12-2020

Role of Herbivore Associated Elicitors in Plant Defense Signaling

Akanksha Gandhi
The University of Texas Rio Grande Valley

ROLE OF HERBIVORE ASSOCIATED ELICITORS IN PLANT DEFENSE SIGNALING

A Thesis

by

AKANKSHA GANDHI

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2020

Major Subject: Biology

ROLE OF HERBIVORE ASSOCIATED ELICITORS IN PLANT DEFENSE SIGNALING

A Thesis
by
AKANKSHA GANDHI

COMMITTEE MEMBERS

Dr. Nirakar Sahoo
Chair of Committee

Dr. Rupesh Kariyat
Committee Member

Dr. Bradley Christoffersen
Committee Member

December 2020

ABSTRACT

Gandhi, Akanksha., Role of Herbivore Associated Elicitors in Plant Defense Signaling

Master of Science (MS), December 2020, 92 pp., 17 figures, 215 references

Chapter 2: In this chapter, I have provided an update on recent developments and advances on early signaling events in plant-herbivore interactions, with a particular emphasis on the membrane potential changes (V_m), calcium (Ca^{2+}), reactive oxygen species (ROS) and plant ion channels involvement in early signaling events initiation and propagation of defense signaling cascade.

Chapter 3: In this chapter, we show that tobacco hornworm (*Manduca sexta*) caterpillar oral secretion (OS) induces ROS in tomato (*Solanum lycopersicum*) protoplasts. By using a dye-based ROS imaging approach, our study showed that the application of Plant-Fed (PF) *M. sexta* OS generates significantly higher ROS while artificial Diet-Fed (DF) *M. sexta* OS failed to induce ROS in isolated tomato protoplasts.

Chapter 4: In this chapter, we demonstrate that hemolymph from *M. sexta* also induce ROS and Ca^{2+} and thereby act as an herbivore associated elicitor (HAE). Using a dye-based imaging technique, our study showed that the application of crude *M. sexta* hemolymph potently increased ROS as well as Ca^{2+} production in isolated tomato protoplasts.

In summary, our results demonstrate that *M. sexta* OS and hemolymph induces cellular defense signals by modulating intracellular ROS and Ca^{2+} in tomato protoplast.

DEDICATION

I dedicate my thesis to my father, Mr. Parveen Gandhi, my mother, Mrs. Kiran Gandhi, and my younger brother Arpit Gandhi. I am thankful for your constant support and for motivating me to work hard to achieve my goals.

ACKNOWLEDGMENT

First and foremost, I would like to thank my advisor, Dr. Nirakar Sahoo, for his unwavering guidance throughout my research. He has given many insightful suggestions and thoughtful comments on my thesis. He was always willing and enthusiastic to assist me and resolve any research-related queries. He has dedicated his time to teach me several techniques that have helped me shape my scientific carrier. His encouragement and motivation have been commendable. Thank you for giving me this wonderful opportunity to be a part of your lab. I am extremely grateful to my graduate committee members Dr. Rupesh Kariyat and Dr. Bradley Christofferson for serving on my committee and their guidance. Special thanks to Dr. Kariyat for his collaboration in this project. This thesis would not have been possible without their constant support.

I owe my deepest gratitude to Dr. Parwinder Grewal and the graduate college for providing me Presidential Graduate Research Assistantship that has helped me to complete my graduate studies. I would also like to thank Dr. Kristine Lowe for providing me a great platform for research and education at the department of biology and for recruiting me as a teaching assistant. I would also acknowledge Dr. Robert Dearth for providing me a great platform and accepting me into the graduate program here at UTRGV. I would also like to thank him for giving me the Biology Graduate Travel Research award to present my research.

I would also like to thank Dr. Priti Sharma, Dr. Gurupkar Singh Sidhu believing in me and writing me recommendation letters to pursue research abroad. I would especially like to thank my family for their unconditional love, guidance, and unending inspiration. I would also like to thank my friend Mandeep Tayal and lab member, Tripti Saini, for their continued support and motivation.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Plants regularly encounter a wide range of abiotic and biotic stresses in nature. Abiotic stress includes drought, salinity, extreme temperatures, radiation, floods, and heavy metals, whereas biotic stress involves the pressure posed on plants by living organisms such as microbes and herbivores. The interactions between plants and insect herbivores (herbivores) are among the most significant environmental associations in nature (Price et al., 1980; Dicke et al., 1990; De Moraes et al., 1998; Stotz et al., 1999; Agrawal et al., 2006; 2012). There is an ongoing battle between plants and herbivores that started millions of years ago. In this war, plants are constantly attacked by a suite of herbivores, a significant factor that limits their growth and fitness (Schafer et al., 2011). It is estimated that every year, herbivory causes a 20% loss in annual plant production (Oerke, 2006; Van der Meijden, 2015). Due to the selection pressure imposed by herbivores, plants can specifically induce defenses based on the type of damage. Plant defenses start right when the adults oviposit (Hilker and Meiners, 2006; Kim et al., 2012; Kim and Felton, 2013). Over time, they have developed diverse mechanisms to ward-off each attacking biotroph (Kariyat et al., 2012a). To withstand the herbivore attack, plants employ physical and chemical defenses. Physical defenses include structural traits such as cuticle, spines, trichomes, which act as a physical barrier for the herbivore, thus providing a fitness advantage to the plant (Agrawal et al., 2009; Kaur and Kariyat, 2020).

Sometimes, the plant can also produce hardened leaves, a phenomenon known as sclerophylly. This will reduce the foraging efficiency of herbivores as the tissue will not be palatable (Handley et al., 2005). On the other hand, the plant can use chemical defenses to hinder herbivore feeding by attracting natural enemies through herbivore-induced plant volatiles and extrafloral nectar (Arimura et al., 2009; Karban 2010; War et al., 2012). Plants can also produce some antifeedants or toxic compounds such as proteinase inhibitors that can adversely affect herbivore growth (Howe and Jander, 2008). Insect components such as saliva, oral secretions (OS) or regurgitant, insect excreta (frass), or oviposition fluids can also elicit specific plant defense responses, thus, priming the plants for future attacks (Howe and Jander, 2008; Acevedo et al., 2015). Considerable progress has been made in understanding this intricate relationship between plants and herbivores with a plethora of field studies. But our knowledge of how plants perceive these cues and how that terminates into specific defense response is still in its infancy.

During herbivore assault, the damaged areas of the plant need to inform the rest of the plant in order to keep them ready for the future attack. Therefore, it is essential to understand the role of key players in long-distance communication and early plant signaling in response to herbivory. It has been proposed that following insect attack, the foremost event that is observed is the depolarization of plasma membrane potential (Bricchi et al., 2010) along with the generation of second messengers such as cytosolic Ca^{2+} (Reddy et al., 2011), reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Miller and Mittler 2006; Bricchi et al., 2010; Arimura et al., 2011; Marino et al., 2012) that contribute to plant defense signal transduction. Although numerous researches have been performed to unravel long-distance communication during herbivore attack, the exact mechanism underlying such signaling remains unclear. In the 2nd chapter of my thesis, I have focused on the literature review about the role that V_m, Ca^{2+}, and

ROS play in long-distance communication and plant defense signaling cascade. I have also discussed the role of ion channels that are involved in plant-herbivore interaction.

Herbivory is caused by two different ways, such as mechanical damage and chemical damage. Plants perceive the insect attack by recognition of herbivore secretions such as OS, oviposition fluids, and frass, which signal them to activate defense responses to deter the herbivore (Kessler and Baldwin, 2002). These herbivore secreted molecules are known as herbivore-associated elicitors (HAEs) (Felton and Tumlinson, 2008; Mithofer and Boland, 2008; Felton et al., 2014). Among them, herbivore OS are so far the most studied HAE. Herbivore OS is a blend of labial and mandibular saliva along with regurgitant (Vadassery et al., 2012). Enzymes, fatty acid-amino acid conjugates and peptides have been identified in OS. Studies have indicated the presence of β-glucosidase, volicitin, inceptin, caeliferins in the OS of caterpillars (Alborn et al.,1997; Schmelz et al., 2007). HAEs have been known to initiate plant cellular signaling via the modulation of intracellular V_m, Ca^{2+}, and ROS. For instance, the OS of Egyptian cotton leafworm (*Spodoptera littoralis*: Lepidoptera) actuated a fast change in the V_m eventually leading to downstream responses (Guo et al., 2013). Similarly, the application of OS from desert locust (*Schistocerca gregaria*: Orthoptera) on wounded leaves of thale cress (*Arabidopsis thaliana*) enhanced the cytosolic Ca^{2+}, mitogen-activated protein kinases (MAPKs) activity, and other defense-related compounds (Schaffer et al., 2011). In a study by Shinya et al. (2016), ROS generation was observed in rice cells in response to the treatment of OS of a chewing generalist herbivore, night feeding rice armyworm (*Mythimna loreyi*: Lepidoptera).

Previous studies had shown that *Manduca sexta* (Lepidoptera, Sphingidae) larval OS induced plant defenses in the form of jasmonic acid and various mono and sesquiterpenes on the wounded leaves of wild tobacco (*Nicotiana attenuata*) (Halitschke et al., 2001; Kariyat et al.,

2012b). However, the effect of *M. sexta* OS on ROS and Ca^{2+} generation has not been thoroughly examined at the cellular level. Therefore, in chapter 3, we have investigated the effect of *M. sexta* OS on intracellular ROS, a defense signaling inducer, on isolated tomato protoplast. We used ROS sensing dye, CM-H_2DCFDA based imaging approach to measure the increase in ROS generation upon *M. sexta* OS application. Our result showed that the application of plant-fed (PF) *M. sexta* OS increased ROS generation while artificial diet-fed (DF) caterpillar OS failed to induce ROS in isolated tomato protoplasts. Membrane-permeable oxidant "tbH_2O_2" showed a strong ROS response to CM-H_2DCFDA loaded tomato protoplasts. We also observed that N-Acetyl- cysteine (NAC), a potent antioxidant commonly used in animals, abolished the ROS generation in isolated tomato protoplasts. Interestingly, our results also showed that the *M. sexta* PF-OS induced ROS increase was abolished in the presence of a Ca^{2+} chelator, BAPTA-AM, suggesting possible crosstalk between Ca^{2+} and ROS signaling.

When insects feed on plants, they come across trichomes, which act as a barrier, deterring their movement. Non-glandular trichomes contain sharp spikes and are sometimes silicified, thus, hampering the herbivore feeding (Lanning and Eleuterius, 1985). This can lead to the release of hemolymph. It is presumed that it might act as a cue for the plants initiating the signaling cascade that augments plant defenses. In the 4th chapter, we found that a newly discovered HAE, *M. sexta* "hemolymph" is capable of inducing ROS and Ca^{2+} in isolated plant protoplast and possibly regulate defenses against insect herbivores. Using a dye-based imaging technique, our study showed that the application of crude *M. sexta* hemolymph potently increased ROS and Ca^{2+} production in isolated tomato protoplasts. The addition of antioxidant NAC antagonized hemolymph-induced ROS generation, indicating that *M. sexta* hemolymph is a ROS inducer in isolated protoplasts. Furthermore, incubating the protoplasts with Ca^{2+} chelator,

BAPTA-AM efficiently abolished the hemolymph-induced ROS production, suggesting possible crosstalk between Ca^{2+} and ROS signaling. Interestingly, the application of crude *M. sexta* hemolymph dramatically increased Ca^{2+} in tomato protoplasts. Also, hemolymph-mediated ROS and Ca^{2+} increase was inhibited in the absence of extracellular Ca^{2+}.

In summary, my thesis shows that *M. sexta* OS and hemolymph induce cellular signaling cascade by increasing the ROS and Ca^{2+} production in tomato protoplast. While insect OS elicits ROS, the hemolymph triggers ROS and Ca^{2+}. Our studies confirmed previous research on cross-talk between ROS and Ca^{2+} as chelating the intracellular Ca^{2+} with BAPTA-AM suppressed the *M. sexta* OS and hemolymph mediated ROS generation. Also, the treatment of tomato protoplasts with NAC inhibited the OS mediated ROS production and the hemolymph-induced ROS and Ca^{2+} generation. This suggests that there is a cellular amplification loop by which ROS leads to Ca^{2+} and vice-versa. Understanding the early signals in plants is vital not only from ecological aspect but also for the development of crops with enhanced resistance to herbivory (Zebelo et al., 2014).

CHAPTER II

DECIPHERING THE ROLE OF EARLY DEFENSE SIGNALING COMPONENTS IN PLANT-HERBIVORE INTERACTIONS

Abstract

Plants and herbivores are in a relentless battle to outwit each other. Plants have evolved a wide range of counter-defense strategies to either manage or resist them. These include physical structural defenses such as trichomes, and spines, and chemical defenses such as toxic secondary metabolites. While behavioral and mechanistic layers of such interactions have been well understood, the physiological link between the host and herbivore is less understood. These interactions are generally initiated at the plant cell membrane, where herbivores physically damage the plant, and the herbivore oral secretions trigger a series of a signaling cascade that leads into the mounting of plant defense mechanisms. Interestingly, the first responder from the host plant in response to herbivore contact at the plasma membrane site is the ion channels or transporters. These transmembrane pore proteins sense the fluctuation in plasma transmembrane potential (V_m) that in turn triggers signal transduction pathways such as calcium (Ca^{2+}) signaling, reactive oxygen species (ROS) generation, reactive nitrogen species, and/or nitric oxide and consequently, defense gene expression and can even lead to growth-defense tradeoffs. In recent years, the studies on deciphering the physiological steps in plant-herbivore interactions have been gaining momentum with the genetic expression of specific GFP reporters such as GCaMP3 (genetically encoded Ca^{2+} sensor) and Ro-GFP (genetical encoded ROS sensor) in plants. In this

review, we have provided an update on recent developments and advances on early signaling events in plant-herbivore interactions, with emphasis on the ion channels that are involved in early signaling events and initiation and propagation of V_m, Ca^{2+} and ROS. This review would be a novel take on understanding plant-herbivore interactions and will gain interest from scientists in both basic and applied plant biologists and physiologists.

