of *Dionaea*'s prey catching control network and would shed light on the question if *Dionaea* is releasing all digestion enzymes at once, or subsequently.

4.3.2 The exogenous application of ABA reduces hormone and transcript levels in intact, but not in truncated plants

ABA functions, among many other things, as the plants drought stress signal (de Ollas and Dodd, 2016). A reduced soil water content for example is perceived by the roots, which react with increased ABA production to protect the plant from water shortage consequences. Upon water shortage, plants react with stomatal closure and the production of osmotically active substances to maintain cell turgor (Finkelstein, 2013; de Ollas et al., 2015; de Ollas and Dodd, 2016). For this increase in ABA levels to occur, jasmonic acid (JA) accumulation leading to JA-isoleucine (JA-Ile) build-up was necessary (de Ollas et al., 2015).

It was analysed in previous publications if the application of ABA to simulate drought would affect the trap closing behavior of *Dionaea muscipula*. The exogenous application of 50 µM active ABA on the traps of *Dionaea muscipula* 48 h prior to mechanostimulation could elevate the trap closing threshold from 2 APs to 3, 5 and sometimes 10 and more APs. Therefore, the application of ABA successfully mimics drought, and *Dionaea* responds by increasing the trap closing threshold in a cost-benefit analysis matter. The opening and pre-tensing of the trap and the digestion process is energy- and water-intensive, as it is using turgor-changes in specific areas, and even more so the digestion process after trap closure. Therefore, in times of drought the trap-closing and digestion process is reserved for larger, nutrient-rich prey (Escalante-Pérez et al., 2011; Escalante-Pérez et al., 2014).

In this thesis it could be observed that the exogenous spray application and incubation of ABA on **intact traps** for a time between 6 hours and 24 hours had no effect on trap closing and reopening behavior (see **3.1.9**). In comparison with the findings of Escalante-Pérez et al., (2011), this difference is probably caused by the difference in pre-incubation time with ABA, as in this thesis the maximum incubation time was 24 h, whereas in Escalante-Pérez et al., (2011) it was 48 h. This shows that the duration of the draught signal is of importance and might point towards the fact that short-term water shortage is not uncommon for *Dionaea*.

Spray application of ABA had, however, a strong inhibiting effect on the transcription levels of the marker hydrolases expressed in the trap tissue. Even after prolonged mechanostimulation of ABA-sprayed intact traps, the marker hydrolase expression was negligible (see **3.1.6, Fig. 3.1.12**). This is in agreement with previous research (Escalante-Pérez et al., 2011) and shows that, under water-stress conditions, *Dionaea* reduces its sensitivity for prey catching and therefore its water consumption. As the open state of the trap is the “energy-intensive” state where the energy has already been invested (Volkov et al., 2008a; Volkov et al., 2014), closing upon prey contact does not result in water consumption. The digestion process however is very water-intensive (Escalante-Pérez et al., 2011), in times of water stress it is reasonable to delay the production of the digestive fluid, as the digestive fluid has a high water content (Scala et al., 1969).

In **truncated plants**, it was observed that the pretreatment with 50 µM active ABA for 24 h had no inhibiting effect on transcription levels of the marker hydrolases after

mechanostimulation (see **3.1.2**), neither had the incubation of truncated traps in 50 µM active ABA for a period between 15 min and 24 hours an effect on trap closing or reopening behavior after trap-closure was elicited (data not shown). The incubation of truncated traps in 200 µM active ABA for a time between 1 h and 6 h had no effect on mechanostimulation-elicited trap closing behavior, but abolished trap reopening for 48 h. After 72 h, most of the traps turned necrotic (Data not shown).

The effectiveness of ABA-mediated inhibition of the response to mechanostimulation in intact plants, and the ineffectiveness to do the same in truncated plants, could have several reasons. In this thesis, it could be shown that, in truncated plants, the cutting procedure elicits a hormonal wounding response (see **3.1.3, Fig. 3.1.3**), shown by a massive increase in JA and JA-Ile-levels within the first 3 hours after cutting. It takes up to 24 hours for JA and JA-Ile levels to return back to basic levels. Those elevated JA and JA-Ile levels could block the effects of exogenous ABA. It is hypothesized that by this wounding event a JA-sensitive pathway could be activated in the trap tissue, which then might be insensitive to ABA signaling. Additionally, ABA is usually produced within the root system as the primary sensor for water deprivation. As described by Gonzalez-Guzman et al., (2012), ABA is required for maintaining lateral root growth during water deficits. Therefore, a root-based signal molecule could be missing in truncated plants to allow the ABA-signal to be perceived. This is further backed up by the fact that hydrolase expression in truncated traps was the same with or without ABA. It is known that guard cells also have ABA receptors, and the gas exchange experiments (see 3.1.4) showed that the guard cells react to exogenous ABA given by the stem even after the trap was truncated from the main plant.

As reviewed by Finkelstein (2013) and Zhang et al., (2015), and published in Dittrich et al., (2019), in *A.th*. a network of proteins of the PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) family has been identified as potential ABA receptors. The PYR/PYL/RCAR family together with protein phosphatases type-2C (PP2Cs), Snf1 (Sucrose-non-fermentation1)-related kinases subfamily2 (SnRK2s) and downstream substrates constitute the core ABA signaling network (Zhang et al., 2015). Generally, PP2Cs inactivate SnRK2s kinases by physical interaction and direct dephosphorylation. Upon ABA binding, PYLs change their conformations and then contact and inhibit PP2Cs, thus activating the SNF1-related kinases (SnRKs) which are required to activate transcription factors, ion channels and numerous other mediators of ABA response (Finkelstein, 2013). Several members of the PYR/PYL/RCAR family mediate intracellular perception, whereas G protein–coupled receptor-type G proteins (GTG receptors) are thought to mediate plasma membrane perception (reviewed by Finkelstein, 2013, Gonzalez-Guzman et al., 2012). This is an important point for future research to further analyze the physiological effects of ABA in *Dionaea muscipula* and can be pursued using transgenic *Dionaea* plants.

Further research is necessary to unravel the intricate complexity of JA/ABA-crosstalk in *Dionaea muscipula*. The effect of the ABA biosynthesis inhibitor Fluridone, which inhibits the carotenoid biosynthesis by inhibiting the enzyme phytoene desaturase (PDS, Gamble and

Mullet, 1986, Hossain et al., 2011) and the JA inhibitor COR-MO (Monte et al., 2014) might shed light on the hormonal interactions. It was observed in this thesis that truncated plants, if they were sprayed with ABA, still strongly expressed the marker hydrolases upon mechanostimulation (see **3.1.2, Fig. 3.1.2**). This might be due to the JA-peak caused by wounding (see **3.1.3, Fig 3.1.3**) which may have primed the tissue and made it insensitive for ABA, and the fact that JA-levels in truncated plants were only slightly affected by ABA-treatment (see **3.1.3, Fig. 3.1.4 A and B),** or might be due to a missing root-based signal molecule in the ABA perception pathway.

It was shown that waiting for 24 h after truncation before proceeding with an experiment did not greatly affect the results, the hydrolase expression was only affected slightly by ABA-spray, (**3.1.2**, **Fig. 3.1.2**) in comparison to almost completely inhibiting hydrolase expression in intact plants (**3.1.6**, **Fig. 3.1.12**). Another approach could be to wait longer (48 h, 72 h) before applying the ABA spray, so that the wounding response has faded further.
Alternatively, the recognition of the JA-peak could be prevented by applying the inhibitor COR-MO to the plant before truncating it. The COR-MO-dose would of course have to be low enough so that the recognition of the JA-peak caused by wounding would be prevented, but not the expression of the marker hydrolases caused by mechanostimulation, or the time span between COR-MO-application and truncation and mechanostimulation has to be long enough for *Dionaea* to degrade all traces of COR-MO. If the truncated trap is then sprayed with ABA and mechanostimulated, the analysis of the expression levels of the marker hydrolases could bring further insight. If the elevated marker hydrolase transcription in truncated ABA-sprayed plants after mechanostimulation is caused by the JA-peak, then in COR-MO treated plants this elevation should not be detectable. Alternatively, this could be achieved by applying molecular photoswitches, for example by selectively expressing a COI1-version in a trap which is inactivated and needs to be activated by a light switch (reviewed in Szymański et al., 2013). This would of course require the existence of transgenic plant lines.

The fact that treatment of intact plants with 50 µM ABA for 4 h and 24 h strongly reduces hydrolase expression (see **3.1.8, Fig. 3.1.16**), but does not inhibit trap closure or re-opening (see **3.1.9, Fig. 3.1.18**), points either to separately controlled pathways, or towards different decision branching points. This would make sense under the presumption that *Dionaea* is used to short-term water shortages in its natural habitat, therefore following the rule that it is beneficial to catch prey even under the influence of short-term water shortage, expecting better conditions soon.

4.3.3 Integration of external stimuli (wounding and drought stress) in digestion regulation

4.3.3.1 Incubation of truncated and intact plants with ABA had different effects on jasmonate levels after mechanostimulation

The *Dionaea* hunting cycle is controlled by jasmonates. Shortly after mechanically induced trap-closure, JA-levels in trap tissue rise (Escalante-Pérez et al., 2011; Böhm et al., 2016a; Pavlovic et al., 2017).

It has been shown that cutting of traps also increases jasmonate levels for up to 24 hours (**3.1.3, Fig. 3.1.3**). Therefore, in a subsequent experiment, 24 hours passed between truncation and the various stimuli in the actual experiment (**3.1.3, Fig. 3.1.4.1**), to let the hormone levels return to basic levels. In truncated plants, the pretreatment with ABA had only a minor effect on JA level characteristics compared to the experiment without ABA treatment (see **3.1.3, Fig. 3.1.4.1 A and B**). This might reflect the results of **3.1.2** where the external application of ABA to truncated mechanostimulated plants did not reduce the marker hydrolase expression. It might also be the case that, although the jasmonate levels have decreased in truncated plants after 24 h, the tissue is unreceptive to ABA signals due to the effects of the JA signals and the de-repression of a multitude of transcription factors (reviewed by Ali and Baek, 2020). It might also be that the ABA signal is mainly not perceived in the green parts of the plant, but in the roots, or that the roots send out additional signal molecules together with ABA, therefore in truncated plants without the roots the spray-application of ABA has only minor effects.

In **intact plants**, ABA had a significant effect on JA levels. **Without ABA**, JA levels were already highly elevated 15 min after mechanostimulation and remained elevated for 1 hour, with high levels still detectable after 3 hours. **With ABA**, JA levels were also elevated 15 minutes after mechanostimulation, but the peak was lower than without ABA, and they declined drastically after 30 minutes and remained low until at least 3 h. (See **Fig 3.1.4.1)**

JA-Ile levels behaved almost similar with or without the influence of ABA, they started to increase slowly beginning at 15 min after mechanostimulation up to the peak value at 3 h, but with ABA the increase was slower and the final value at 3 h was also reduced (See **Fig. 3.1.5.1**).

Therefore, it can be concluded that ABA has a pronounced dampening effect on JA levels after mechanostimulation in intact plants, and to a lesser extent on JA-Ile-levels.

In short, this can be summarized:

- In **Fig. 3.1.3** JA levels in intact plants after mechanostimulation **without ABA-treatment** reached their maximum after 15 minutes and remained elevated up to 1 h. After 3 h the levels were reduced again. In comparison, JA-Ile levels slowly and constantly rose up to 3 h.

- In **Fig. 3.1.4.1/3.1.5.1**, JA-levels in intact plants after mechanostimulation **with ABA-treatment** reached their maximum after 15 minutes but dropped sharply at 30 minutes and remained low. But JA-Ile-levels slowly and constantly rose up to 3 h, similar to without ABA-treatment.

Keeping in mind the drastically altered JA levels with and without ABA, but the very similar JA-Ile levels in intact plants after mechanostimulation (**3.1.3, Figs. 3.1.3, 3.1.4.1 and 3.1.5.1)**, the regulation of JA-Ile-synthesis from JA has to be regulated by more factors than the amount of existing JA.

Koo et al., (2014) described the turnover and oxidization of JA-Ile to hydroxy- (12OH-JA-Ile) and dicarboxy- (12COOH-JA-Ile) derivatives in *A. thaliana*. In further experiments, the amount of inactivated JA-Ile-derivatives after ABA treatment should be examined to assess if ABA speeds up JA-Ile-deactivation or slows down JA-conversion to JA-Ile.

As Pavlovic et al., (2017) claimed that *Dionaea* could not distinguish between wounding and prey, it comes to mind to analyze hydrolase expression in truncated, but otherwise untouched plants. If in traps with the leaf attached, which were truncated at the base of the leaf, but remained open even after truncation and would not have been mechanostimulated, the expression of marker hydrolases could be detected, in combination with the elevation of jasmonate levels, it would be known that jasmonates, regardless of their origin, are the key trigger to activate the digestive pathway. This would mimic the spray application of COR, where expression of the marker hydrolases and onset of the digestive fluid secretion was observed without mechanostimulation, and sometimes even without trap closure (Escalante-Pérez et al., 2011). Scherzer et al., (2017) could show that the observed cytosolic Ca^{2+}-spikes in gland cells after 3 trigger hair bendings are not elicited by methyl jasmonate-spray alone, but marker hydrolase expression was induced. It has been shown by Dr. Christina Larisch that various JA-derivatives like 12-OH-JA-Ile and coronalon were also able to induce trap closure and marker hydrolase expression, but in a lower extent than the external application of JA-Ile or COR. It is important to notice that those derivatives were only able to induce marker hydrolase expression if trap closure was also elicited. If the trap did not close, marker hydrolase expression could not be observed (personal communication Dr. Christina Larisch). The control plants in the experiment described in **3.1.2** (**Fig. 3.1.2**) after 4 h could give a hint to the answer of the question if all jasmonates, no matter their origin, elicit the digestive pathway in *Dionaea*. They were cut in total 28 h prior to marker transcript expression analysis, and no marker hydrolase expression could be detected, and in **Fig. 3.1.3** it could be seen that jasmonate levels were almost back to normal 24 h after truncation. The findings of the experimental part with the control plants in **3.1.4 (Fig. 3.1.8)** finally answer the question if jasmonates always trigger hydrolase expression. The results from the control plants showed, 7 to 8 hours after truncation, no expression of digestion marker hydrolases, although their trap closure was elicited by applying the paper clip between their trap lobes . This data indicates that *Dionaea* does not automatically react with triggering the digestive pathway upon contact with jasmonates no matter where their origin is.

The general remark that *Dionaea* cannot distinguish between wounding and prey, has to be regarded with great care. Pavlovic et al., (2017) massively wounded the trap tissue by repeatedly piercing it with a needle, thereby eliciting trap closure, and also eliciting the firing of APs, and afterwards they found highly increased JA- and JA-Ile-levels, and the traps started secretion of the digestive fluid. Elevated jasmonate levels were also observed in this thesis after truncation of the whole leaf (**3.1.3, Fig. 3.1.3**), but truncated plants in which only the trap closure was elicited and no further APs were triggered did not show expression of the digestion marker transcripts. The wounding experiment described in Pavlovic et al., (2017) should be carefully repeated without eliciting APs by using Cs^+ treatment to electrically silence the trigger hairs and the entire trap surface (Iosip et al., 2020). But the experiment shown in Pavlovic et al., (2017) as it was performed there, cannot be taken as proof that *Dionaea* can´t distinguish between wounding and prey capture. There, wounding of the trap tissue short-circuited the digestion cycle due to AP generation, but wounding on other parts of the plant does not. The outcome of the experiment would be better phrased as: *Dionaea* cannot distinguish the origin of APs elicited in trap tissue.

This shows that the way of how, when, at what location and in which concentration the signaling phytohormone is introduced into *Dionaea's* physiology is of great importance, as well as the stimuli preceding the introduction of the phytohormone. The spray application of the JA-mimic coronatine, as well as the spray application of 12-OPDA, 12-OH-JA-Ile, JA, MeJA and coronalon is sufficient to trigger marker hydrolase expression (sometimes after mechanical trap closure is elicited, sometimes they elicit trap closure) if sprayed onto the trap. But the generation of JA and JA-Ile in trap tissue caused by the truncation of the plant is not sufficient to elicit marker hydrolase expression. It is possible that, if the jasmonates are delivered via spray-application to the trap tissue, they elicit unique pathways, for example in the gland cells, and they trigger a signaling cascade. Therefore, without this signaling cascade, it would be apparent why the appearance of JA and JA-Ile after truncation does not elicit marker transcript expression and trap closure. Another possibility is that the concentration within the trap tissue after spray application is just much higher than after truncation and is therefore able to elicit trap closure and marker hydrolase expression. This could be tested by establishing the minimal concentration of COR required to elicit trap closure by spraying traps with different concentrations of COR. If the minimum concentration has been determined, the trap could be sprayed, incubated for some time, then excessive liquid is rinsed from the trap and the JA and JA-Ile – concentration within the trap tissue is determined.

As it has been shown in **3.1.2**, even waiting for 24 h after truncation (with **3.1.3** in mind where JA levels had returned to normal after 24 hours) before applying ABA and mechanostimulation to truncated plants to assess the effect of ABA on marker hydrolase expression was not sufficient to simulate a non-truncated plant. The effects of ABA in truncated plants might therefore be masked by factors other than jasmonates. One reason could be the damage-associated molecular patterns (DAMPs) elicited by truncation, which results in systemic wounding signalling like Ca^{2+}, ROS, ATP, glutamate and electrical signaling, which activates

downstream phosphorylation cascades to activate defense gene expression (Reviewed in Vega-Muñoz et al., 2020). It was shown that truncation elicits JA synthesis, and it was also observed that truncated plants had a systemic increase in ABA concentration even 24 h after truncation, when the JA peak was already gone again (Data not shown). This might be a wounding response in *Dionaea*, but might as well be a physical response to the truncation and the impaired water transport. It could be hypothesized that there is an interplay of the JA- and the ABA-pathway, and that the elevated JA and ABA levels triggered by truncating the plant prevents the recognition of the exogenous ABA signal, maybe by inactivation of ABA receptors or by inhibiting ABA-responsive gene expression by de-sensitizing the tissue.