Introduction

Plants are under continued stress of various abiotic factors such as cold, salinity and biotic factors such as pathogens and herbivores. Among these, it is estimated that insect-herbivory causes a total of 20% annual crop yield loss (Oerke, 2006; Van der Meijden, 2015). Keeping this in mind and to ensure global food security, it is imperative to understand such interactions at the cellular level. These interactions generally initiate at the plasma membrane where herbivores physically damage the plant (wounding) and chemically through HAEs such as oral secretions (OS), frass, and ovipositional fluids (Felton and Tumlinson, 2008**;** Mithofer and Boland, 2008*;* Felton et al., 2014). Wounding by herbivores exposes the protoplasts and these plant cells interact with HAEs that trigger a series of signaling cascades and activates changes in V_m, Ca^{2+}, and ROS signals (Maffei et al., 2004; Wu and Baldwin, 2009; Bonaventure, 2012).

In order to protect themselves from the herbivore attack, the plant alerts its unaffected parts by long-distance communication from the site of perception. Cell to cell communication is indispensable for cellular processes such as differentiation, morphogenesis, homeostasis, growth, and plant defense (Raven et al., 2014). So, systemic signaling is an efficient way for plants to deal with impending damage and enhance their survival chances by expressing defense genes

(Choi et al., 2017). There has been considerable research for identifying the factors that are involved in long-distance signaling. The plant can appraise its unaffected parts by propagation of three intracellular regulators, namely V_m, Ca^{2+}, and ROS waves (Choi et al., 2016; Gilroy et al., 2016). These waves transmission rate can range from ~100 to > 1000 μm/sec (Choi et al., 2016; Shao et al., 2020). The propagation of long-distance electrical signals occurs as a result of variation in membrane potential due to potassium (K^+) and Ca^{2+} flux. The membrane potential is a gradient generated due to the flow of ions across the membrane. Variation in membrane potential (V_m) plays an essential role in wounding responses in plants (Maffei et al., 2004). Ca^{2+} is a versatile second messenger used by plants to sense the external stimuli and translate them into the adaptive intracellular response (Jammes et al., 2011). Insect feeding and its OS can trigger cytosolic Ca^{2+} changes, and these spatiotemporal changes are known to generate Ca^{2+} signatures (McAinsh and Hetherington, 1998; Reddy, 2001; Moore et al., 2002; Hetherington & Brownlee, 2004). ROS are highly toxic and reactive molecules that are derived from oxygen and includes O_2, H_2O_2, OH. ROS have been shown to act as a long-distance rapid wound signal that is self-propagating (Miller et al., 2009). Growing evidence has begun to implicate Ca^{2+} and ROS as signaling molecules involved in plant defense response.

Long-distance communication in plants has been linked with the ion channels or membrane transporters. These are transmembrane pore proteins involved in the movement of ions across the cell membrane. They play several important roles in animals, such as setting membrane potential, signal transduction, and membrane trafficking. The presence of the nervous system in animals facilitate the transmission of signals throughout their body. A repertoire of ion channels is responsible for this electrical excitation in nerve cells which are connected through axons (Hille et al., 1999). However, plants do not have a nervous system, but they can still

communicate and transmit signals from wounded to unwounded parts of the plant. In recent years, with the use of electrophysiological tools, the research on ion channels in plants has gained momentum. Studies have reported that ion channels facilitate long-distance communication via V_m, Ca^{2+}, and ROS (Fig.1,2). For instance, one of the recent studies by Toyota et al. (2018) has led to the discovery of glutamate-like receptor channels in *Arabidopsis thaliana,* which are homologous to mammalian ionotropic glutamate receptors. In plants, these ion channels are involved in Ca^{2+} signaling, nutrient uptake, root gravitropism, and plant defense (Miller et al., 2010; Manzoor et al., 2013). However, ion channels in mammals are involved in neurotransmission, and their opening is facilitated by the binding of glutamate on the postsynaptic neuron, resulting in the influx of Ca^{2+} and other cations. The signal is transmitted as a result of changes in V_m that occur due to ion flux (Muday and Harding, 2018). It is fascinating that in plants, these channels are responsible for long-distance Ca^{2+} signaling in response to herbivory or mechanical damage and effectively communicate neighboring cells about the herbivore attack.

Membrane potential (V_m)

In animals, the V_m is responsible for generating action potentials in tissues, muscles, nerves in animals and plays a key role in diverse biological functions such as biological sensing, hearing, cell cycle, proliferation, contractility, circadian rhythm, etc. (Kadir et al., 2018). However in plants the V_m regulate plant cellular functions such as maintaining turgor pressure, osmotic balance, and stomatal closure. The difference in ionic distribution between inside and outside of protoplasts leads to the generation of V_m. In equilibrium, there is no net flux of ions through the membrane, called the resting membrane potential. Changes in the resting membrane potential occur due to an unbalanced ions movement, leading to V_m being more positive

(depolarization) or more negative (hyperpolarization). Plants maintain a negative resting membrane potential in the order of -110 to-150 mV. Herbivory causes perturbations in the membrane potential in plants followed by fast electrical signals and hence activation of signal transduction pathways (Maffie et al., 2012). An interesting study by Maffei et al. (2004) showed that both mechanical wounding and OS of African cotton leafworm (*Spodoptera littoralis*; Lepidoptera) affect V_m in lima bean (*Phaseolus lunatus*) where V_m was measured at increasing distances i.e., at 5, 30, and 60 mm from the bite zone. V_m depolarization was observed within the first 15 minutes of feeding by *S. littoralis* in the palisade cells. The effect of *S. littoralis* regurgitate and its components were also tested on V_m in lima bean leaf and the findings showed that V_m alterations were independent of regurgitate concentration (Maffei et al., 2004). The same research group also observed the changes in V_m in response to the application of different H_2O_2 concentrations on mechanically damaged and herbivory-wounded lima bean leaves. The H_2O_2 application stimulated a strong V_m that was higher in herbivory-wounded plants in comparison to mechanically damaged leaves (Maffei et al., 2006). V_m depolarization was studied in both wild type and *A. thaliana pdko3* line, which was mutated in genes encoding plasmodesmata proteins. Plasmodesmata are the channels in the plant cells that allow the passage of molecules, forming a route for the cell to cell communication. V_m depolarization was observed within 7 to 8 minutes after herbivory in wild type, whereas root V_m depolarization was not observed in the *pdko3* mutant in response to herbivory or application of OS from *S. littoralis*. However, Ca^{2+} elevation was observed in both wildtypes as well as in *pdko3.* This observation ruled out the possibility of Ca^{2+} channels being involved in V_m depolarization. To dissect the dependence of V_m depolarization on potassium (K^+) channels, the K^+ channel activity was measured using the fluorescent indicator, FluxOR ™. A significant increase was seen in K^+ channel activity in wild-

type plants, whereas a complete loss of K^+ channel activity was observed in *pdko3* plants (Bricchi et al., 2013).

The fluctuation in V_m has been known to be induced by the binding of specific components from herbivore OS with the receptors present at the plasma membrane in plants (Zebelo and Maffei., 2012). These components can alter ion channel activities that cause an imbalance in ion movement and influence the membrane potential of the plasma membrane (Maffei et al., 2004). A study by Mohanta et al. (2012) showed that the maidenhair tree (*Ginkgo biloba*), a living fossil plant, responds to herbivory by *S. littoralis* by inducing V_m depolarization, which was evident up to 6 hours. A study conducted by using the model plant, *A. thaliana* showed that the extent of V_m depolarization was the same for *S. littoralis*, green peach aphid (*Myzus persicae*: Hemiptera) and rod-shaped, gram-negative bacterium (*Pseudomonas syringe*) but the timing of the occurrence of V_m depolarization was different for each of these biotrophs (Bricchi et al., 2012). One of the perplexing questions is that why V_m depolarization is observed at a different period. Intriguingly, the magnitude of early defense response depends upon the amount of tissue damage by these biotrophs. V_m depolarization was rapid upon the attack of chewing herbivore, *S. littoralis* (30 min to 2 h) as it caused substantial tissue loss. On the other hand, less damage was caused by a phloem feeder, *M. persicae* (4 to 6 h) that delayed the plant defense response (Bricchi et al., 2012). Upon herbivory, V_m depolarization is followed by an increase in cytosolic Ca^{2+} and ROS production.

Calcium (Ca^{2+})

Ca^{2+} is a ubiquitous molecule that plays a pivotal role in many physiological processes in plants such as stomatal opening and closure, root growth, fertilization, nutrient signaling, and plant immunity (Edel et al., 2017). Plants respond to various biotic and abiotic stresses, including

insect attacks, by inducing changes in cytosolic Ca^{2+} levels. Ca^{2+} signatures are responsible for long-distance signal transduction that leads to downstream plant defense responses. The main stores of Ca^{2+} in plants include cytosol, vacuole, apoplast, mitochondria, and endoplasmic reticulum (Arimura and Maffei, 2010). A vacuole is the largest storage center of Ca^{2+} (Peiter, 2011). The cytosolic Ca^{2+} ranges from 0.1 to 1 μM while the vacuolar free Ca^{2+} varies from 0.2-5 mM. This difference in concentration between Ca^{2+} stores and cytosol makes Ca^{2+} a secondary messenger that is involved in intracellular signal propagation (Pottosin and Schönknecht, 2007).

Ca^{2+} enters from the apoplast to the cytosol via plasma membrane Ca^{2+} channels after herbivore attack. Increased cytosolic Ca^{2+} triggers the release of Ca^{2+} from the vacuole through the two pore channel (TPC1), thus increasing the levels of cytosolic Ca^{2+} (Dodd et al., 2010). This increase is vital for the generation of physiological responses (Tuteja et al., 2007). Membrane potential and Ca^{2+} concentration gradient are the driving forces for vacuolar Ca^{2+} transport. To maintain the optimal levels of Ca^{2+}, the cell uses ion channels and transporter. One of the open questions is what mechanism drives the transport of Ca^{2+} in and out of the apoplast and tonoplast membrane. This is possibly due to the presence of specific ion channels/ receptors that get activated upon depolarization of the plasma membrane, which triggers the Ca^{2+} release (Edel et al., 2017). Only a handful of studies have been conducted to identify these ion channels. Additional work using molecular and electrophysiological techniques is needed to characterize these ion channels.

Plant Ion Channels

Ion channels are macromolecular pores in the membrane that regulate the flux of ions along their electrochemical gradient at a rate of 10^6 ions per second. Electrophysiological

methods have led to the characterization of ion channels into four different types based on their gating activity. The opening or closing of a channel in response to a specific stimulus is called gating. These can be classified as ligand-gated ion channels, voltage-gated, stretch-activated, and light-activated. Ligand-gated ion channels are activated by binding of molecules such as ATP, amino acids, cyclic nucleotides, or lipids such as ionotropic glutamate receptor (iGluR). Voltage-dependent channels become excited after sensing the change in membrane potential, for example plant-Shaker type channels. Light-activated channels are gated by sensory stimuli such as light. Stretch-activated ion channels detect physical forces such as pressure, membrane tension. These channels are responsible for setting up membrane potential, signal transduction, and membrane trafficking in animals, while in plants, they are responsible for water and solute transport (Johansson et al.,1996), stomatal opening and closure (Armstrong et al.,1995; Suh et al., 1998), pollination (Holdaway-Clarke et al., 1997), salt tolerance (Katsuhara and Tazawa, 1992) and plant defense (Luo et al., 2017). The first plant ion channel that was discovered is a K^+ channel, Stelar K^+ outward rectifier (SKOR) (Gaymard et al., 1998). However, three families of ion channels have been identified so far that play a role in plant-herbivore interactions such as cyclic nucleotide gated channels (CNGCs), glutamate receptor-like channels (GLRs), and two pore channel 1 **(**TPCs) (Edel et al., 2017).

Cyclic Nucleotide Gated Channels (CNGC)

CNGC is the well-studied ligand- gated Ca^{2+} channel that was first discovered in retinal photoreceptors and olfactory neurons (Zagotta et al., 1996). They play a role in signal transduction in animals and are also present in other non-neuronal tissues (Kaupp et al., 2002). These ion transport proteins have also been identified in plants (Kohler et al., 1999; Leng et al., 1999). With the discovery of the first CNGC in barley (Schuurink et al., 1998), the plant CNGC

research field has been expanded. These non-selective cation channels are involved in plant development, thermotolerance (Finka et al., 2012), salt stress (Kugler et al., 2009). These channels confined at the plasma membrane consist of 20 family members in *A. thaliana* and have six membrane-spanning regions and a pore domain (Fig.3) (Duszyn et al., 2019). They are activated by binding of cyclic nucleotides such as cAMP (cyclic adenosine monophosphate), cGMP (cyclic guanosine monophosphate) (Talke et al., 2003; Kudla et al., 2010; Swarbreck et al., 2013) and inhibited by calmodulin binding (Wang et al., 2013). These channels show similarity with shaker-like K^+ channels (Kaplan et al., 2007). Voltage clamp studies have demonstrated that plant CNGCs are voltage-gated and require more hyperpolarizing potentials (more negative than -120 MV) for activation of this channel (Leng et al., 2002).

There is a cyclic-nucleotide binding (CNB) and a calmodulin-binding domain (CaMB) present at the C-termini of the channel (Dietrich et al., 2010). On the contrary, the animal system has a CaMB domain at the N-termini (Liu et al., 1994; Grunwald et al., 1998). The plant and the animal CNGC differ in their pore amino acid sequence as well as the selectivity for various cations (Kaplan et al., 2007; Chin et al., 2009). The amino acids that form the CaM binding domain overlap with the polypeptide region that forms the CNBD (Jha et al., 2016). This overlapping affects the channel activation as the binding of CaM at the C termini hinders cyclic nucleotide-binding suggesting variability in plant and animal CNGC channel regulation (Varnum et al.,1997; Trudeau et al., 2002).

Studies have shown that these ion channels also play a vital role in modulating biotic stress (Ma et al., 2012). A growing body of evidence suggests its role in elevating cytosolic Ca^{2+} that is a crucial signaling event during biotic stress. A recent study by Meena et al. (2019) identified that upon herbivory, CNGC19 is responsible for generating and transmitting Ca^{2+}

signals in local and systemic leaves. Its role in defense was confirmed by generating a loss-of-function mutant in which the Ca^{2+} signals were attenuated. This *A. thaliana* mutant was more susceptible to attack by cotton leafworm (*Spodoptera litura*). Moreover, lower jasmonic acid levels were also observed in this mutant. This observation suggests that the CNGC channel plays a significant role in plant defense against herbivores(Meena et al., 2019).