4.3.3.2 Different incubation periods of ABA have no effect on hydrolase expression repression, but expression of different hydrolases is regulated independently

In **3.1.8** intact traps were sprayed with 50 µM active ABA and incubated for 4 h or 24 h prior to receiving mechanostimulation (10x, 1/min). Afterwards they were harvested after either 4 or 24 hours.

The results show (**Fig. 3.1.16**) that mRNA expression levels of the marker hydrolases VF CHITINASE I and SAG 12 were already reduced most pronounced after 4 h of ABA incubation compared to the water control treatment, indicating that the ABA signal has already been perceived by the respective receptors and elicited the subsequent reactions in the *Dionaea* digestion-controlling pathway.

The levels of SCPL 49 were apparently not affected as strongly by the ABA treatment, neither after 4 h nor after 24 h of incubation. Whereas a reduction is suggested for VF CHITINASE I and SAG 12, a reduction is not apparent for SCPL 49. If the data reflected a difference between VF CHITINASE I and SAG 12 on the one hand and SCPL 49 on the other, which remains to be shown in further experiments, a regulation by different pathways could be concluded, or by separate branching points of one pathway instead of an “all at once”-expression. This was also discussed in Libiaková et al., (2014), the authors proposed a multi-control point-system, in which after the animal is trapped and many APs are mechanically triggered, the elevation of OPDA levels lead to the formation of JA and slow secretion of digestive fluid, with only a fraction of the possible digestive enzymes. If nutrients are starting to be obtained from the prey, for example uric acid as the principle constituent of insect excretion, which is released by trapped insects and strongly induces secretion of the digestive fluid (Robins, 1976), these chemical signals from the prey are perceived, phytohormone levels are elevated stronger, the JA level is increased, and this leads to the formation of JA-Ile, and the secretion of the digestive fluid is in turn increased again (Libiaková et al., 2014).

Neither the 4 h nor the 24 h of incubation had an apparent effect on the traps closing ability (the majority of traps closed after 2 stimuli, only a small fraction closed after 3 stimuli). The same is true for truncated plants submerged in 50 µM ABA for 3 h, 6 h or 24 h: the traps´ ability to close is not impeded by this incubation period (own observation, data not shown). This experimental design and these samples could be used in the future to identify ABA-responsive or mechanostimulation-responsive genes and analyse the interplay of ABA and mechanostimulation.

4.3.3.3 Effect of trap incubation with either ABA or a jasmonate pathway inhibitor are similar

In **3.1.8** (**Figs. 3.1.16 and 3.1.17)**, the effects of treatment of traps with ABA on marker hydrolase transcript expression as well as COI1- and JAZ1-expression after 10x mechanostimulation were shown. In accordance with the findings in Böhm et al., (2016a), COI1 transcript numbers were elevated in untreated control plants, and were strongly reduced 4 h after mechanostimulation in water-treated control plants, whereas JAZ1 transcript numbers were initially low in untreated control plants but were strongly elevated in water-treated control plants after mechanostimulation.

For COI1, the effect of ABA was clearly visibly 4 h after mechanostimulation.

- When the plants had been incubated with ABA for 4 h prior to the mechanostimulation,
    - 4 h after mechanostimulation the COI1 transcript reduction observed in water-treated control-plants could not be detected, the COI1 transcript levels remained elevated (approximately 2750 transcripts/10.000 molecules Actin, **Fig 3.1.17 A**.
    - 24 hours after the mechanostimulation, the COI1 levels were both similarly low (approx. 750 transcripts/10.000 molecules Actin) in plants which had been treated with water or with ABA.
  - ➢ The inhibiting effect of ABA was only observed 4 h after mechanostimulation

- When the plants had been incubated with ABA for 24 hours prior to the mechanostimulation, and transcript levels were analysed
    - 4 h after mechanostimulation, the COI1 levels were strongly reduced to about 250 transcripts, but this was also observed in the water-treated control plants.
    - 24 h after mechanostimulation, the results were similar to those obtained after 4 h after mechanostimulation.
  - ➢ No difference between water-treated or ABA-treated plants was observed in COI1-levels 4 h or 24 h after mechanostimulation when the plants had been incubated for 24 h prior to the mechanostimulation. Only the reduced COI1-levels both after 24 h water-incubation and 24 h ABA-incubation in plants 24 h after mechanostimulation were striking. This means that, 24 h after mechanostimulation, the COI1-levels had not returned to basic levels in water-treated plants, and the fact that they did not remain strongly elevated in ABA-treated plants shows that the ABA-repressive effect is not absolute, because then the levels would not have decreased.

For JAZ1, the repressive effect of ABA was statistically significant 24 h after mechanostimulation. In water-treated control plants, JAZ1-levels are initially low and rise drastically after 2 APs.

- In **Fig. 3.1.17**, JAZ1 levels rose to approximately 12000 transcripts after 4 h of mechanostimulation and 4 h of water-treatment, but in 4 h ABA-treated plants, JAZ1-levels also rose to about 6000 transcripts. As the error bar for the JAZ1 levels in water-treated plants was quite large, the difference was not statistically significant.
- In plants that were pretreated with either water or ABA for 24 hours, 4 h after mechanostimulation the JAZ1-levels were comparable.
- 24 h after mechanostimulation, JAZ1-levels in water-treated plants are elevated, and in plants which were treated with ABA for either 4 h or 24 h, the JAZ1-levels were negligible. This difference was statistically significant.

In **3.1.7, Fig. 3.1.13** and **Fig**. **3.1.14** the effect of an inhibitor for the jasmonate pathway, COR-MO, was assessed. The inhibiting effect of COR-MO on the hydrolase expression was evident (**Fig. 3.1.13**). The induction of the hydrolases in water-treated control traps after 60 mechanostimulations was very strong, with about 10.000 to 50.000 transcripts/10.000 molecules Actin, but in traps which were treated with COR-MO, 60 mechanostimulations resulted in hardly any hydrolase transcription induction.

The effect was also evident at the COI1 transcript level. COI1 transcript levels in water-treated plants were drastically reduced after 60 mechanostimulations, whereas in COR-MO treated plants the reduction was not as expressed. JAZ1 levels were elevated 10fold in water-treated plants after 60 mechanostimulations, whereas there was no significant elevation in COR-MO treated plants. Therefore, COR-MO effectively blocks all jasmonate signals which would lead to hydrolase transcript expression from entering the digestion pathway.

COI1 (CORONATINE INSENSITIVE 1) and its co-receptor JAZ1 (JASMONATE ZIM DOMAIN 1) are the first elements in the JA pathway. The triggering of action potentials is known to start the production of JA-Ile (Escalante-Pérez et al., 2011; Böhm et al., 2016a), which then binds to COI1, which then forms a complex with JAZ1 and leads to JAZ-degradation. It is known that COR-MO mimics JA-Ile but does not activate the JA-pathway. The fact that the application of COR-MO inhibits the COI1-JAZ1-complex formation (Monte et al., 2014), which is also reflected in the mRNA-levels (upon normal JA-Ile binding to COI1, COI1-mRNA-levels are strongly decreased, and JAZ1-mRNA-levels are strongly increased, but under the influence of COR-MO, the levels stay unchanged (Böhm et al., 2016a)), and the fact that the application of COR-MO almost completely blocks the expression of the marker hydrolases after as much as 60 APs, show that gene expression of the hydrolases is downstream in the JA-pathway and comes after the recognition of JA-Ile and the subsequent signaling cascade.

4.3.4 ABA leads to partial stomatal closure in truncated *Dionaea* traps and reduces COR-induced secretion, but not marker hydrolase expression

In **3.1.4** the effects of COR- and ABA-treatment on truncated traps are presented. ABA was shown to reduce humidity levels in gas exchange measurements performed on illuminated plants; therefore it was concluded that the application of ABA reduced stomatal aperture of *Dionaea muscipula* traps, even after truncation. Stomata were not completely closed by the administration of 50 µM active ABA, as switching off the lights afterwards led to a much more expressed drop in humidity (**Fig. 3.1.7**).

As COR-spray could induce secretion in the trap (**Fig. 3.1.8**), it was analyzed if the administration of ABA via the xylem to the trap could counteract the actions of COR. In **3.1.4**, **Fig 3.1.6** it was shown that the transpirational pull in a truncated trap is strong enough to distribute substances from the liquid in the reaction vessel via the cut site and the xylem throughout the entire leaf and trap tissue. As it was shown that the administration of ABA via the xylem could partly reduce COR-induced secretion (**Fig. 3.1.9**) and did not reverse COR-induced hydrolase expression (**Fig. 3.1.10**), together with the findings from the inhibitor and ABA experiments (**3.1.7** and **3.1.8**) one might conclude that, once COR has acted upon the jasmonate-induced digestion pathway, the effects are very hard to reverse by using ABA. This is probably due to a strong binding of coronatine to the COI1-receptor, and due to the use of unphysiological concentrations, as COR has been shown to be effective from as low concentrations as 15 pM (Kenyon and Turner, 1992). This conclusion is supported by the fact that COR-induced hydrolase expression in intact plants could also not be reduced by subsequent ABA-treatment (**Fig. 3.1.11**). It has also to be considered that, for the gas-exchange measurements, the spray application of ABA to the intact trap is not feasible during a running measurement, as this would result in a strong increase of relative humidity in the air stream and would therefore mask the reaction of the plant itself. Therefore, the gas exchange measurements with COR and ABA were performed with truncated traps. Only later in the course of this thesis it was discovered that the truncation of the traps renders them partly insusceptible to subsequent ABA-treatment. Future gas exchange experiments have to be performed on intact traps, it should therefore be assessed if the spray-application of ABA via the petiole is possible and effective.