Glutamate Receptor-like channels

Glutamate-like receptor (GLR) is a non-selective ion channel responsible for permeating Ca^{2+} ions across the plasma membrane of animals and plants. Mammalian ionotropic glutamate receptors (iGluRs) have been linked with the central nervous system, where they play a vital role in synaptic transmission (Dingledine et al., 1999). It is perplexing that GLRs also exist in plants despite the absence of the central nervous system (Lam et al., 1998). In plants, GLRs play a crucial role in carbon and nitrogen metabolism (Kang and Turano, 2003), root gravitropism (Miller et al., 2010), pollen tube growth (Michard et al., 2011; Wudick et al., 2018), immune defense reactions (Kang et al., 2004; Kwaaitaal et al., 2011; Li et al., 2013; Manzoor et al., 2013; Forde and Roberts, 2014) and wound-induced intracellular signaling (Mousavi et al., 2013). *A. thaliana* consists of 20 GLR genes, each containing N-terminal domain, 2 extracellular ligand-binding sites (S1, S2), transmembrane domains (M1-M4) including a pore region (P) and the C-terminal domain (Davenport et al., 2002) (Fig. 4)

In mammals, iGluRs are divided into three groups according to their sequence diversity and ligand specificities (Traynelis et al., 2010). These include N-methyl-d-aspartate (NMDA), α-amino-3- hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and Kainate receptors. Plant GluRs share a high degree of similarity with the NMDA receptors that ranges from 16 to 63% in

the ligand-binding domains and the transmembrane domains (Lam et al., 1998). These are not only present at the plasma membrane but can also be found in chloroplasts, mitochondria (Teardo et al., 2011, 2015) and vacuolar membranes (Wudick et al., 2018). Unlike their mammalian counterparts, the plant GLRs have much broader ligand selectivity. The major difference in plant and animal iGLR is the pore region. These non-selective cation channels are activated by an amino acid, glutamate, that acts as a metabolite, energy source, and a neurotransmitter in animals (Young and Ajami, 2000; Forde and Lea, 2007).

Electrophysiological studies have shown the involvement of GLRs in inducing Ca^{2+} fluxes and plasma membrane depolarizations in plant cells (Dennison and Spalding, 2000). It is one of the few Ca^{2+} channels mediating herbivore-associated defense signaling that has been characterized in plants (Vasta et al., 2011) (Fig. 1, 2). Previous studies have indicated a strong and rapid cytosolic Ca^{2+} fluctuation upon the application of GLR agonists such as glutamate (Demidchik et al., 2004). In a dose-dependent study by Vasta et al. (2011), 0.1 mM glutamate application leads to an increase in cytosolic Ca^{2+} that reaches 3-4 µM in tobacco (*Nicotiana tabacum* var xanthi). This glutamate-induced Ca^{2+} increase was abolished by treatment with 2 mM of Ca^{2+} channel inhibitor, lanthanum and a Ca^{2+} chelator, BAPTA. These observations suggest that GLRs play an important role in plant defense signaling.

Studies have indicated that GLR3.3 is a key player in transmitting signals in the form of Ca^{2+} waves from wounded to unwounded parts of the plant. Wound induced surface potential changes were observed upon feeding of *S. littoralis* larvae on *A. thaliana*. However, the surface potential changes decreased in the four mutants, glr 3.1, glr3.2, glr 3.3, and glr 3.6 upon wounding. (Mousavi et al., 2013). A recent study by Toyota et al. (2018) showed that GLRs are activated by wounding and upon herbivory by Cabbage butterfly (*Pieris rapae;* Lepidoptera) in

A. thaliana leaves expressing GCaMP3. Cytosolic Ca^{2+} elevation and subsequent defense gene expression was evident only after the application of glutamate and not with other amino acids such as sorbitol. The Ca^{2+} signals were completely abolished in the glr3.3 glr3.6 double mutant in *A. thaliana* which indicates the role that GLR plays in inducing systemic defense responses. Although these two GLRs exist in different locations as the GLR 3.3 is present at the phloem and GLR 3.6 at the contact cells of xylem parenchyma, the study shows that both these clades are responsible for long-distance Ca^{2+} signaling (Toyota et al., 2018). Another recent study by Shao et al. (2020) showed that upon wounding of the main root, the Ca^{2+} elevation and surface wave potential (SWP) were observed in GCaMP6 expressing *A. thaliana* , which was at 2 cm distance from the root-shoot junction. Furthermore, the application of 100 mM glutamate at the wound site in the root also triggered a Ca^{2+} increase as well as SWP in all the leaves. Interestingly, this wound and glutamate-induced root to shoot Ca^{2+} increase was attenuated in the glr3.3-glr3.6 double mutant suggesting the role that these channels play in propagating systemic signals from leaf to leaf and root to shoot.

Two Pore Channel 1 (TPC1)

Two-pore channel (TPC1), a slow vacuolar-type cation channel, is present in the vacuolar membrane (Narloch et al., 2011). In humans, it can be found at the endolysosomal membrane (Cang et al., 2014). In plants, the vacuolar TPC1 channel has been known to be responsible for diverse roles ranging from nutrient sensing, pH homeostasis, control of the membrane potential in plants. The first plant TPC1 gene was cloned in *A. thaliana* (AtTPC1) that consisted of 733 amino acids and is homologous to rat TPC1 (Furuichi et al., 2001). The protein is composed of two shaker-like units , with each unit having six transmembrane domains, two Ca^{2+} binding EF domains, and one putative 14-3-3 site (Peiter et al., 2005) (Fig.5). Ca^{2+} binding to the cytosolic

EF-hand domain leads to conformational changes coupled to the pair of pore-lining inner helices from the first 6-TM domains, whereas membrane potential only activates the second voltage-sensing domain whose conformational changes are coupled to the pair of inner helices from the second 6-TM domains (Guo et al., 2016). The slow vacuolar channel transports Ca^{2+} along with Na^{+} and K^{+} and has a $Ca^{2+/}K^{+}$ permeability ratio of 3:1 (Ward and Schroeder, 1999; Pitt et al., 2010). The release of Ca^{2+} strongly depends on cytosolic free Ca^{2+} concentration indicating that this channel participates in Ca^{2+} induced Ca^{2+} release (Ward and Schroeder, 1994; Bewell et al., 1999).

A study by Kiep et al. (2015) reported the real-time imaging in *A. thaliana* expressing Ca^{2+} reporter aequorin that was carried out in response to wounding and herbivory by *S. littoralis* resulting in the generation of local and systemic cytosolic Ca^{2+} signals. However, upon mechanical wounding, this systemic Ca^{2+} signal got suppressed in the tpc1-2 knockout mutant. This observation implicates that TPC1 is required to trigger the cytosolic Ca^{2+} changes that are a hallmark for a successful herbivore recognition leading to a subsequent defense response (Fig. 1 and 2). To confirm that, tpc 1-2 mutant expressing GCaMP3 was generated and Ca^{2+} signals decreased significantly but not completely as compared to 35S: GCaMP3 plants indicating that the release of Ca^{2+} from vacuole by TPC1 relies on extracellular Ca^{2+} release by GLRs and it is downstream of the GLRs. It has been suggested that the binding of Ca^{2+} to the cytosolic EF-hands leads to the opening of TPC1 and may sense the increase in cytosolic Ca^{2+} via the opening of plasma membrane Ca^{2+} channels (Peiter et al., 2005).

However, future work is still needed to fully explore the role of the TPC1 channel in regulating Ca^{2+} levels in response to an attack by herbivores. Furthermore, there is a need to

dissect the regulatory mechanism that governs its gating during such a response. The interplay between the plasma membrane and vacuolar Ca^{2+} channels in generating Ca^{2+} fluxes upon herbivory remains obscure.

Tools used in research on Ca^{2+} signaling in plant-herbivore interactions

In recent years, the research on Ca^{2+} signaling has gained momentum due to the use of genetically encoded Ca^{2+} reporters in plants such as GCaMP3, GCaMP6, cameleon YC3.6 etc. (genetically encoded Ca^{2+} indicator) (Fig. 6). For instance, the GFP-based yellow cameleon (YC 3.6) Ca^{2+} reporter has been used to monitor Ca^{2+} variations in *A. thaliana* leaf upon herbivory (HW) by 3rd and 5th instar larvae of *S. littoralis*. A strong Ca^{2+} response in plants was observed when it was fed by 3rd instar larvae (330 nM) in comparison to those fed by 5th instar (about 150 nM). Cytosolic Ca^{2+} levels were also quantified by application of OS from 5th instar larvae on mechanically damaged leaves. There was a significant increase in cytosolic Ca^{2+} levels up to 290 nM (Verrilo et al., 2014). This indicates that changes in Ca^{2+} concentrations are an indicator of long-distance signaling in plant-herbivore interactions. In addition to the genetically encoded sensor, Ca^{2+} sensing dye has also been used to dissect the role of Ca^{2+} in plant-herbivore interactions. For instance, Ca^{2+} indicator, Ca^{2+} orange ^{TM} was used to detect the variations in cytosolic Ca^{2+} concentrations in Lima bean after herbivory by *S. littoralis.* The changes in Ca^{2+} concentration induced by single wounding (MD) event as well as by continuous mechanical damage caused by a robotic worm (MecWorm, MW) were compared. After 30 minutes, a significant elevation in Ca^{2+} fluorescence was observed due to herbivory around the wounding zone, which was evident up to 4 hours, whereas only a faint fluorescence was seen both in MD and MW plants (Bricchi et al., 2010). By utilizing the genetically encoded Ca^{2+} sensor and dye-

based Ca^{2+} imaging approach, the research would dissect the mechanistic role that Ca^{2+} plays in plant herbivore signaling cascade.

Role of Ca^{2+} sensors in defense responses

Ca^{2+} sensors are the Ca^{2+} binding proteins that play a crucial role in decoding Ca^{2+} signals during herbivory. These Ca^{2+} dependent effectors encipher the frequency, amplitude, and signal localization of Ca^{2+} signatures. It is estimated that there are over 250 Ca^{2+} sensor proteins in *A. thaliana* (Day et al., 2002). These can be classified into 3 main families, i.e., the calcineurin B-like proteins (CBLs) (Luan, 2009), the calmodulin (CaM), and calmodulin-like proteins (CMLs) (Yang and Poovaiah, 2003), the Ca^{2+} dependent protein kinases (CPKs) and the Ca^{2+} and calmodulin-dependent protein kinase (CCPK) (Cheng et al., 2002). All these sensors are equipped with EF-hand motifs that facilitate the binding of $Ca^{2+,}$ resulting in conformational changes in its structure (Batistič and Kudla, 2012). CaM is a Ca^{2+} sensor relay protein as it has no enzymatic function of its own. *A. thaliana* genome consists of 7 CaM genes that encode four isoforms (CaM1/4; CaM2/3/5; CaM6; CaM7)(McCormack and Braam, 2003). Certain transcription factors, protein kinases, phosphatases, metabolic enzymes, ion pumps, ion exchangers are regulated by CaM/CaM- like proteins (CML)(Bouché et al., 2005). Research has demonstrated that *A. thaliana* signal responsive (AtSR1) proteins (Yang and Poovaiah, 2000) also known as the CaM-binding transcription activators (AtCAMTAs) (Bouche et al., 2002) are involved in wounding mediated defense responses. *A. thaliana* mutant *atsr1*, defective in the CaM-binding ability, was susceptible to attack by fungus gnat (*Bradysia impatiens:* Diptera). Thus, confirming the role of CaM binding in wound-signaling (Qiu et al., 2012). In addition to CaM, the plant also harbors CML that changes its secondary structure upon Ca^{2+} binding and

functions as Ca^{2+} relays/sensors (Köhler and Neuhaus, 2000). CML share 16% amino acid similarity with CAM and contain 2-6 EF-hand motif (McCormack and Braam, 2003). Two CMLs, CML 37 and CML42 are involved in defense responses to herbivory. A gene encoding CML42 was shown to be upregulated upon application of OS by *S. littoralis* in *A. thaliana,* suggesting its role in early defense plant signaling (Vadassery et al., 2012). CPKs have been classified as sensor responders as they are comprised of Ca^{2+} binding domain as well as serine/threonine kinase domain in a single protein serving the basics for translating Ca^{2+} signals into phosphorylation events (Tena et al., 2011; Boudsocq and Sheen, 2013). *A. thaliana* consists of 34 genes of the CPK family that are involved in plant defense responses. CPK 3 and CPK 13 both participate in signaling after Ca^{2+} influx upon *S. littoralis* attack through regulation of plant defensin gene (PDF1.2) by phosphorylation of the transcription factor, HsfB2a (Kanchiswamy et al., 2010). The cpk3 and cpk13 mutants showed reduced transcript levels of plant defensin gene PDF1.2 compared to wild-type plants.

Reactive Oxygen Species (ROS)

ROS are highly reactive byproducts of plant metabolism which are generated by partial reduction of oxygen (Miller 2008). Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-) are the three major forms of ROS. These highly reactive molecules are generated mainly by NADPH oxidases, respiratory burst oxidase homologs (RBOHs) (Torres and Dangl 2005; Suzuki et al., 2011). These are produced by mitochondria, chloroplast, and peroxisomes by leakage of electrons onto O_2. ROS plays an important role in ABA-induced stomatal closure, gravitropism, programmed cell death. In recent years, it has become evident

that ROS acts as an important signaling molecules in plant defense response against herbivores (Maffei et al., 2006).

It is widely accepted that insect feeding can lead to oxidative burst, which is the rapid generation of free radicals such as superoxide (O^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-) (Lamb and Dixon, 1997). Previously, it was assumed that ROS is a toxic molecule that causes cellular damage of macromolecules (Asada and Takahashi, 1987) but in recent years, it has emerged as a critical player in plant defense against herbivores. ROS acts as secondary messengers that can penetrate up to 8.4 cm/min in *A. thaliana* (Gilroy et al., 2014). Plants use these molecules as arms to alert the plant against invading insects to prevent further damage caused by herbivory. It has been shown that ROS can be either detrimental or beneficial depending upon their concentration in plant cells (Sharma et al., 2012).

A study by Miller et al. (2009) showed that ROS generation was inhibited in *rbohD* mutant since these plants were deficient in NADPH oxidases, which are the enzymes responsible for ROS generation. The role of NADPH oxidases in orchestrating defenses against chewing insect herbivores was validated in *N. attenuate,* where the application of OS from *M. sexta* increased *Narboh D* (*Nicotina attenuata* NADPH oxidase homologue D) transcripts on wounded leaves. ROS accumulation was diminished after OS treatments in *Narboh D*-silenced *N. attenuata* plants without affecting the other crucial defense hormones. Moreover, the performance of the herbivore, *S. littoralis* was reduced in *Narboh D*-silenced plants (Wu et al., 2013).

Compelling evidence suggests that ROS production by RBOHD (RBOH protein homologs) is dependent on the Ca^{2+} binding (Ogasawara et al., 2008; Kimura et al., 2012).

Studies are required to unravel the mechanistic link between Ca^{2+} and ROS generation. Quantification of ROS levels, produced in response to herbivory has become an area of interest with the discovery of redox-sensitive probes (Janků et al., 2019). These molecules have gained attention as they are robust and promising tools that can measure ROS in real-time with high sensitivity. One such probe is H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate). This dye has been proved effective in a recent study where they detected ROS signals by whole plant- live imaging (Fichman et al., 2019). A study by Christensen et al. (1997) revealed the production of H_2O_2 upon the attack of *S. littoralis* larvae on lima bean leaves using the dye 3,3-diaminobenzidine. H_2O_2 may reach intracellular concentrations up to 1 M in about 13 minutes after wounding (Jacks and Davidonis, 1996). Studies have shown that the production of ROS is an indication of successful recognition of insect attack and it plays a central role in transmitting the signals from wounded to unwounded leaves of the plants. ROS production is indispensable for the systemic induction of defense responses in plants (Baxter et al., 2014).

While most of the studies have demonstrated ROS production following an attack by sucking insects, there are fewer studies related to ROS generation by chewing herbivores. A study by Shinya et al. (2016) showed that application of OS isolated from generalist herbivore, night feeding rice armyworm (*Mythimna loreyi;* Lepidoptera) on rice cells resulted in ROS generation. This study also showed the effect of synthetically prepared N-linolenoyl-L-Glu, the most abundant FAC present in OS of *M. loreyi* on ROS accumulation. Another interesting study has shown that even lower plants like arsenic accumulating fern, *Pteris vittata* responds to the attack of *S. littoralis* by activating peroxidases and generating H_2O_2 near the wounded area. Levels of H_2O_2 were lower in mechanically wounded young leaves in comparison to herbivory

wounded plants. This indicates that the fern can distinguish between mechanical and herbivory wounding by modulating the amount of ROS production (Imbiscuso et al., 2009).

Plants can anticipate damage by activating direct and indirect defenses in response to oviposition by herbivores. In the model plant, *A. thaliana*, the production of H_2O_2, a form of ROS was detected by staining the leaves with 3,3-diaminobenzidine (DAB) 72 hours after oviposition by Cabbage butterfly (*Pieris brassicae*; Lepidoptera). This was evident by the formation of a reddish-brown precipitate after polymerization with H_2O_2. This data indicates that oviposition can trigger a localized response that resembles the hypersensitive response induced by pathogens. It is speculated that this plant response is due to the presence of certain elicitors in the insect eggs (Little et al., 2007). The role of ROS in plant resistance to herbivores has been demonstrated in resistant and near-isogenic susceptible wheat after the attack of Russian wheat aphid (*Diuraphis noxia;* Hymenoptera). A strong burst of H_2O_2 as well as NADPH oxidase was observed in resistant plants 3 hours after infestation in comparison to susceptible plants. Treatments of plants with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase suppressed the H_2O_2 production. Elevation in H_2O_2 levels (47%) was evident by treating resistant wheat plants with a mixture of glucose and glucose oxidase (Moloi and van der Westhuizen, 2006).

Figures

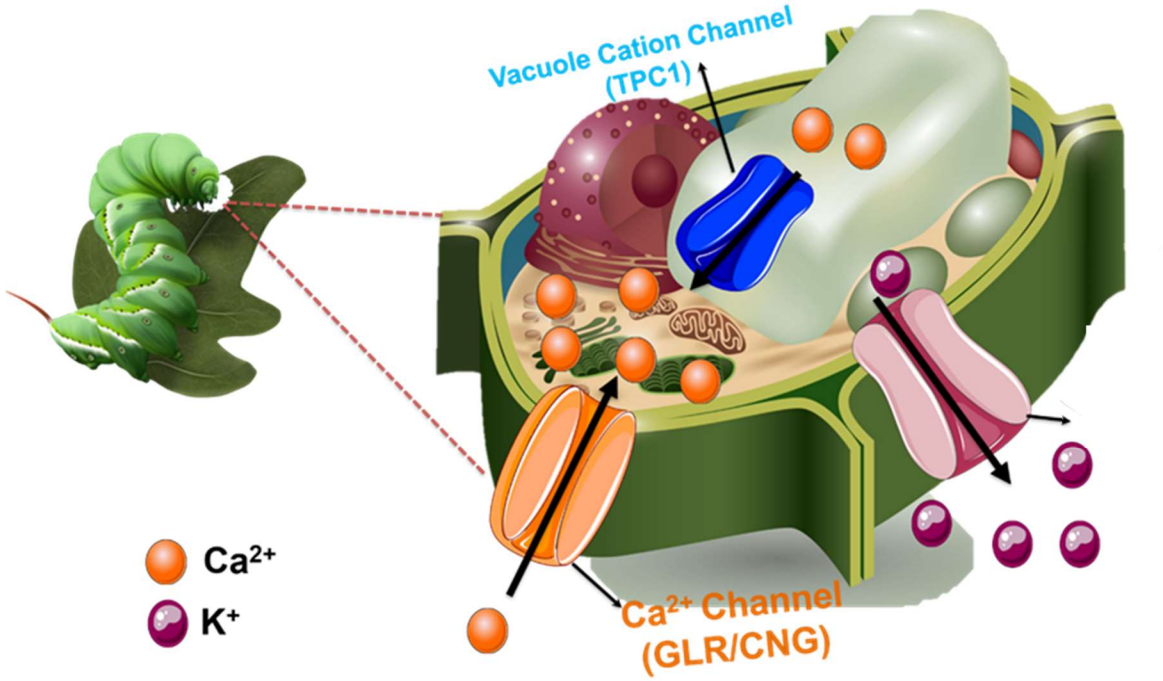

Figure 1: Plant-herbivore interactions at the cellular level. Schematic diagram showing *Manduca sexta* attack induced signaling cascade in host plants. During insect attack, plasma membrane Ca^{2+} channels, GLR and CNGC triggers Ca^{2+} influx. Vacuolar cation channel, TPC1 releases Ca^{2+} from the intracellular store, vacuole.

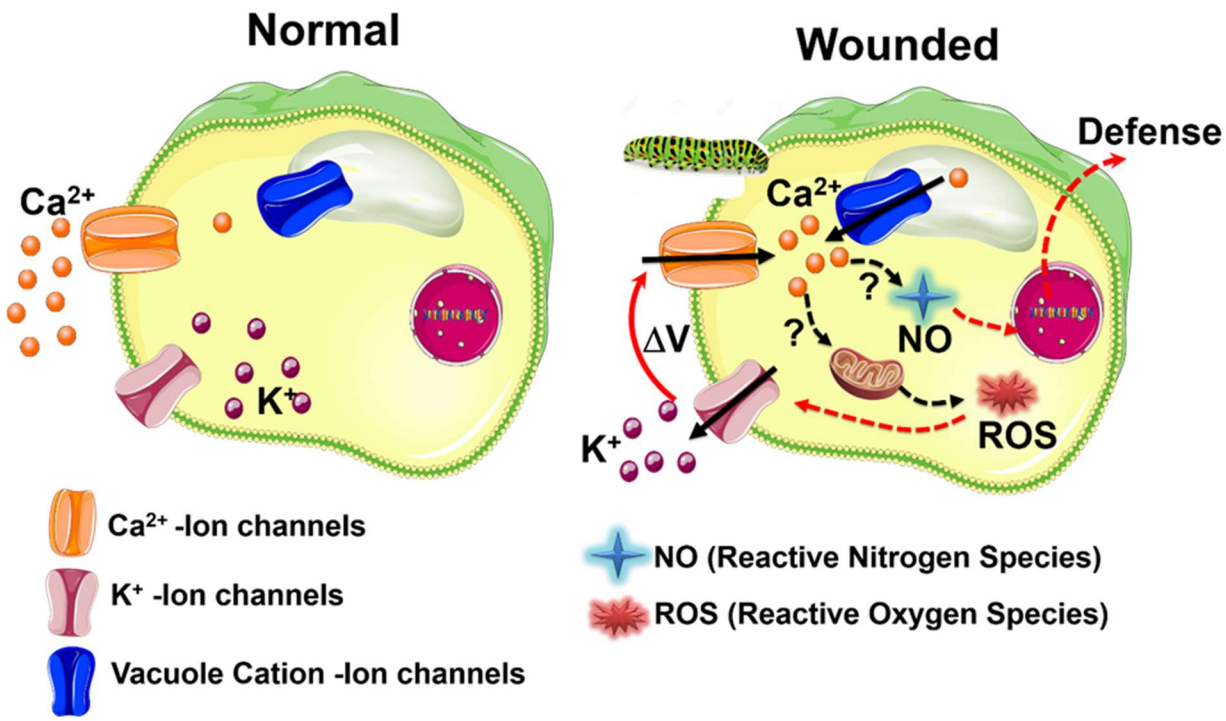

Figure 2: Schematic representation of the plant signaling pathways after insect attack. Ion channels such as Ca^{2+} and K^{+} channel at the plasma membrane serve as first responder in the herbivore attack. The wounded cell initiates a diverse set of molecular regulators that include changes in membrane potential by modifying K^{+} channels, propagation of Ca^{2+} waves triggers intracellular Ca^{2+} increase and intracellular ROS affects K^{+} channel function through negative feedback homeostasis mechanism.

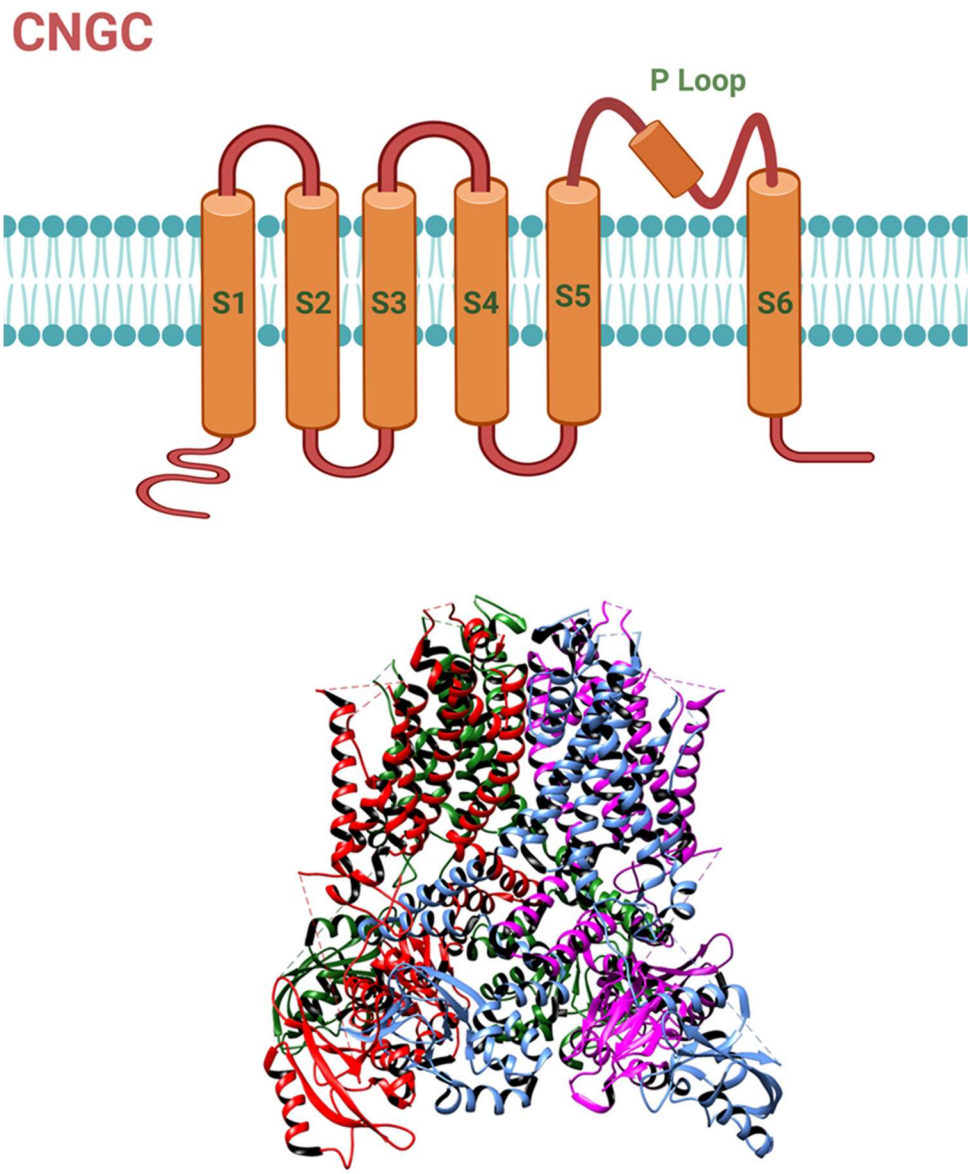

Figure 3: Putative structure of cyclic nucleotide gated channel (CNGC). Schematic representative figure of a single subunit of plasma membrane Ca^{2+} channel, CNGC 19 showing six transmembrane and one pore, P-loop of the channel (top). Four subunits make a functional channel. 3D structure of CNGC19 channel showing 4 subunits (bottom).