In summary, those experiments proved that COR is very much suited to activate the jasmonate-induced digestion pathway, but with the concentrations used, it is not reasonably possible to perform any inhibition studies. As it was shown by Monte et al., (2014) that COR-MO could counteract the effects of COR in *A. th*, it might be possible that higher concentrations of JA-Ile can displace COR from COI1 in *Dionaea*. Further researchers should experiment with much lower concentrations to assess if the binding of COR to COI1 can be disturbed by high concentrations of JA-Ile, or if ABA-treatment can counteract the effects of COR in intact plants, to assess if inhibition studies are even possible.

Some conclusions which can be drawn from the partly reduced COR-induced secretion after ABA-treatment in **Fig. 3.1.9** and **Fig 3.1.10** are that

- the ABA-signal is, at least partly, received in the green parts of the plants and not exclusively in the roots, as the secretion reduction was observed in truncated plants.
- the process of secreting the digestive fluid is regulated together with the transcription of the digestive marker transcripts, as the transcript levels were still highly elevated at the end of the experiment without ABA, and here humidity levels were also high. In the experiment where secretion was reduced after ABA was given, it was observed that the transcript levels were also reduced, although the difference was not significant.
- once the digestion process has started*, Dionaea* is still able to integrate external stimuli in the decision-network and react accordingly, as the ABA-treatment simulates drought stress and *Dionaea* reacts with decreased secretion of the digestive fluid to prevent water loss. Therefore, it seems reasonable to assume that the digestion process can be stopped by water shortage.

4.3.5 Summary of transcriptional and hormonal regulation

To summarize the different findings described above, the following model (**Fig. 4.1.1**) was created, based on Libiaková et al., (2014), with additional details from Bemm et al., (2016), Böhm et al., (2016a), Hedrich and Neher (2018), own observations and the findings described in this thesis.

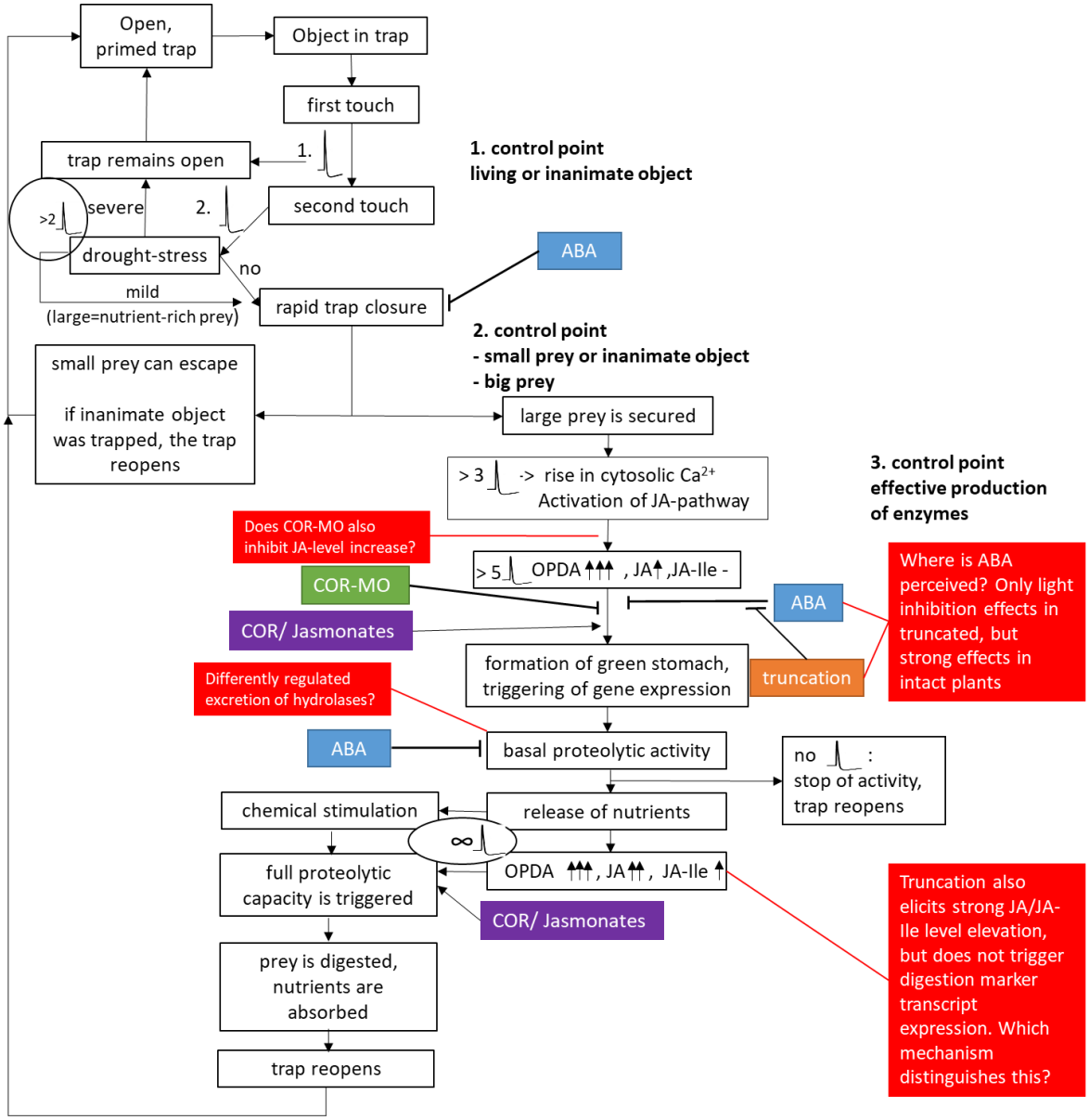


Fig. 4.1.1 Integrated model of *Dionaea muscipula* trapping behavior with indicated hormone intervention
Based on Libiaková et al., (2014), this model was enhanced with subsequent findings. Boxes in black show the normal regulation of *Dionaea* trapping mechanism, with the decision and control points indicated in bold letters. If an object enters the trap, rapid closure is elicited if 2 APs are triggered and the trap does not experience drought stress. If mild drought-stress is present, the trap will close after several APs. The third AP increases cytosolic Ca^{2+} levels, and further APs start the production of Jasmonates and the formation of the green stomach. Basic proteolytic activity is triggered, and if nutrients are released and further APs are fired, the full proteolytic capacity is triggered. Colored boxes indicate the effect of exogenously applied substances or actions, and the apparent interaction point. Arrows indicate promoting effects, blocked lines indicate inhibiting effects. Red boxes and lines indicate open questions which remain after this thesis and will be answered by future research.

APs are represented by the image 

Escalante-Pérez et al., (2011) could show that pre-incubation of the trap with ABA (for 48 h) interferes with or strongly inhibits trap closure (**Fig. 4.1.1**, first blue box, ABA). In this thesis it could be shown that pre-incubation of the trap with ABA for 24 h strongly reduced the expression of the digestion marker transcripts after mechanostimulation (**Fig 4.1.1**, second blue box) in intact plants, but truncation cancelled these effects (**Fig. 4.1.1,** brown box). It would therefore be interesting to know if this inhibition is caused by the missing perception of ABA in truncated plants, or by interfering signals caused by the wounding event (**Fig. 4.1.1**, second red box**)**. As the truncation of the plants elicited elevation of JA and JA-Ile-levels, but did not trigger the expression of digestion marker hydrolases, *Dionaea* seems to be able to distinguish between wounding and prey. The exact mechanism remains to be revealed (**Fig. 4.1.1,** fourth red box). The external application of Jasmonates, or COR, short-circuits the regulatory circuit (**Fig. 4.1.1,** violet boxes). The application of the inhibitor COR-MO successfully blocks the expression of the digestion marker transcripts (**Fig. 4.1.1,** green box), COR-MO probably also blocks the elevation of OPDA, JA and JA-Ile, but this has to be proven (**Fig. 4.1.1,** first red box)**.** As VF CHITINASE I and SAG 12 reacted sometimes differently than SCPL 49, it might be worth investigating further how the fine-tuning of the composition of the digestive fluid is regulated and which feedback loops are involved (**Fig. 4.1.1,** third red box).