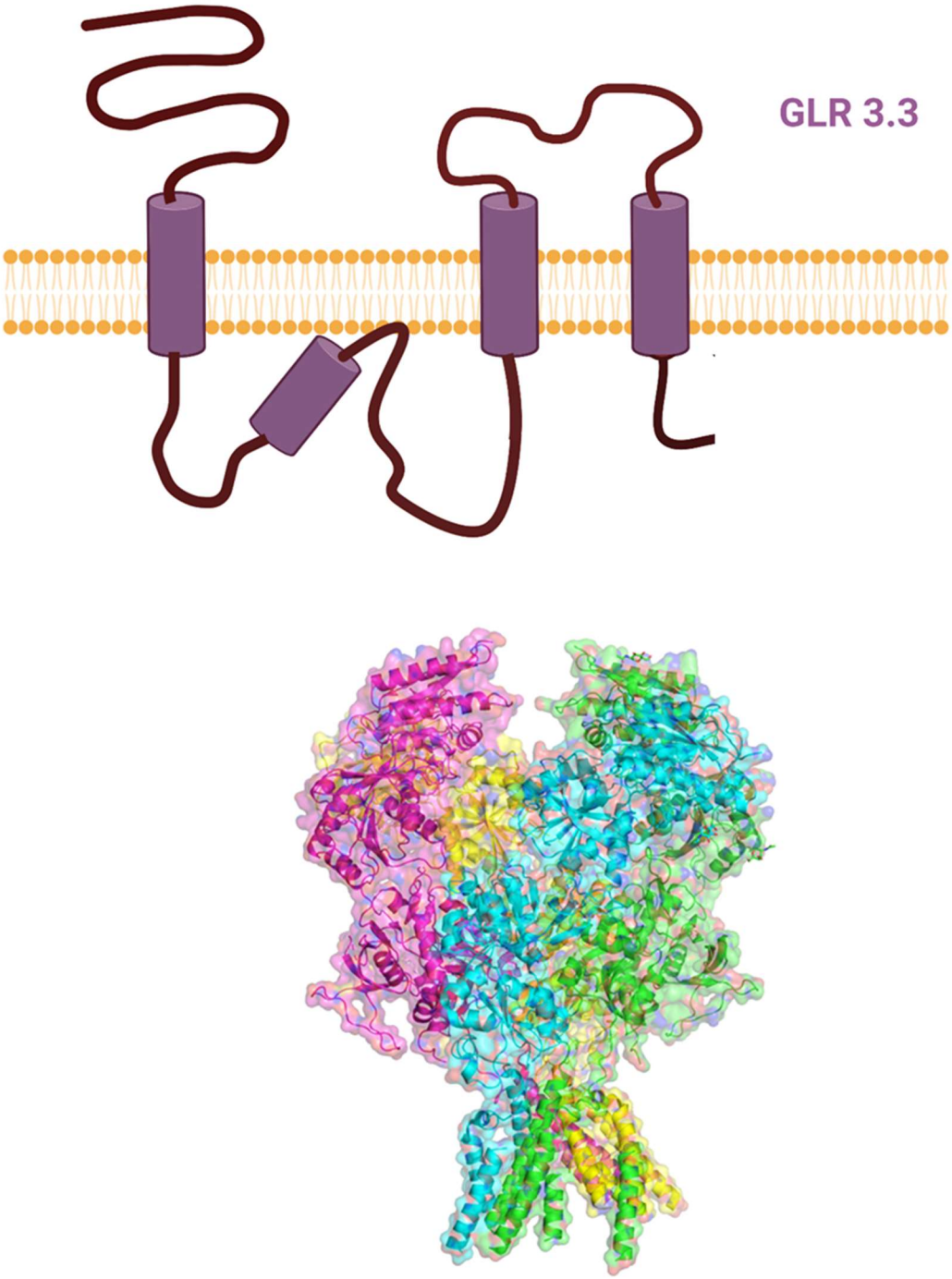

Figure 4: Putative structure of glutamate receptor like channels (GLR 3.3). Schematic representative figure of plasma membrane Ca^{2+} channel, GLR 3.3 showing four transmembrane domains (top). 3D structure of channel showing 4 subunits (bottom).

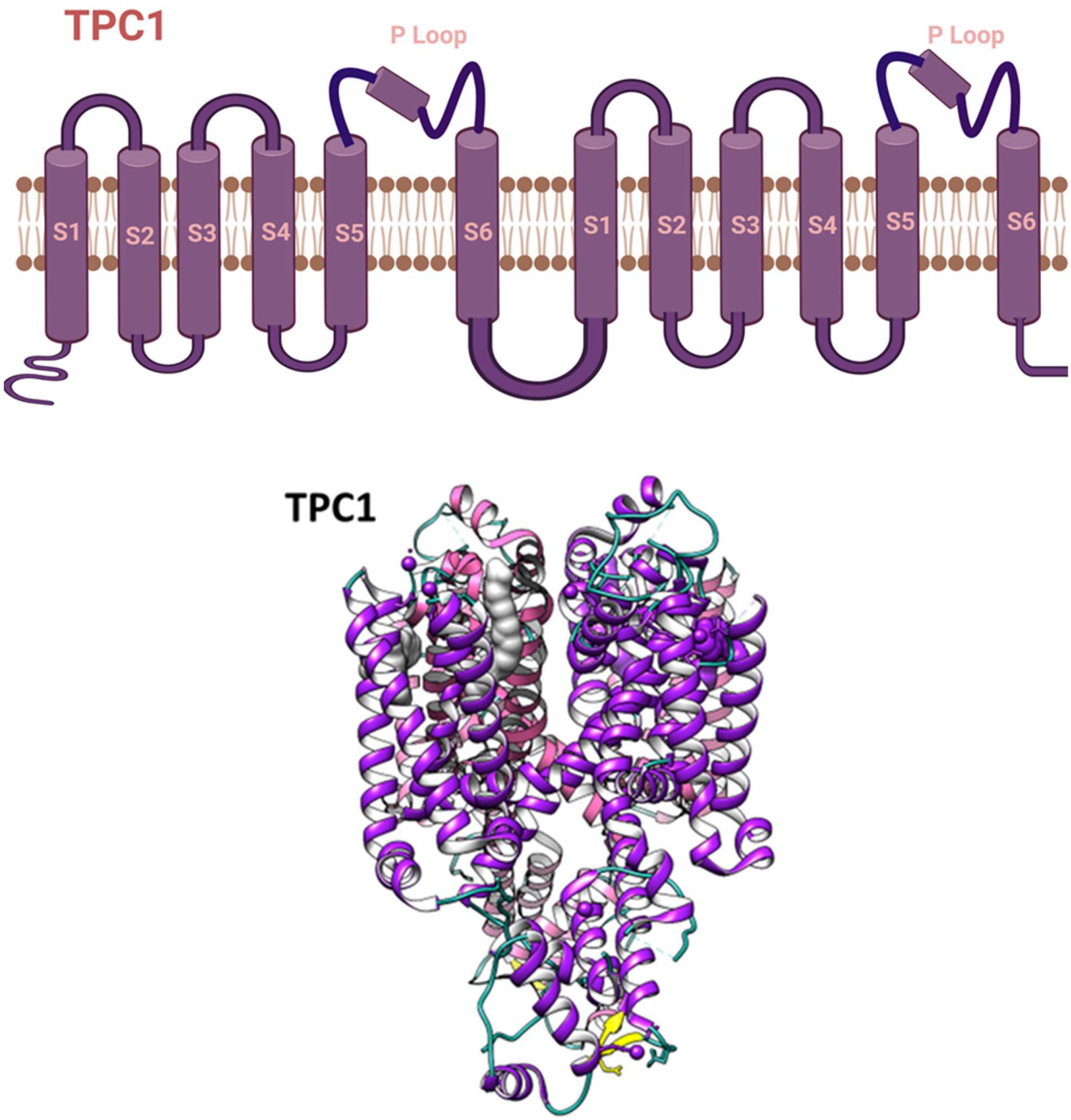

Figure 5: Putative structure of two pore channel (TPC1) channels. Schematic representative figure of vacuolar Ca^{2+} channel, TPC1 showing the two, 6 transmembrane subunits and two pore helices. Cartoon illustration (top); 3D structure (bottom).

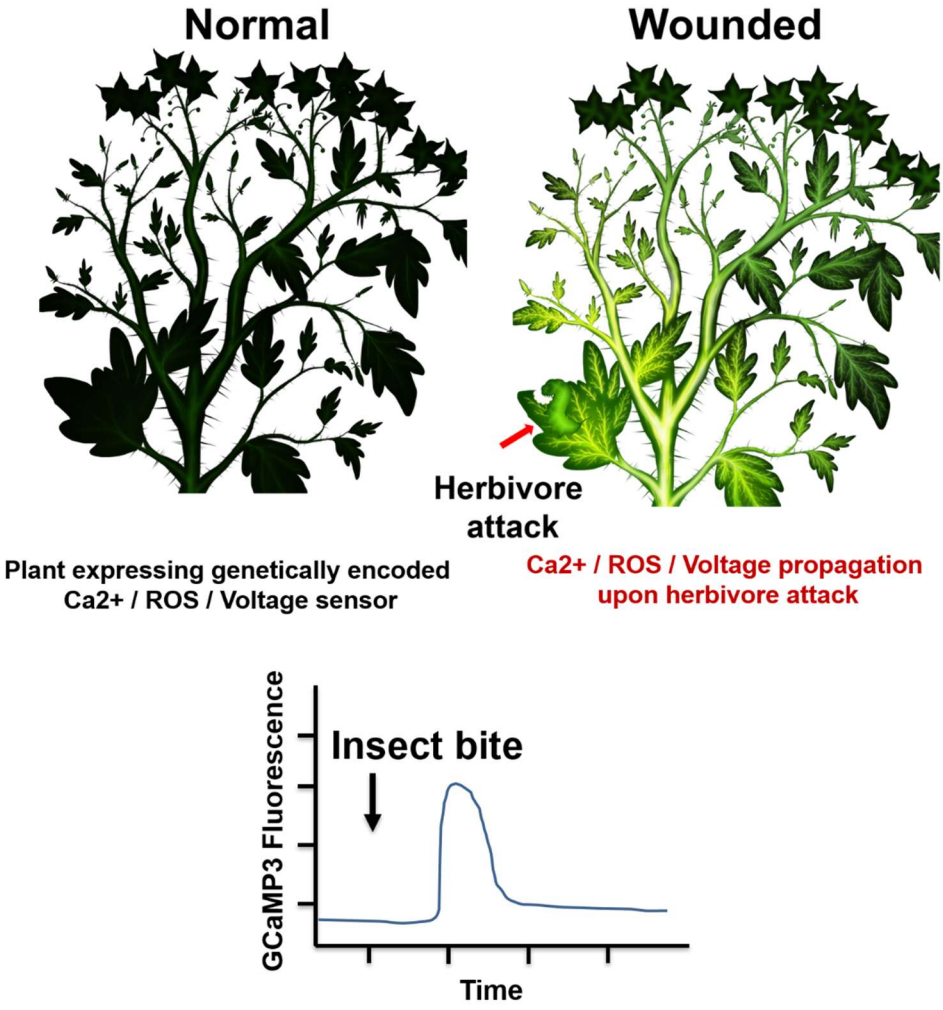

Figure 6: Tools used in plant long-distance signaling research. Wounding triggers long-distance propagation of Ca^{2+}, V_m and ROS signals that result in systemic defense responses**.** Genetically encoded Ca^{2+}, voltage, ROS sensor is expressed in *Arabidopsis thaliana* showing the intracellular signaling occurring in plant when it is attacked by herbivore (top); Schematic representation of a graph showing Ca^{2+} or V_m or ROS imaging readout (bottom).

CHAPTER III

TOBACCO HORNWORM (*MANDUCA SEXTA*) ORAL SECRETIONS ELICIT REACTIVE OXYGEN SPECIES IN ISOLATED TOMATO PROTOPLASTS

Abstract

Plants are under constant attack by a suite of insect herbivores. Over the millions of years of co-existence, plants have evolved the ability to sense insect feeding via herbivore associated elicitors in oral secretions, which can mobilize defenses responses. However, our understanding of herbivore associated elicitors and the intrinsic downstream modulator of such interaction remains less understood. In this study, we show that tobacco hornworm caterpillar (*Manduca sexta*) OS induces ROS in tomato (*Solanum lycopersicum*) protoplasts. By using a dye-based ROS imaging approach, our study shows that the application of Plant-Fed (PF) *M. sexta* OS generates significantly higher ROS while artificial Diet-Fed (DF) caterpillar OS failed to induce ROS in isolated tomato protoplasts. The elevation in ROS generation was saturated after ~140 seconds of PF-OS application. ROS production was also suppressed in the presence of an antioxidant NAC (N-acetyl-L-cysteine). Interestingly, the PF-OS induced ROS increase was abolished in the presence of a Ca^{2+} chelator, BAPTA-AM. These results indicate a potential signaling cascade involving herbivore associated elicitors, Ca^{2+}, and ROS in plants during insect feeding. In summary, our results demonstrate that plants incorporate a variety of independent signals connected with their herbivores to regulate and mount their defense responses.

Introduction

Herbivory is an unavoidable part of plant's life. Over the millions of years, plants and herbivorous insects (herbivores) have been involved in a relentless war where plants are actively attacked by herbivores reducing plant growth, development, and, consequently, their fitness (Schaffer et al., 2011). It is estimated that insect herbivory leads to about ~20 percent losses in annual plant growth (Oerke, 2006; Sharma et al., 2017). To counter this, although sessile, plants have evolved several defense approaches, which include morphological, biochemical, and molecular mechanisms (Zhao et al., 2009; Karban et al., 2010; War et al., 2012; Kariyat et al., 2012a; Kariyat et al., 2013). During an insect attack, the host plant perceives at least two types of signals- 1) physical injury or wounding known as Damage-Associated Molecular Patterns (DAMPs) and 2) chemical cues found in herbivore oral secretions (OS) or oviposition fluid (OF) known as herbivore associated molecular patterns (HAMPs) (Howe et al., 2008; Wu et al., 2009; Felton et al., 2018; Erb et al., 2019).