5. Literature

5.1 Weblinks

International Union for Conservation of Nature - IUCN 2016. The IUCN Red List of Threatened Species. Version 2016-2. http://www.iucnredlist.org. Downloaded on 04 September 2016

***International Carnivorous Plant Society* - ICPS 2022**
Registered Cultivar Names for *Dionaea muscipula* (Accessed on 01. January 2022 - http://cpnames.carnivorousplants.org/Cultivars.php?name=Dionaea

www.WolframAlpha.com, Input query "Average temperature Wilmington, USA", temperature history (setting "all"). Downloaded 21.10.2016. Data from weather station KILM (Wilmington international airport)

Applied Biosystems – Application note regarding qPCR (ThermoFisher)
http://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1503-PJ9169-CO019879-Re-brand-Real-Time-PCR-Understanding-Ct-Value-Americas-FHR.pdf
Downloaded 20.09.16

Jasmonic acid: https://pubchem.ncbi.nlm.nih.gov/compound/5281166
Jasmonic acid isoleucine: https://pubchem.ncbi.nlm.nih.gov/compound/5497150,
Downloaded 12.11.2017

NCBI/GenBank resources

	mRNA	Protein
SAG 12	https://www.ncbi.nlm.nih.gov/nuccore/KT223141	https://www.ncbi.nlm.nih.gov/protein/953131590
SCPL 49	https://www.ncbi.nlm.nih.gov/nuccore/KT223142	https://www.ncbi.nlm.nih.gov/protein/953131592

Carnivorome transcriptome browser (TBro)
Browser containing the Venus Flytrap Transcriptome
https://tbro.carnivorom.com/tbro/

BBC
Hungry Venus flytraps snap shut on a host of unfortunate flies | Life – BBC
Narrated by David Attenborough
https://www.youtube.com/watch?v=O7eQKSf0LmY
Accessed 20.05.2018, uploaded by BBC on 02.12.2009

5.2 Bibliographic literature

Adamec, L., Matušíková, I., and Pavlovič, A. (2021). Recent ecophysiological, biochemical and evolutional insights into plant carnivory. Annals of Botany *128*, 241–259.

Ahmad, P., Rasool, S., Gul, A., Sheikh, S.A., Akram, N.A., Ashraf, M., Kazi, A.M., and Gucel, S. (2016). Jasmonates: Multifunctional Roles in Stress Tolerance. Frontiers in Plant Science *7*.

Ali, Md.S., and Baek, K.-H. (2020). Jasmonic Acid Signaling Pathway in Response to Abiotic Stresses in Plants. IJMS *21*, 621.

Ankenbrand, M.J., Weber, L., Becker, D., Förster, F., and Bemm, F. (2016). TBro: visualization and management of *de novo* transcriptomes. Database *2016*, baw146.

Bailey, T.S., and McPherson, S. (2012). Dionaea: the Venus's flytrap (Poole, Dorset, England: Redfern Natural History Productions).

Baird, D.D., Upchurch, R. P., Homesley, W. B., and Franz, J. E. (1971). Introduction of a new broad spectrum post emergence herbicide class with utility for herbaceous perennial weed control. Proceedings of the North Central Weed Science Society *26*, 64–68.

Barthlott, W., Porembski, S., Seine, R., and Theisen, I. (2007). The curious world of carnivorous plants: a comprehensive guide to their biology and cultivation (Portland, Or: Timber Press).

Bartsch, M., Mahnkopp, F., and Winkelmann, T. (2014). In vitro propagation of Dionaea muscipula ELLIS. Propagation of Ornamental Plants *Vol 14*, 117–124.

Bauer, H., Ache, P., Lautner, S., Fromm, J., Hartung, W., Al-Rasheid, K.A.S., Sonnewald, S., Sonnewald, U., Kneitz, S., Lachmann, N., et al. (2013). The Stomatal Response to Reduced Relative Humidity Requires Guard Cell-Autonomous ABA Synthesis. Current Biology *23*, 53–57.

Beebe, J.D. (1980). Morphogenetic Responses of Seedlings and Adventitious Buds of the Carnivorous Plant Dionaea muscipula in Aseptic Culture. Botanical Gazette *141*, 396–400.

Bemm, F., Becker, D., Larisch, C., Kreuzer, I., Escalante-Pérez, M., Schulze, W.X., Ankenbrand, M., Van de Weyer, A.-L., Krol, E., Al-Rasheid, K.A., et al. (2016). Venus flytrap carnivorous lifestyle builds on herbivore defense strategies. Genome Research *26*, 812–825.

Böhm, J. (2016). Die Nährstoffresorption in den Fallen von Dionaea muscipula weist Parallelen zur Nährsalzaufnahme in Wurzeln auf. Würzburg : Universität Würzburg.

Böhm, J., Scherzer, S., Shabala, S., Krol, E., Neher, E., Mueller, T.D., and Hedrich, R. (2016a). Venus Flytrap HKT1-Type Channel Provides for Prey Sodium Uptake into Carnivorous Plant Without Conflicting with Electrical Excitability. Molecular Plant *9*, 428–436.

Böhm, J., Scherzer, S., Krol, E., Kreuzer, I., von Meyer, K., Lorey, C., Mueller, T.D., Shabala, L., Monte, I., Solano, R., et al. (2016b). The Venus Flytrap Dionaea muscipula Counts Prey-Induced Action Potentials to Induce Sodium Uptake. Current Biology *26*, 286–295.

Breddam, K. (1986). Serine carboxypeptidases. A review. Carlsberg Res. Commun. *51*, 83–128.

Brown, W.H. (1916). THE MECHANISM OF MOVEMENT AND THE DURATION OF THE EFFECT OF STIMULATION IN THE LEAVES OF DIONAEA. American Journal of Botany *3*, 68–90.

Brown, W.H., and Sharp, L.W. (1910). The Closing Response in Dionaea. Botanical Gazette *49*, 290–302.

Bu, Q., Jiang, H., Li, C.-B., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q., and Li, C. (2008). Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Res *18*, 756–767.

Cucurou, C., Battioni, J.P., Thang, D.C., Nam, N.H., and Mansuy, D. (1991). Mechanisms of inactivation of lipoxygenases by phenidone and BW755C. Biochemistry *30*, 8964–8970.

Darwin, C. (1875). Insectivorous plants (London: John Murray).

Davis, A.L., Babb, M.H., Lowe, M.C., Yeh, A.T., Lee, B.T., and Martin, C.H. (2019). Testing Darwin's Hypothesis about the Wonderful Venus Flytrap: Marginal Spikes Form a "Horrid Prison" for Moderate-Sized Insect Prey. The American Naturalist *193*, 309–317.

De Buck, S., Jacobs, A., Van Montagu, M., and Depicker, A. (1998). *Agrobacterium tumefaciens* Transformation and Cotransformation Frequencies of *Arabidopsis thaliana* Root Explants and Tobacco Protoplasts. Molecular Plant-Microbe Interactions *11*, 449–457.

DiPalma, J.R., McMichael, R., and DiPalma, M. (1966). Touch receptor of venous flytrap, Dionaea muscipula. Science *152*, 539–540.

Dittrich, M., Mueller, H.M., Bauer, H., Peirats-Llobet, M., Rodriguez, P.L., Geilfus, C.-M., Carpentier, S.C., Al Rasheid, K.A.S., Kollist, H., Merilo, E., et al. (2019). The role of Arabidopsis ABA receptors from the PYR/PYL/RCAR family in stomatal acclimation and closure signal integration. Nat. Plants *5*, 1002–1011.

Ellis, J. (1768). Letter about Venus's fly-trap. The St. James's Chronicle 4.

Ellison, A., and Adamec, L. (2018). Carnivorous Plants (Oxford University Press).

Ellison, A.M., and Gotelli, N.J. (2009). Energetics and the evolution of carnivorous plants--Darwin's "most wonderful plants in the world." Journal of Experimental Botany *60*, 19–42.

Escalante-Pérez, M., Krol, E., Stange, A., Geiger, D., Al-Rasheid, K.A.S., Hause, B., Neher, E., and Hedrich, R. (2011). A special pair of phytohormones controls excitability, slow closure, and external stomach formation in the Venus flytrap. Proceedings of the National Academy of Sciences *108*, 15492–15497.

Escalante-Pérez, M., Scherzer, S., Al-Rasheid, K.A.S., Döttinger, C., Neher, E., and Hedrich, R. (2014). Mechano-Stimulation Triggers Turgor Changes Associated with Trap Closure in the Darwin Plant Dionaea muscipula. Molecular Plant *7*, 744–746.

Fagerberg, W.R., and Howe, D.G. (1996). A quantitative study of tissue dynamics in Venus's flytrap *Dionaea muscipula* (Droseraceae). II. Trap reopening. American Journal of Botany *83*, 836–842.

Farmer, E.E., Gasperini, D., and Acosta, I.F. (2014). The squeeze cell hypothesis for the activation of jasmonate synthesis in response to wounding. New Phytologist *204*, 282–288.

Finkelstein, R. (2013). Abscisic Acid Synthesis and Response. The Arabidopsis Book *11*, e0166.

Forterre, Y., Skotheim, J.M., Dumais, J., and Mahadevan, L. (2005). How the Venus flytrap snaps. Nature *433*, 421–425.

Gao, P., Loeffler, T.S., Honsel, A., Kruse, J., Krol, E., Scherzer, S., Kreuzer, I., Bemm, F., Buegger, F., Burzlaff, T., et al. (2015). Integration of trap- and root-derived nitrogen nutrition of carnivorous Dionaea muscipula. New Phytologist *205*, 1320–1329.

Geiger, D., Maierhofer, T., AL-Rasheid, K.A.S., Scherzer, S., Mumm, P., Liese, A., Ache, P., Wellmann, C., Marten, I., Grill, E., et al. (2011). Stomatal Closure by Fast Abscisic Acid Signaling Is Mediated by the Guard Cell Anion Channel SLAH3 and the Receptor RCAR1. Science Signaling *4*, ra32–ra32.

Gfeller, A., Liechti, R., and Farmer, E.E. (2010). Arabidopsis Jasmonate Signaling Pathway. Science Signaling *3*, cm4–cm4.

Gimenez-Ibanez, S., Boter, M., and Solano, R. (2015). Novel players fine-tune plant trade-offs. Essays in Biochemistry *58*, 83–100.

Givnish, T.J. (2015). New evidence on the origin of carnivorous plants. Proceedings of the National Academy of Sciences *112*, 10–11.

Givnish, T.J., Burkhardt, E.L., Happel, R.E., and Weintraub, J.D. (1984). Carnivory in the Bromeliad Brocchinia reducta, with a Cost/Benefit Model for the General Restriction of Carnivorous Plants to Sunny, Moist, Nutrient-Poor Habitats. The American Naturalist *124*, 479–497.

Gogoi, B., and Bhau, B.S. (2018). DNA barcoding of the genus Nepenthes (Pitcher plant): a preliminary assessment towards its identification. BMC Plant Biol *18*, 153.

Gonzalez-Guzman, M., Pizzio, G.A., Antoni, R., Vera-Sirera, F., Merilo, E., Bassel, G.W., Fernández, M.A., Holdsworth, M.J., Perez-Amador, M.A., Kollist, H., et al. (2012). Arabidopsis PYR/PYL/RCAR Receptors Play a Major Role in Quantitative Regulation of Stomatal Aperture and Transcriptional Response to Abscisic Acid. The Plant Cell *24*, 2483–2496.

Gregorio, G.J.D., and Dipalma, J.R. (1966). Anthocyanin in Dionaea muscipula Ellis (Venus Flytrap). Nature *212*, 1264–1265.

Hauser, F., Waadt, R., and Schroeder, J.I. (2011). Evolution of Abscisic Acid Synthesis and Signaling Mechanisms. Current Biology *21*, R346–R355.

Hedrich, R. (2015). Carnivorous plants. Current Biology *25*, R99–R100.

Hedrich, R., and Fukushima, K. (2021). On the Origin of Carnivory: Molecular Physiology and Evolution of Plants on an Animal Diet. Annu. Rev. Plant Biol. *72*, 133–153.

Hedrich, R., and Neher, E. (2018). Venus Flytrap: How an Excitable, Carnivorous Plant Works. Trends in Plant Science *23*, 220–234.

Henarejos-Escudero, P., Guadarrama-Flores, B., García-Carmona, F., and Gandía-Herrero, F. (2018). Digestive glands extraction and precise pigment analysis support the exclusion of the carnivorous plant Dionaea muscipula Ellis from the Caryophyllales order. Plant Science *274*, 342–348.

Heubl, G., Bringmann, G., and Meimberg, H. (2006). Molecular Phylogeny and Character Evolution of Carnivorous Plant Families in Caryophyllales — Revisited. Plant Biology *8*, 821–830.

Hilu, K.W., and Liang, H. (1997). The matK gene: sequence variation and application in plant systematics. American Journal of Botany *84*, 830–839.

Hodick, D., and Sievers, A. (1988). The action potential of Dionaea muscipula Ellis. Planta *174*, 8–18.

Hollingsworth, P.M., Forrest, L.L., Spouge, J.L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M.W., Cowan, R.S., Erickson, D.L., Fazekas, A.J., et al. (2009). A DNA barcode for land plants. Proceedings of the National Academy of Sciences *106*, 12794–12797.

Hoshi, Y., and Kondo, K. (1998). A Chromosome Phylogeny of the Droseraceae by Using CMA-DAPI Fluorescent Banding. Cytologia *63*, 329–339.

Hossain, M.A., Munemasa, S., Uraji, M., Nakamura, Y., Mori, I.C., and Murata, Y. (2011). Involvement of Endogenous Abscisic Acid in Methyl Jasmonate-Induced Stomatal Closure in Arabidopsis. PLANT PHYSIOLOGY *156*, 430–438.

Iosip, A.L., Böhm, J., Scherzer, S., Al-Rasheid, K.A.S., Dreyer, I., Schultz, J., Becker, D., Kreuzer, I., and Hedrich, R. (2020). The Venus flytrap trigger hair–specific potassium channel KDM1 can reestablish the K+ gradient required for hapto-electric signaling. PLoS Biol *18*, e3000964.

Jaffe, M.J. (1973). The role of ATP in mechanically stimulated rapid closure of the Venus's flytrap. Plant Physiology *51*, 17–18.

Jensen, M.K., Vogt, J.K., Bressendorff, S., Seguin-Orlando, A., Petersen, M., Sicheritz-Pontén, T., and Mundy, J. (2015). Transcriptome and Genome Size Analysis of the Venus Flytrap. PLOS ONE *10*, e0123887.

Juniper, B.E., Robins, R.J., and Joel, D.M. (1989). The carnivorous plants (London ; San Diego: Academic Press).

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y., and Howe, G.A. (2008a). COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences *105*, 7100–7105.

Katsir, L., Chung, H.S., Koo, A.J., and Howe, G.A. (2008b). Jasmonate signaling: a conserved mechanism of hormone sensing. Current Opinion in Plant Biology *11*, 428–435.

Kenyon, J.S., and Turner, J.G. (1992). The Stimulation of Ethylene Synthesis in Nicotiana tabacum Leaves by the Phytotoxin Coronatine. Plant Physiol. *100*, 219–224.

Koo, A.J.K., and Howe, G.A. (2009). The wound hormone jasmonate. Phytochemistry *70*, 1571–1580.

Koo, A.J., Thireault, C., Zemelis, S., Poudel, A.N., Zhang, T., Kitaoka, N., Brandizzi, F., Matsuura, H., and Howe, G.A. (2014). Endoplasmic Reticulum-associated Inactivation of the Hormone Jasmonoyl-l-Isoleucine by Multiple Members of the Cytochrome P450 94 Family in Arabidopsis. Journal of Biological Chemistry *289*, 29728–29738.

Kreuzwieser, J., Scheerer, U., Kruse, J., Burzlaff, T., Honsel, A., Alfarraj, S., Georgiev, P., Schnitzler, J.-P., Ghirardo, A., Kreuzer, I., et al. (2014). The Venus flytrap attracts insects by the release of volatile organic compounds. Journal of Experimental Botany *65*, 755–766.

Krol, E., Dziubinska, H., Stolarz, M., and Trebacz, K. (2006). Effects of ion channel inhibitors on cold-and electrically-induced action potentials in Dionaea muscipula. Biologia Plantarum *50*, 411–416.

Libiaková, M., Floková, K., Novák, O., Slováková, L., and Pavlovič, A. (2014). Abundance of Cysteine Endopeptidase Dionain in Digestive Fluid of Venus Flytrap (Dionaea muscipula Ellis) Is Regulated by Different Stimuli from Prey through Jasmonates. PLoS ONE *9*, e104424.

Lloyd, F.E. (1942). The Carnivorous Plants (Lloyd Press).

Lorenzo, O. (2004). JASMONATE-INSENSITIVE1 Encodes a MYC Transcription Factor Essential to Discriminate between Different Jasmonate-Regulated Defense Responses in Arabidopsis. THE PLANT CELL ONLINE *16*, 1938–1950.

Maierhofer, T., Lind, C., Huttl, S., Scherzer, S., Papenfuss, M., Simon, J., Al-Rasheid, K.A.S., Ache, P., Rennenberg, H., Hedrich, R., et al. (2014). A Single-Pore Residue Renders the Arabidopsis Root Anion Channel SLAH2 Highly Nitrate Selective. The Plant Cell *26*, 2554–2567.

Malcheska, F., Ahmad, A., Batool, S., Müller, H.M., Ludwig-Müller, J., Kreuzwieser, J., Randewig, D., Hänsch, R., Mendel, R.R., Hell, R., et al. (2017). Drought-Enhanced Xylem Sap Sulfate Closes Stomata by Affecting ALMT12 and Guard Cell ABA Synthesis. Plant Physiology *174*, 798–814.

Melotto, M., Underwood, W., and He, S.Y. (2008). Role of Stomata in Plant Innate Immunity and Foliar Bacterial Diseases. Annual Review of Phytopathology *46*, 101–122.

Merzendorfer, H. (2006). Insect chitin synthases: a review. Journal of Comparative Physiology B *176*, 1–15.

Monte, I., Hamberg, M., Chini, A., Gimenez-Ibanez, S., García-Casado, G., Porzel, A., Pazos, F., Boter, M., and Solano, R. (2014). Rational design of a ligand-based antagonist of jasmonate perception. Nature Chemical Biology *10*, 671–676.