Herbivore-plant interactions are generally initiated at the plant cell membrane where herbivore associated elicitors trigger a series of signaling cascades that induces plant response (Arimura et al., 2005; Maffei et al., 2007; Arimura et al., 2010, 2011). It has been proposed that following insect attack; the foremost event is the plasma membrane depolarization (V_m) (Bricchi et al., 2010; Zebelo et al., 2012) followed by the generation of second messengers such as cytosolic Ca^{2+} (Reddy et al., 2011) and ROS (Shin et al., 2004; Shin et al., 2005; Steffens et al., 2013; Halliwell et al., 2015) that facilitates plant defense signal transduction. This leads to a suite of defense related traits including the induction of trichomes, spines, and secondary metabolites (e.g., alkaloids, phenolics, volatile organic compounds) that negatively impacts herbivore fitness

and mediate multi-trophic interactions (Turlings et al., 2018; Kaur et al., 2020). While the ecological aspects of plant-herbivore interactions, and their molecular mechanisms are well understood, the early initiation mechanisms associated with alterations in the V_m, Ca^{2+}, and ROS production immediately after herbivore assault warrants more empirical testing in various systems (Bonaventure, 2012).

ROS represents one of the significant biomolecules which play a crucial role in defense signaling in plants (Mittler et al., 2011; Noctor et al., 2018). It is well known that there is a rapid generation of molecules such as superoxide (O^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO^-) upon insect attack and leads to an oxidative burst (Lamb et al., 1997). Previous studies have shown that plants can identify herbivore OS that leads to the oxidative burst and facilitates in transmitting the long-distance signals (Chung et al., 2013; Schmelz et al., 2015; Zebelo and Maffei, 2012; Molassiotis and Fotopoulos, 2011). ROS production is indispensable for the systemic induction of defense responses in plants (Mittler et al., 2011; Noctor et al., 2018). Regardless of their significance in several facets of cell biology, our knowledge of oxidative signaling, as well as their regulation, remains limited (Molassiotis and Fotopoulos, 2011).

In this study, we identified that *Manduca sexta* (tobacco hornworm) (Lepidoptera; Sphingidae) OS stimulates the ROS generation in isolated tomato protoplasts. *M. sexta* is a crucial insect model used to test both ecological effects and molecular mechanisms underlying plant-herbivore interactions research (Howe and Jander, 2008; Kariyat et al., 2012a; 2019; Portman et al., 2015; Tayal et al., 2020b). *M. sexta* is a specialist on Solanaceae, that includes tomato (*Solanum lycopersicum*) which also serves as a good cellular model for plant defense-

related studies (Portman et al., 2020). By utilizing ROS sensing dye 2',7' dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) based cell imaging technique, we efficiently measured the transient elevation in ROS generation upon application of *M. sexta* OS. This ROS sensing dye has been previously used in studying *in vivo* ROS production in root cells and hairs (Kristiansen et al., 2009). Our investigation demonstrates that the *M. sexta* OS induces ROS production in tomato protoplasts and the OS effect is altered based on the diet choices of the insect. Moreover, we identified that *M. sexta* OS-mediated ROS generation is dependent on the intracellular Ca^{2+}.

Material and Methods

Plant Material

F1 tomato hybrid seeds (Variety: Valley Girl, Johnny's Selected Seeds, Maine, USA) were grown in pots in a growth chamber at 25 °C with a relative humidity of 65%. The seeds were sown in Sunshine professional growing mix (Sun Gro Horticulture Canada Ltd., MA, USA). Seedlings were transplanted two weeks after germination, and OMRI (Organic Material Review Institute, OR, USA) listed organic fish emulsion fertilizer (NPK 5:1:1, Alaska Fish Fertilizer, Pennington Seed, Inc., USA) was added once in two weeks. Plants were watered regularly and grown in controlled conditions without herbivores (Tayal et al., 2020a). All plants used in the study were 4 weeks old after transplanting.

Protoplast isolation

Protoplasts were isolated by modifying the method described by Zhai et al. (2009). Briefly, 0.5 grams of the leaf material from 4 weeks old tomato plants were collected and sliced using a fresh

razor blade in 3.75 ml of the TVL solution (0.3 M sorbitol and 50 mM $CaCl_2$). This solution was stored at -20°C until further use. Following this, 5 ml of the enzyme solution containing 0.5 M sucrose, 10 mM MES-KOH [pH 5.7], 20 mM $CaCl_2$, 40 mM KCl, 0.9% macerozyme and 1.5 % cellulase (Research Products International Corp, Mt. Prospect, IL, USA) was prepared and heated at 55 °C to inactivate proteases and increase enzyme solubility. Finely chopped leaf tissue was transferred to a beaker with enzyme solution that was freshly prepared to retain the efficiency of the enzymes. The beaker was covered with aluminum foil and parafilm and was subjected to vacuum for 15 minutes (He et al., 2007). The plant tissues were then kept on a shaker at 35 rpm in the dark for 12-14 hours. After overnight shaking, the digested material was filtered through 8 layered-cheese cloth, pre-wet in W5 solution (0.1% (w/v) glucose, 0.08% (w/v) KCl, 0.9% (w/v) NaCl, 1.84% (w/v) $CaCl_2$, 2 mM MES-KOH pH 5.7). The cheesecloth was washed again with 3.75 ml of W5 solution to sieve the remaining protoplasts. The protoplasts were centrifuged for 7 minutes at 100 g. The supernatant was discarded, and the collected pellet was dissolved in 500 µl of W5 solution.

Manduca sexta rearing and oral secretion collection

Eggs of *M. sexta* (Lepidoptera: Sphingidae) were obtained from a commercial vendor (Great Lake Hornworm Ltd. Romeo, Michigan, USA) and were hatched in Petri dish containing moist filter paper in a growth chamber (16:8 h light: dark; 25: 22°C day: night; 65% RH). In order to collect DF and PF OS, half of the Ist instar larvae were reared on wheat-germ-based artificial diet (wheat germ, casein, sucrose, cholesterol, salts, vitamins, agar, preservatives) purchased from Carolina Biological, Burlington, North Carolina, USA while other half was reared on tomato plants (Kariyat et al., 2013). Regurgitant was collected from the oral cavity of newly

molted fourth instar larvae by holding the *M. sexta* and gently squeezing its head into a capillary tube and or an eppendorf tube was placed at the mouth of *M. sexta*. The collected OS was centrifuged and stored at -80°C until further use.

ROS Measurements

ROS measurement was performed at room temperature with the PTI EasyRatioPro system (HORIBA Scientific). Isolated protoplasts were incubated with 2 µM ROS-sensing dye, CM-H_2DCFDA (Invitrogen™ Molecular Probes™) for 1 hour in the dark. A small drop of the protoplast sample carrying ~30-50 protoplasts was placed on a glass coverslip under an Olympus IX71 inverted microscope attached with PTI EasyRatioPro system. A change in fluorescence of a single protoplast was recorded with EasyRatioPro software with an excitation wavelength at 494 nm and emission wavelength at 520 nm. All the chemicals such as *M. sexta* OS (crude), tbH_2O_2 (1 M), NAC (1 mM) of 1- 4ul were dropped into the protoplast sample during the live measurements to test their effect on the intracellular ROS generation. For Ca^{2+} dependent ROS generation experiment, Isolated protoplasts were preincubated with 1.5 µM of BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid) (Invitrogen™ Molecular Probes™) for 1 hr prior to the ROS measurement.

Data Analysis and Presentation

ROS imaging data were analyzed with EasyRatioPro (PTI, HORIBA Scientific) software and further processed with Excel (Microsoft, Redmond, WA, USA) and Igor Pro v8.0 (Wavemetrics, Lake Oswego, OR, USA) software. Protoplast images were processed with ImageJ (NIH). Figures were prepared with Origin Pro v2020 (Originlab, Northampton, MA, USA) and Adobe

Illustrator v24.1 (Adobe, San Jose, CA, USA). Averaged data are presented as means±SEM (N = number of protoplasts from 3-5 independent measurements). For comparisons with two groups such as Basal ROS levels and ROS levels from DF *M. sexta* OS and Tomato PF *M. sexta* OS, we used the non-parametric Mann-Whitney U test, and for comparisons with three groups as depicted in Figure 4; Basal, OS/ tbH_2O_2, and NAC, we used a non-Parametric Kruskal-Wallis test followed by Dunn's pairwise post hoc comparisons. Non-parametric tests were used since data failed to meet normality assumptions after transformations. For all analyses, data from extractions were pooled to attain a sample size of 66-124 protoplasts and was repeated for at least three replications. All analyses were carried out using GraphPad Prism v9.0 (La Jolla, California, USA).

Results

M. sexta OS induced ROS generation in tomato protoplasts

While herbivores prey on the plant, protoplasts come into contact with oral secretions that induce plant defense signaling, and ROS has been known to play a critical role in these defense responses. To determine if herbivore OS would modulate ROS levels in the plant, we performed CM-H2DCFDA dye-based ROS imaging of tomato protoplast and tested the effect of herbivore *M. sexta* crude OS (Figure 1A). We found that the application of *M. sexta* OS induced a drastic increase of ROS generation in isolated tomato protoplasts. After a lag of 134.2 ± 11.4 s, the ROS level reached a maximum after 140.5 ± 5.9 s of *M. sexta* OS application (Figure 1B, C; $N = 74$). These data indicate that *M. sexta* OS is a potent elicitor of ROS in plant protoplasts.

Diet dependent *M. sexta* OS effect on ROS production in tomato protoplasts

Many herbivores have coevolved with specific plant host, and typically exhibit preferences to the diet of the same host plant. On the other hand, plants can sense the herbivore derived elicitor such as OS, comprising of regurgitant and saliva of host plant and facilitates in plant defense signal transduction. To investigate whether the *M. sexta* OS mediated ROS increase is diet-dependent, we tested the effect of OS derived from tomato Plant-Fed (PF) and artificial Diet-Fed (DF) *M. sexta*. Our ROS imaging recording from tomato protoplasts showed that the application of the tomato PF *M. sexta* OS increased ROS generation (basal: 0.035±0.003; PF OS: 0.292±0.018; $P<0.0001$; Mann-Whitney test) (Fig. 2A, 2C, 2D; N=86) while artificial DF *M. sexta* OS failed to induce ROS in isolated tomato protoplasts (basal: 0.015±0.002; DF OS: 0.021±0.007; $P=0.3846$) (Fig. 2B, 2C, 2D; N=90). These results suggest that the herbivore OS diet plays an essential role in the generation of ROS in host plants.

Membrane-permeable oxidant "tbH_2O_2" induced ROS in tomato protoplasts

Previous studies have found an increase in the production of ROS, such as H_2O_2 in less than 5 minutes of herbivore-induced wounding (Bolwell et al., 2002; Maffei et al., 2007). This observation is in line with our findings, which showed that the maximum ROS generation in tomato protoplasts was achieved in less than 3 minutes of *M. sexta* OS application. To investigate whether our ROS imaging approach could detect the H_2O_2 induced ROS, we applied a membrane-permeable “tert butyl hydrogen peroxide” (tbH_2O_2) to the CM-H_2DCFDA dye loaded tomato protoplasts. As shown in Fig. 3, the increase in maximum ROS production was

observed after 2 minutes of the application of tbH_2O_2 (basal: 0.064±0.005; tbH_2O_2: 0.665±0.084; P<0.0001; Mann-Whitney test) (Figure 3A,D; N = 100). These results indicate that our ROS imaging approach could efficiently detect intracellular ROS either by H_2O_2 or herbivore OS.

Antioxidant N-acetylcysteine (NAC) abolished *M. sexta* OS, and oxidant tbH_2O_2 induced ROS generation in tomato protoplasts

The evidence presented so far suggests that *M. sexta* OS and tbH_2O_2 induced ROS generation in isolated tomato protoplasts. To further validate these observations, we used an antioxidant NAC, a glutathione (GSH) precursor, that boosts GSH content in cells. As shown in Fig. 4, the application of NAC to the tomato protoplasts efficiently quenched the ROS generated by *M. sexta* OS (basal: 0.048±0.006; PF OS: 0.319±0.019; NAC: -0.552±0.026; P<0.0001; Kruskal-Wallis test followed by Dunn's pairwise posthoc analysis) (Fig. 4A, 4C; N=115) and tbH_2O_2 (basal: 0.043±0.006; tbH_2O_2: 0.460±0.034; NAC: -0.619±0.016; P<0.0001; Kruskal-Wallis test followed by Dunn's pairwise posthoc analysis) (Fig. 4B, 4C; N=71). However, NAC treatment leads to a negative baseline, suggesting that protoplasts were partially oxidized in our experimental conditions (Fig. 4). This finding further supports that *M. sexta* OS is a ROS inducer in isolated protoplasts.

Ca^{2+} chelator BAPTA-AM inhibited *M. sexta* OS induced ROS generation in tomato protoplasts

Ca^{2+} has been known to serve as a second messenger in plant-herbivore interactions. Several studies have shown that herbivore-induced wounding triggers a dramatic Ca^{2+} cytosolic ion influx, which further regulates the formation of ROS (Maffei et al., 2007). To investigate

whether *M. sexta* OS-induced ROS generation is dependent on cytosolic Ca^{2+}, we preincubated the tomato protoplast in BAPTA-AM, a membrane-permeable Ca^{2+} chelator and tested the effect of *M. sexta* OS on ROS generation. As shown in Fig. 5A, 5C, the application of *M. sexta* OS completely abolished the ROS production in BAPTA-AM preincubated tomato protoplasts (basal: 0.028±0.003; PF OS: 0.042±0.013; P=0.786; Mann-Whitney test) (Figure 5A,C; N = 66) However, tbH_2O_2 - induced ROS was not affected by Ca^{2+} chelator BAPTA-AM (basal: 0.066±0.006; tbH_2O_2: 0.618±0.028; P<0.001; Mann-Whitney test) (Figure 5B,C; N = 124). These results indicate that *M. sexta* OS induced ROS generation was mediated by cytosolic Ca^{2+}.