Mur, L.A.J., Prats, E., Pierre, S., Hall, M.A., and Hebelstrup, K.H. (2013). Integrating nitric oxide into salicylic acid and jasmonic acid/ ethylene plant defense pathways. Frontiers in Plant Science *4*.

Nagy, J.I., and Maliga, P. (1976). Callus Induction and Plant Regeneration from Mesophyll Protoplasts of Nicotiana sylvestris. Zeitschrift Für Pflanzenphysiologie *78*, 453–455.

Nap, J.P., Bijvoet, J., and Stiekema, W.J. (1992). Biosafety of kanamycin-resistant transgenic plants. Transgenic Res. *1*, 239–249.

Nelson, E.C. (1989). Dionaea D. Solander ex J. Ellis (Droseraceae): notes on the nomenclature and typification of Venus's Fly-trap. Botanical Journal of the Linnean Society *99*, 249–254.

Nguyen, C.T., Kurenda, A., Stolz, S., Chételat, A., and Farmer, E.E. (2018). Identification of cell populations necessary for leaf-to-leaf electrical signaling in a wounded plant. Proc Natl Acad Sci USA *115*, 10178–10183.

Noguero, M., and Lacombe, B. (2016). Transporters Involved in Root Nitrate Uptake and Sensing by Arabidopsis. Frontiers in Plant Science *7*.

de Ollas, C., and Dodd, I.C. (2016). Physiological impacts of ABA–JA interactions under water-limitation. Plant Molecular Biology *91*, 641–650.

de Ollas, C., Arbona, V., and GóMez-Cadenas, A. (2015). Jasmonoyl isoleucine accumulation is needed for abscisic acid build-up in roots of Arabidopsis under water stress conditions: JA-ABA interaction in roots. Plant, Cell & Environment *38*, 2157–2170.

Palfalvi, G., Hackl, T., Terhoeven, N., Shibata, T.F., Nishiyama, T., Ankenbrand, M., Becker, D., Förster, F., Freund, M., Iosip, A., et al. (2020). Genomes of the Venus Flytrap and Close Relatives Unveil the Roots of Plant Carnivory. Current Biology *30*, 2312-2320.e5.

Pan, J., Hu, Y., Wang, H., Guo, Q., Chen, Y., Howe, G.A., and Yu, D. (2020). Molecular Mechanism Underlying the Synergetic Effect of Jasmonate on Abscisic Acid Signaling during Seed Germination in Arabidopsis. Plant Cell tpc.00838.2019.

Park, S.H., Rose, S.C., Zapata, C., Srivatanakul, M., and Smith, R.H. (1998). Cross-protection and selectable marker genes in plant transformation. In Vitro Cellular & Developmental Biology - Plant *34*, 117–121.

Paszota, P., Escalante-Pérez, M., Thomsen, L.R., Risør, M.W., Dembski, A., Sanglas, L., Nielsen, T.A., Karring, H., Thøgersen, I.B., Hedrich, R., et al. (2014). Secreted major Venus flytrap chitinase enables digestion of Arthropod prey. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics *1844*, 374–383.

Pavlovic, A., Demko, V., and Hudak, J. (2010). Trap closure and prey retention in Venus flytrap (Dionaea muscipula) temporarily reduces photosynthesis and stimulates respiration. Annals of Botany *105*, 37–44.

Pavlovic, A., Slovakova, L., Pandolfi, C., and Mancuso, S. (2011). On the mechanism underlying photosynthetic limitation upon trigger hair irritation in the carnivorous plant Venus flytrap (Dionaea muscipula Ellis). Journal of Experimental Botany *62*, 1991–2000.

Pavlovič, A., Jakšová, J., and Novák, O. (2017). Triggering a false alarm: wounding mimics prey capture in the carnivorous Venus flytrap (*Dionaea muscipula*). New Phytologist *216*, 927–938.

Roberts, P.R., and Oosting, H.J. (1958). Responses of Venus Fly Trap (Dionaea muscipula) to Factors Involved in Its Endemism. Ecological Monographs *28*, 193–218.

Robins, R.J. (1976). The Nature of the Stimuli Causing Digestive Juice Secretion in Dionaea muscipula Ellis (Venus's Flytrap). Planta *128*, 263–265.

Sauer, M., Robert, S., and Kleine-Vehn, J. (2013). Auxin: simply complicated. Journal of Experimental Botany *64*, 2565–2577.

Scala, J., Iott, K., Schwab, D.W., and Semersky, F.E. (1969). Digestive Secretion of Dionaea muscipula (Venus's Flytrap). Plant Physiol. *44*, 367–371.

Scherzer, S., Krol, E., Kreuzer, I., Kruse, J., Karl, F., von Rüden, M., Escalante-Pérez, M., Müller, T., Rennenberg, H., Al-Rasheid, K.A.S., et al. (2013). The Dionaea muscipula Ammonium Channel DmAMT1 Provides NH4+ Uptake Associated with Venus Flytrap´s Prey Digestion. Current Biology *23*, 1649–1657.

Scherzer, S., Böhm, J., Krol, E., Shabala, L., Kreuzer, I., Larisch, C., Bemm, F., Al-Rasheid, K.A.S., Shabala, S., Rennenberg, H., et al. (2015). Calcium sensor kinase activates potassium uptake systems in gland cells of Venus flytraps. Proceedings of the National Academy of Sciences *112*, 7309–7314.

Schulze, W., Schulze, E.D., Schulze, I., and Oren, R. (2001). Quantification of insect nitrogen utilization by the venus fly trap Dionaea muscipula catching prey with highly variable isotope signatures. J. Exp. Bot. *52*, 1041–1049.

Schulze, W.X., Sanggaard, K.W., Kreuzer, I., Knudsen, A.D., Bemm, F., Thogersen, I.B., Brautigam, A., Thomsen, L.R., Schliesky, S., Dyrlund, T.F., et al. (2012). The Protein Composition of the Digestive Fluid from the Venus Flytrap Sheds Light on Prey Digestion Mechanisms. Molecular & Cellular Proteomics *11*, 1306–1319.

Seckbach, J., and Dubinsky, Z. (2011). All flesh is grass: plant-animal interrelationships (Dordrecht [Netherlands] ; New York: Springer).

Seki, M., Umezawa, T., Urano, K., and Shinozaki, K. (2007). Regulatory metabolic networks in drought stress responses. Current Opinion in Plant Biology *10*, 296–302.

Seo, P.J., and Park, C.-M. (2011). Cuticular wax biosynthesis as a way of inducing drought resistance. Plant Signaling & Behavior *6*, 1043–1045.

Sheard, L.B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T.R., Kobayashi, Y., Hsu, F.-F., Sharon, M., Browse, J., et al. (2010). Jasmonate perception by inositol-phosphate-potentiated COI1–JAZ co-receptor. Nature *468*, 400–405.

Sickel, W., Van de Weyer, A.-L., Bemm, F., Schultz, J., and Keller, A. (2019). Venus flytrap microbiotas withstand harsh conditions during prey digestion. FEMS Microbiology Ecology *95*.

STUDENT (1908). THE PROBABLE ERROR OF A MEAN. Biometrika *6*, 1–25.

Suda, H., Mano, H., Toyota, M., Fukushima, K., Mimura, T., Tsutsui, I., Hedrich, R., Tamada, Y., and Hasebe, M. (2020). Calcium dynamics during trap closure visualized in transgenic Venus flytrap. Nat. Plants *6*, 1219–1224.

Suhita, D. (2004). Cytoplasmic Alkalization Precedes Reactive Oxygen Species Production during Methyl Jasmonate- and Abscisic Acid-Induced Stomatal Closure. PLANT PHYSIOLOGY *134*, 1536–1545.

Szymański, W., Beierle, J.M., Kistemaker, H.A.V., Velema, W.A., and Feringa, B.L. (2013). Reversible Photocontrol of Biological Systems by the Incorporation of Molecular Photoswitches. Chem. Rev. *113*, 6114–6178.

Tzfira, T., Tian, G.-W., Lacroix, B., Vyas, S., Li, J., Leitner-Dagan, Y., Krichevsky, A., Taylor, T., Vainstein, A., and Citovsky, V. (2005). pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Molecular Biology *57*, 503–516.

Vadassery, J., Reichelt, M., Jimenez-Aleman, G.H., Boland, W., and Mithöfer, A. (2014). Neomycin Inhibition of (+)-7-Iso-Jasmonoyl-L-Isoleucine Accumulation and Signaling. Journal of Chemical Ecology *40*, 676–686.

Vega-Muñoz, I., Duran-Flores, D., Fernández-Fernández, Á.D., Heyman, J., Ritter, A., and Stael, S. (2020). Breaking Bad News: Dynamic Molecular Mechanisms of Wound Response in Plants. Front. Plant Sci. *11*, 610445.

Veleba, A., Šmarda, P., Zedek, F., Horová, L., Šmerda, J., and Bureš, P. (2017). Evolution of genome size and genomic GC content in carnivorous holokinetics (Droseraceae). Ann Bot 119, 409–416.

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R.K., Kumar, V., Verma, R., Upadhyay, R.G., Pandey, M., et al. (2017). Abscisic Acid Signaling and Abiotic Stress Tolerance in Plants: A Review on Current Knowledge and Future Prospects. Frontiers in Plant Science *08*.

Volkov, A.G., Adesina, T., Markin, V.S., and Jovanov, E. (2008a). Kinetics and Mechanism of Dionaea muscipula Trap Closing. PLANT PHYSIOLOGY *146*, 694–702.

Volkov, A.G., Adesina, T., and Jovanov, E. (2008b). Closing of Venus flytrap by electrical stimulation of motor cells. Plant Signaling & Behavior *2*, 139–145.

Volkov, A.G., Pinnock, M.-R., Lowe, D.C., Gay, M.S., and Markin, V.S. (2011). Complete hunting cycle of Dionaea muscipula: Consecutive steps and their electrical properties. Journal of Plant Physiology *168*, 109–120.

Volkov, A.G., Forde-Tuckett, V., Volkova, M.I., and Markin, V.S. (2014). Morphing structures of the Dionaea muscipula Ellis during the trap opening and closing. Plant Signaling & Behavior *9*, e27793.

Volkov, A.G., Xu, K.G., and Kolobov, V.I. (2017). Cold plasma interactions with plants: Morphing and movements of Venus flytrap and Mimosa pudica induced by argon plasma jet. Bioelectrochemistry *118*, 100–105.

Walker, J.F., Yang, Y., Moore, M.J., Mikenas, J., Timoneda, A., Brockington, S.F., and Smith, S.A. (2017). Widespread paleopolyploidy, gene tree conflict, and recalcitrant relationships among the carnivorous Caryophyllales. American Journal of Botany *104*, 858–867.

Wasternack, C., and Strnad, M. (2016). Jasmonate signaling in plant stress responses and development – active and inactive compounds. New Biotechnology *33*, 604–613.

Wasternack, C., and Xie, D. (2010). The genuine ligand of a jasmonic acid receptor: improved analysis of jasmonates is now required. Plant Signal Behav *5*, 337–340.

Weiler, E.W., Kutchan, T.M., Gorba, T., Brodschelm, W., Niesel, U., and Bublitz, F. (1994). The Pseudomonas phytotoxin coronatine mimics octadecanoid signalling molecules of higher plants. FEBS Lett. *345*, 9–13.

Widholm, J.M. (1972). The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol *47*, 189–194.

Williams, S.E., and Bennet, A.B. (1982). Leaf Closure in the Venus Flytrap: An Acid Growth Response. SCIENCE *218, 10*, 1120–1122.

Wolf, B., Lesnaw, J.A., and Reichmann, M.E. (1970). A mechanism of the irreversible inactivation of bovine pancreatic ribonuclease by diethylpyrocarbonate. A general reaction of diethylpyrocarbonate . A general reaction of diethylpyrocarbonate with proteins. Eur. J. Biochem. *13*, 519–525.

Xiong, L., Ishitani, M., Lee, H., and Zhu, J.K. (2001). The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell *13*, 2063–2083.

Yan, J., Zhang, C., Gu, M., Bai, Z., Zhang, W., Qi, T., Cheng, Z., Peng, W., Luo, H., Nan, F., et al. (2009). The Arabidopsis CORONATINE INSENSITIVE1 Protein Is a Jasmonate Receptor. Plant Cell *21*, 2220–2236.

Yoshida, T., Mogami, J., and Yamaguchi-Shinozaki, K. (2014). ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. Current Opinion in Plant Biology *21*, 133–139.

Youngsteadt, E., Irwin, R.E., Fowler, A., Bertone, M.A., Giacomini, S.J., Kunz, M., Suiter, D., and Sorenson, C.E. (2018). Venus Flytrap Rarely Traps Its Pollinators. The American Naturalist *191*, 539–546.

Zhang, X.L., Jiang, L., Xin, Q., Liu, Y., Tan, J.X., and Chen, Z.Z. (2015). Structural basis and functions of abscisic acid receptors PYLs. Frontiers in Plant Science *6*.

6. Attachments

6.1 List of abbreviations

RT	room temperature
AP	Action potential
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
e.g.	*exempli gratia*, lat. “for example”
et al.	et al*ii*, lat. “and others”
FW	Fresh weight
ABA	Abscisic acid
JA	Jasmonic acid
JA-Ile	Jasmonic acid - isoleucine
min	minute
h	hour
RCF	relative centrifugal force
g	earth gravitational acceleration (9,801 m/s^2)
ON	over night
Vol	volumes
MeOH	Methanol
EtOH	Ethanol
cDNA	complementary DNA
GOI	gene of interest
H_20	water
ddH_20	double destilled water
Fig.	Figure

Bp	DNA base pair(s)
Kbp	Kilo base pairs= 1,000 bp
Mbp	Mega base pairs= 1,000,000 bp
Gbp	Giga base pairs = 1,000,000,000 bp
Rh	Relative humidity
L	Liter
ml	Milliliter (1/1000 Liter)
µL	Mikroliter (1/100.000 Liter)
mOsmol/kg	Osmotic concentration, molarity of osmotic active ions in a solution
K_D	The equilibrium dissociation constant
N	Nitrogen
NH_4^+	Ammonium
NO_3^-	Nitrate
Ad (xx ml/L with ddH_20)	„fill up to the specified final volume with the liquid indicated", in this example with double-distilled water

6.2 Affidavit/Eidesstattliche Erklärung

Eidesstattliche Erklärungen nach §7 Abs. 2 Satz 3, 4, 5 der Promotionsordnung der Fakultät für Biologie

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation: „Die Venus Fliegenfalle – Die Rolle von Oxilipinen im Fallenverhalten von *Dionaea muscipula*“, eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Weiterhin erkläre ich, dass bei allen Abbildungen und Texten bei denen die Verwertungsrechte (Copyright) nicht bei mir liegen, diese von den Rechtsinhabern eingeholt wurden und die Textstellen bzw. Abbildungen entsprechend den rechtlichen Vorgaben gekennzeichnet sind sowie bei Abbildungen, die dem Internet entnommen wurden, der entsprechende Hypertextlink angegeben wurde.

Affidavit

I hereby declare that my thesis entitled: „ The Venus flytrap - Role of oxylipins in trap performance of Dionaea muscipula” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Besides I declare that if I do not hold the copyright for figures and paragraphs, I obtained it from the rights holder and that paragraphs and figures have been marked according to law or for figures taken from the internet the hyperlink has been added accordingly.

Marburg, den _____

_____

Unterschrift

6.3 List of own publications / Liste eigener Publikationen

Scherzer, S., Krol, E., Kreuzer, I., Kruse, J., Karl, F., von Rüden, M., Escalante-Pérez, M., Müller, T., Rennenberg, H., Al-Rasheid, K.A.S., et al., (2013). The Dionaea muscipula Ammonium Channel DmAMT1 Provides NH_4^+ Uptake Associated with Venus Flytrap´s Prey Digestion. Current Biology *23*, 1649–1657.

6.4 Danksagung

Zuerst möchte ich Professor Rainer Hedrich für die Möglichkeit danken, dieses hochinteressante Doktorarbeitsthema in seinem Institut bearbeiten zu dürfen.

Weiterhin möchte ich Dr. Ines Kreuzer für Ihre konstante und exzellente Betreuung, hilfreichen Ratschläge und Schubser in die richtige Richtung danken.

Prof. Dirk Becker möchte ich für Ratschläge bezüglich *Dionaea*-Transkriptom-Fragestellungen und Tbro-Tipps danken.

Der Dank an Brigitte ist selbstredend und kann nicht groß genug sein. Sie hat mir alles beigebracht, was ich im Labor wissen musste. Nebenher war Ihre freundliche und aufmunternde Art und ihr reichhaltiger praktischer Erfahrungsschatz mehr als einmal eine echte Stütze bei erneuten unerwarteten Ergebnissen.

Joachim möchte ich für stets freundliche Worte und gute Ratschläge zur Pflanzenaufzucht und -pflege danken. Ein großer Dank auch an Matze für manche Unterstützung, die längst nicht seine Aufgabe, aber sehr nötig waren! Danke dafür.

Weiterhin gilt mein großer Dank allen anderen Doktoranden, mit denen man Probleme besprechen, sich über Unglücke austauschen und daraus seine Lehren ziehen konnte. Es hat gutgetan, stets zu wissen, in eine Gruppe eingebettet zu sein. Eine Doktorarbeit schreibt man zwar selber, aber nicht allein. Das sind Nadine und Alex, Hanna und Anna-Lena, Julian, Dawid und Justyna, Antonella, Doro und Lena, Sönke, Jenny, Christof und Dennis, Ronnie und Hubert, Steffi, Eva und Katha, Sarah, Timo, Tobi, Hannes und Heike, Anda, Alicja, Melli und Karimi.

Den Müller-Mädels besten Dank für die sportlichen Ausflüge

Und zu guter Letzt meinen Dank an meine Frau (Die mich auch ohne den Doktortitel geheiratet hat), meine Familie und meine Freunde für die kontinuierliche Motivation und Unterstützung.