Discussion

Identification of herbivore elicitors and their regulation of the intracellular ROS production is vital for unraveling the non-self-recognition signaling cascades in plants. In this study, we show that "OS" from the *M. sexta* is effective in producing ROS in tomato protoplasts and OS-induced intracellular ROS production is dependent on intracellular Ca^{2+}. Our results of ROS imaging of single protoplast to understand the kinetics of the ROS initiation upon herbivore OS application will be critical in understanding early initiation events in herbivore defenses in plants. Our cellular approach of dissecting the ROS plays an essential part in various pathways, including physiological, hormonal and developmental aspects of plant growth (Felton et al., 2018; Erb et al., 2019). In addition, ROS also plays a crucial role in defense signaling cascade against abiotic and biotic stress conditions (Miller et al., 2008; Kwon et al., 2013; Choudhury et al., 2013, 2016; Rejeb et al., 2014). Hence, understanding of ROS in plants remains an emerging field of research. More recently, several studies have used the fluorescent reporter molecules to measure the ROS levels *in vivo,* and have collectively documented that these molecules are

robust and promising tools that can measure ROS in real-time with high sensitivity (Maffei et al., 2006; Wooley et al., 2013; Wojtala et al., 2014; Oparka et al., 2016). However, these probes, including Diaminobenzidine (DAB), Nitro blue tetrazolium (NBT), Amplex Red, have certain limitations of being toxic and susceptible to degradation by light (Swanson et al., 2011). However, use of CM-H_2DCFDA in protoplasts is a valuable general ROS indicator to study plant-herbivore interactions (Kristiansen et al., 2009: Oparka et al., 2016). A study by Maffei et al. (2004) showed that ROS (H_2O_2) accumulation was observed in lima bean leaves (*Phaseolus lunatus*) incubated with DAB, upon attack by Egyptian cotton leafworm (*Spodoptera littoralis;* Lepidoptera) as well in mechanically damaged leaves. However, the H_2O_2 production was more in the herbivore-wounded zones in comparison to the mechanically damaged leaves. To further validate the finding, CM-H_2DCFDA dye with confocal laser scanning microscopy was used, which confirmed the variation in H_2O_2 generation in mechanically damaged and herbivore-wounded leaves. In addition, a recent study by Fischman et al. (2019) showed the local and systemic ROS signals accumulation upon wounding and was evaluated by using CM-H_2DCFDA dye-based ROS sensing in whole-plants. This new method of examining the ROS generation on whole mature plants in real-time could unravel systemic signaling in plants and greatly facilitate the identification of new pathways for ROS signaling. Our study clearly demonstrates that CM-H_2DCFDA dye-based ROS imaging approach on single tomato protoplast was able to quantify and visualize ROS generation without any toxic effects on cell health.

Among the signaling molecules leading to defense induction, ROS has been found to be crucial, and the timing of ROS generation plays a vital role in initiating plant responses. For example., ROS (H_2O_2) was produced in less than 5 minutes after herbivore-induced wounding

(Bolwell et al., 2002; Bhattacharjee et al., 2005; Maffei et al., 2007; Kerchev et al., 2011). In another study by Mohanta et al. (2012), the generation of ROS (H_2O_2) in the maidenhair tree (*Ginkgo biloba*) was observed after 30 minutes upon herbivory Egyptian cotton leafworm (*Spodoptera littoralis*). These observations are in line with our findings; our cellular approach found that the maximum ROS generation in tomato protoplast was achieved in less than 3 minutes of *M. sexta* OS application. Clearly, regardless of the feeding habit (chewing or sucking mouthparts), ROS is critical. This is in addition to the upregulation of certain genes associated with oxidative stress, along with Ca^{2+} signaling (Rae et al., 2000; Chen et al., 2005; Cabrera et al., 2008). Previous studies have reported that plants perceive the components that are mainly of plant origin once they are encountered by the herbivore (Kant et al., 2015). Our results have added a new dimension to the previously known fact depicting that oral secretions from plant origin (PF OS) can induce ROS signals while OS from artificial diet (DF OS) do not generate ROS in protoplasts thus, giving an indication that PF OS contains components that are responsible for stimulating ROS in protoplasts. It is possible that differences in ROS responses to OS from plant feeding and diet feeding could be due to plant components such as fragments of the cell wall. It will be interesting to find out which OS component is mainly responsible for ROS generation. Clearly, herbivore diet plays a crucial role in plant defense signaling, an area we are currently exploring in detail using mass Spectrometry to examine the differences in the composition of both the PF and DF OS, from *M. sexta* and other herbivore species feeding on different plant species.

Elicitor-dependent production of secondary messengers such as ROS and Ca^{2+} is critical to several signaling processes in plants (Davies et al., 2006; Jeworutzki et al., 2010; Zebelo et al.,

2014). Nevertheless, the details of mechanisms that control the mutual interrelation of ROS and Ca^{2+} signaling merely start to emerge. One of the fascinating questions is whether the ROS production is interconnected to Ca^{2+} signaling. In order to unravel this, we used BAPTA-AM, which is the most used Ca^{2+} chelator in the mammalian cell. The application of PF-OS in the presence of BAPTA-AM on the isolated tomato protoplasts failed to show ROS accumulation, indicating the mechanistic link between Ca^{2+} and ROS production. Studies have shown that ROS is regulated by intracellular Ca^{2+} (Yan et al., 2006; Gorlach et al., 2015; Liao et al., 2017). Upon insect attack, a first 'priming' Ca^{2+} inflow occurs, followed by the release of Ca^{2+} from intracellular stores such as vacuole, mitochondria via Ca^{2+} channels. An increase in cytoplasmic Ca^{2+} activates NADPH oxidases, an enzyme responsible for ROS generation upon binding of Ca^{2+} to its EF-hands resulting in plant defense responses (Takeda et al., 2008; Kimura et al., 2012; Drerup et al., 2013). ROS could also activate Ca^{2+} channels and facilitate ROS mediated Ca^{2+} fluxes (Yan et al., 2006; Görlach et al., 2015). These ROS- dependent events could initiate a cellular amplification loop, resulting in the Ca^{2+} wave propagation from cell to cell. Our results support the possible connection between ROS-Ca^{2+} signaling pathway that might be helpful in understanding the plant-herbivore interactions at the cellular level.

We choose tbH_2O_2 over H_2O_2 to study the ROS response in isolated tomato protoplasts. Since H_2O_2 gets quickly oxidized and produces small bubbles in solutions containing protoplasts which rendered difficulties in measuring the ROS responses in our experimental condition. In addition, H_2O_2 is very slowly permeable across the membrane. Therefore, we used a membrane-permeable version "tbH_2O_2", which showed a strong ROS response to CM-H_2DCFDA loaded tomato protoplasts. To investigate further the ROS response mediated via *M. sexta* OS and

tbH_2O_2, we used a membrane-permeable antioxidant NAC, that has a free radical scavenging property and frequently used in animal ROS research. It resulted in the suppression of *M. sexta* OS and tbH_2O_2 -induced ROS production. Our study has depicted, for the first time, use of these two chemicals 1) membrane-permeable oxidant "tbH_2O_2" and (2) membrane-permeable antioxidant "NAC" in a plant system and could be used in plant-herbivore interaction research.

Figures

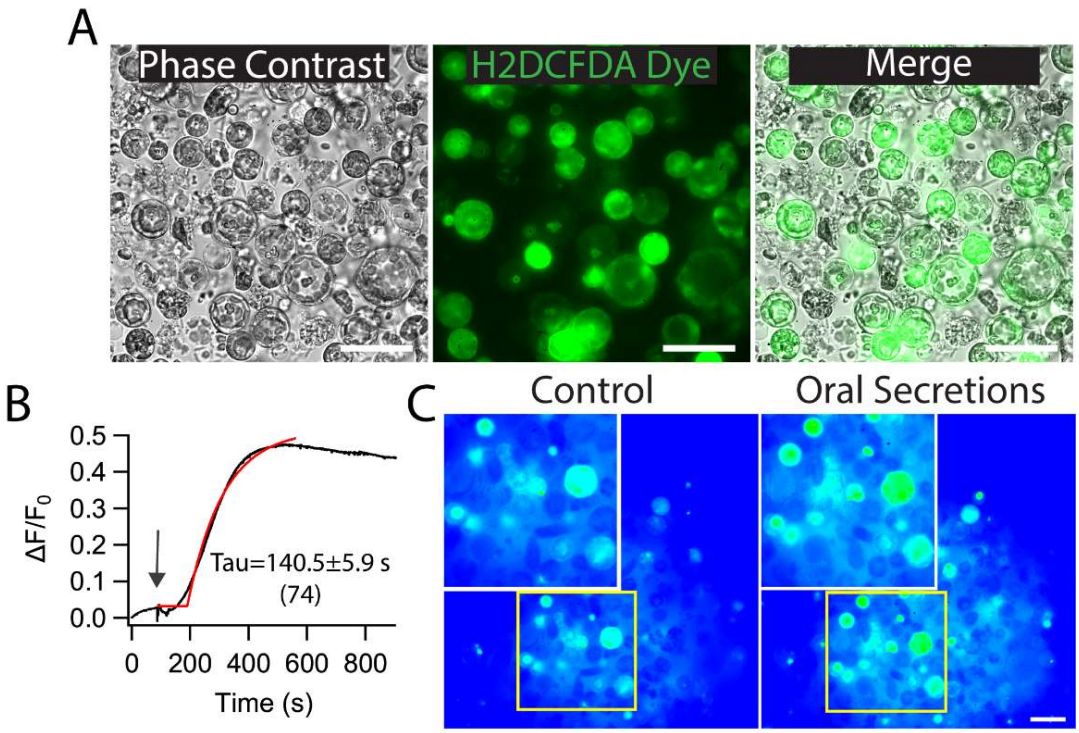

Figure 1: Effect of *Manduca sexta* oral secretion on ROS production in tomato (*Solanum lycopersicum*) protoplasts. (A) Representative Phase Contrast image (left) of protoplast at 100x magnification isolated from tomato leaves. Isolated protoplasts were loaded with ROS sensing dye CM-H_2DCFDA (middle). (B) Representative ROS imaging trace showing an increase in protoplast ROS level upon application of *M. sexta* OS. The data were fitted with a single exponential fit function with a lag of 134.2±11.4 s and Tau of 140.5±5.9 s. (C) Representative image of ROS generation in tomato protoplast at 40X magnification before and after 400 s of *M. sexta* OS application. Scale bar: 10 µm. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in B.

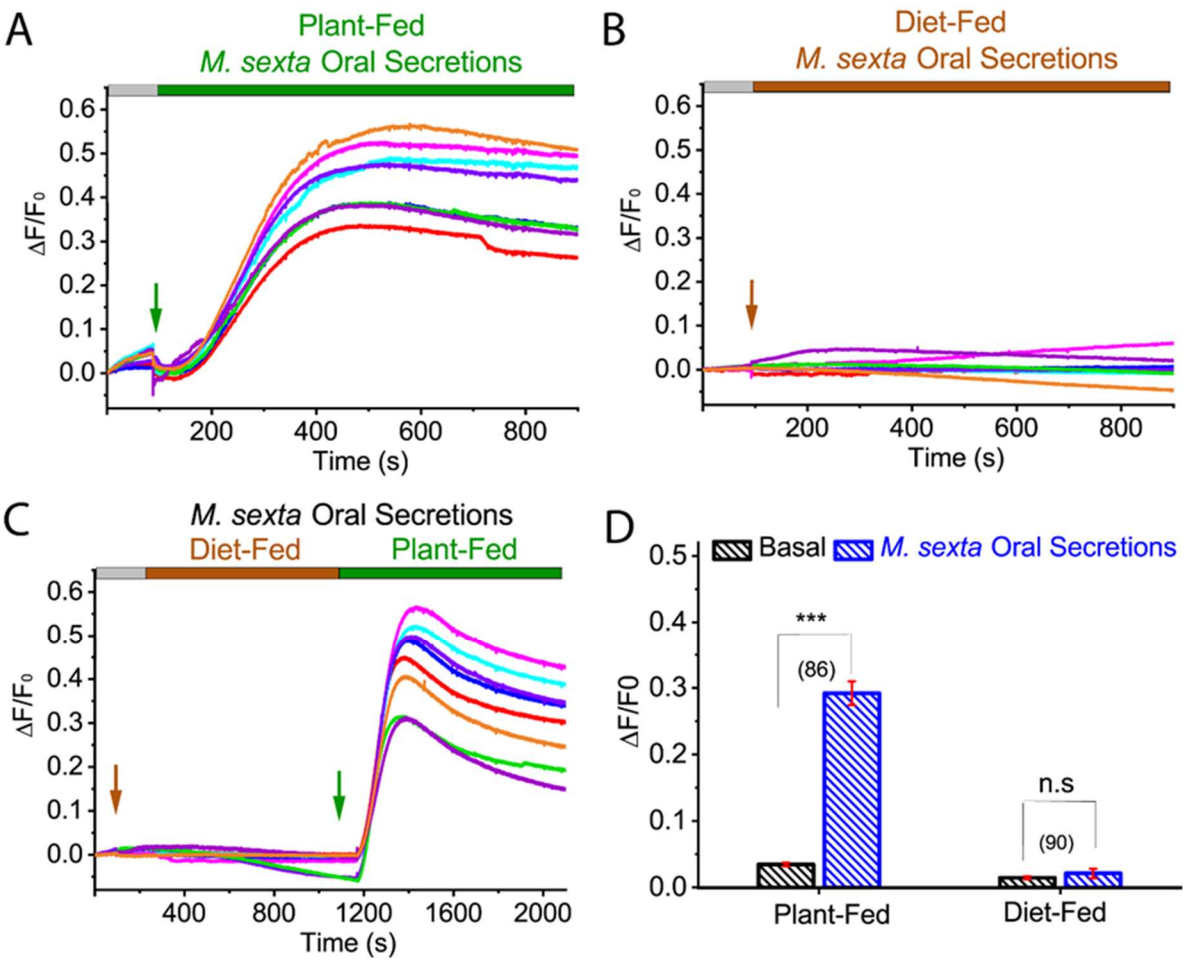

Figure 2: Effect of Plant-Fed *M. sexta* oral secretion on ROS elevation in tomato (*S. lycopersicum*) protoplasts. Representative ROS imaging of tomato protoplasts with the application of the tomato PF *M. sexta* OS (A) DF *M. sexta* OS (B) and combination of both (C). (D) Bar graph analysis of data shown in (A) &(B) depicting the maximum ROS generation after PF and DF *M. sexta* OS application. Statistical indicators reflect the non-parametric Mann-Whitney test, measuring for an effect of PF and DF *M. sexta* OS on ROS production: n.s, not significant; ***P < 0.0001. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in D.

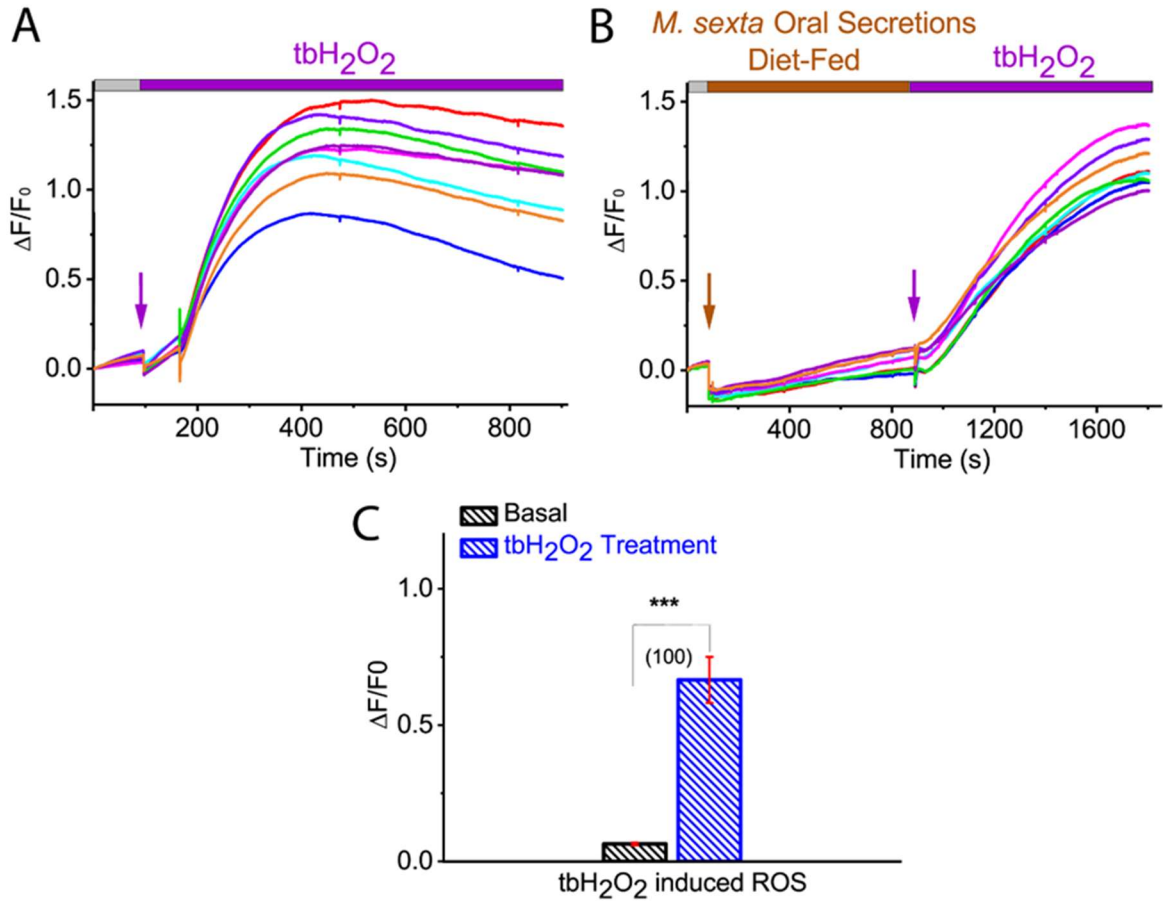

Figure. 3: Effect of membrane-permeable oxidant "tbH_2O_2" on ROS production in tomato (*S. lycopersicum*) protoplasts. Representative ROS imaging of isolated tomato protoplasts with the application of the membrane-permeable oxidant "tbH_2O_2" (A) and after the application of the DF *M. sexta* OS (B). (C) Bar graph analysis of data shown in (A) illustrating the maximum ROS generation after the tbH_2O_2 application. Statistical indicators reflect the non-parametric Mann-Whitney test, measuring for an effect of tbH_2O_2 on ROS production: ***$P < 0.0001$. Different color traces in the graph (A,B) reflect the OS-induced ROS response in individual protoplasts from a single replicate. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in C.

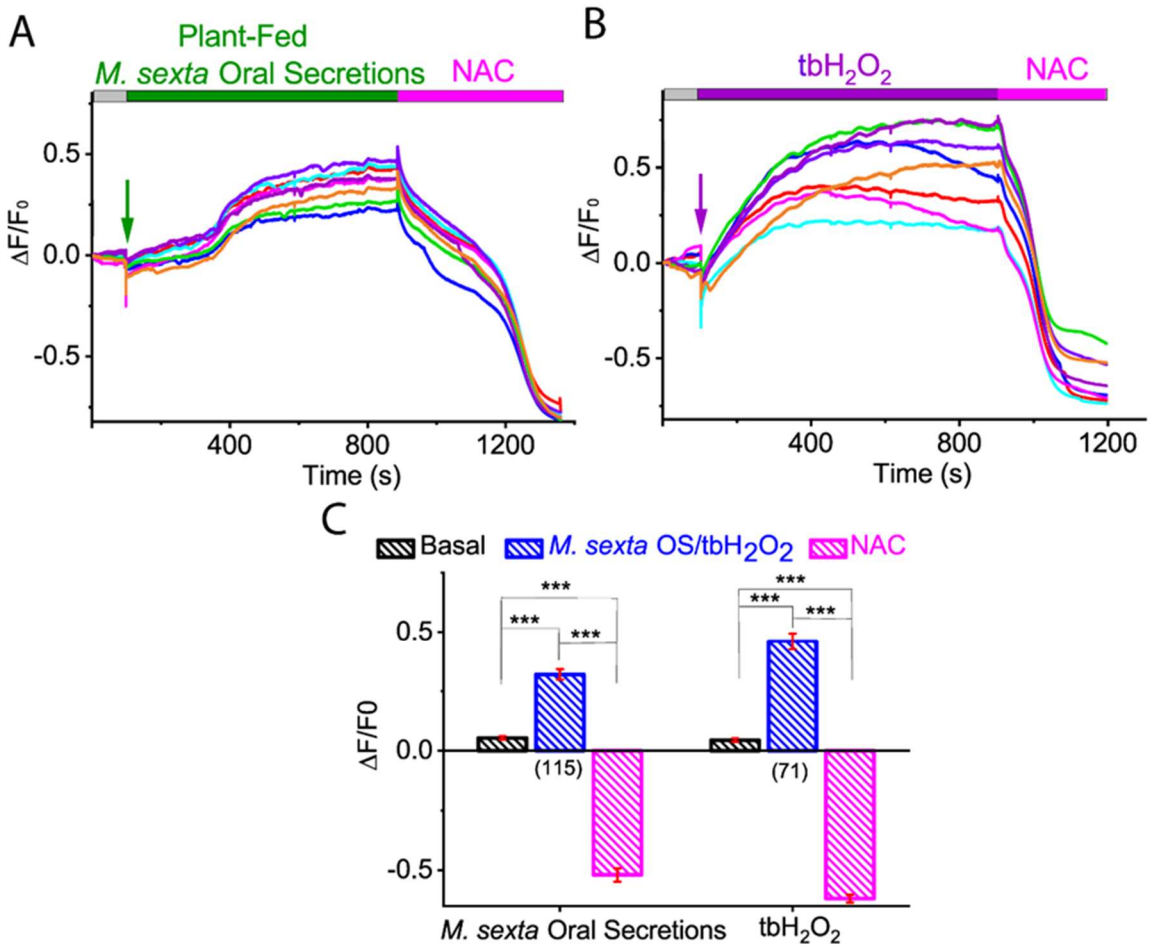

Figure 4: Effect of antioxidant NAC on *M. sexta* OS and oxidant tbH_2O_2 on ROS production in tomato (*S. lycopersicum*) protoplasts. Representative ROS imaging of isolated tomato protoplasts with the application of the PF *M. sexta* OS (A) and tbH_2O_2 (B), followed by the application of antioxidant NAC. (C) Bar graph analysis of data shown in (A & B) illustrating the maximum ROS generation after the PF *M. sexta* OS and tbH_2O_2 application and the minimum ROS level after NAC application. Statistical indicators reflect the non-Parametric Kruskal-Wallis test followed by Dunn's pairwise post hoc comparisons, testing for an effect of PF *M. sexta* OS, tbH_2O_2 and NAC on ROS level in the isolated protoplasts: ***$P < 0.0001$. Different color traces in the graph (A,B) reflect the OS-induced ROS response in individual protoplasts from a single replicate. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in C.

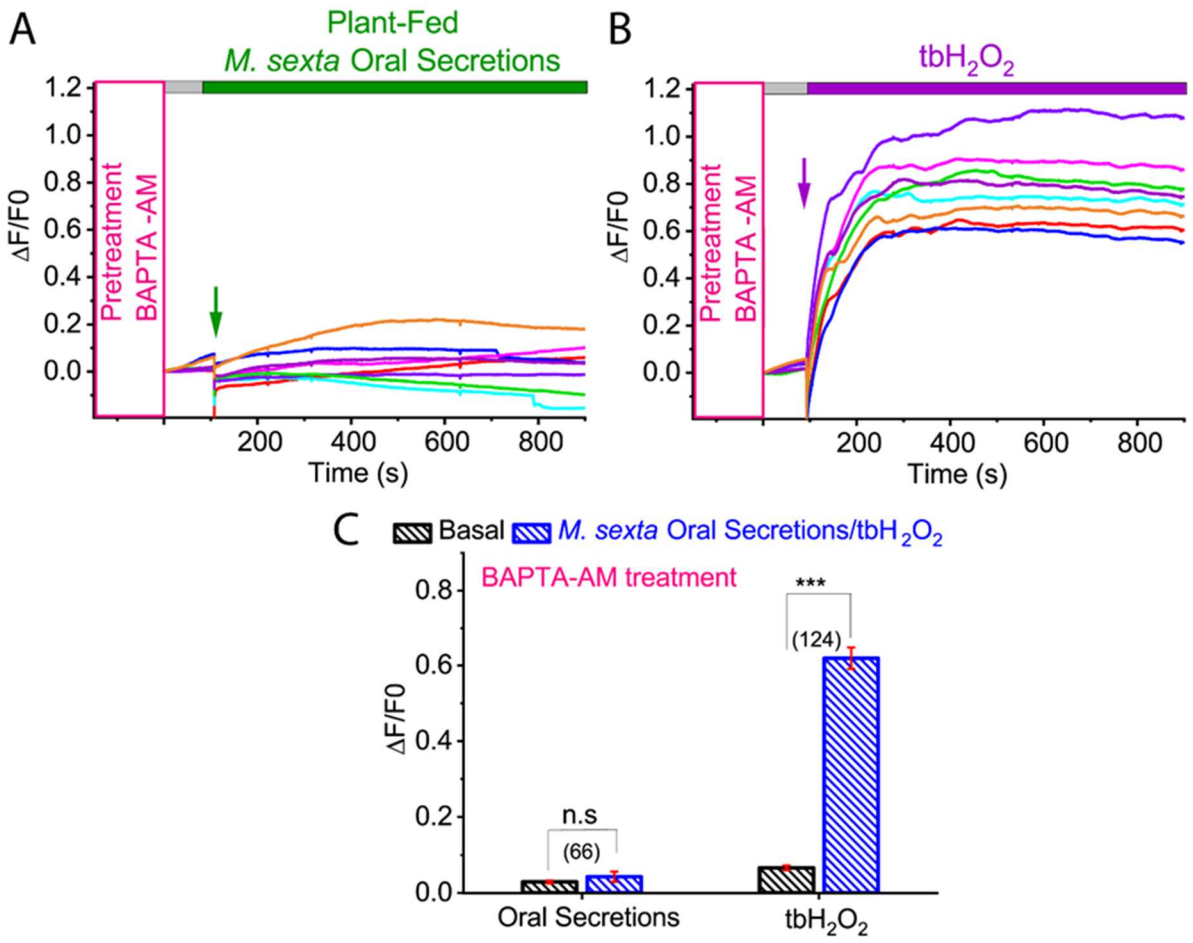

Figure 5: Effect of Ca^{2+} chelator BAPTA-AM on *M. sexta* OS and tbH_2O_2 on ROS generation in tomato (*S. lycopersicum*) protoplasts. Representative ROS imaging of isolated tomato protoplast in the presence of BAPTA, with the application of the PF *M. sexta* OS (A) and tbH_2O_2 (B). (C) Bar graph analysis of data shown in (A & B) illustrating the maximum ROS generation after the PF *M. sexta* OS and tbH_2O_2 application. Statistical indicators reflect the non-parametric Mann-Whitney test, measuring for an effect of PF *M. sexta* OS and tbH_2O_2 on ROS level in the BAPTA-AM preincubated isolated protoplasts: n.s, not significant; ***P < 0.0001. Different color traces in the graph (A,B) reflect the OS-induced ROS response in individual protoplasts from a single replicate. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in C.

CHAPTER IV

TOBACCO HORNWORM (*MANDUCA SEXTA*) HEMOLYMPH MODULATES REACTIVE OXYGEN SPECIES AND CALCIUM GENERATION IN TOMATO PROTOPLASTS

Abstract

Plants have been at war with herbivorous insects for millions of years and have developed a set of highly regulated defense strategies to sense herbivore attack using chemical cues known as herbivore-associated elicitors (HAEs), including oral secretions, ovipositional fluids, and frass. These HAEs induce a series of signaling cascades, which ultimately provide induced defenses against them. Despite the existing HAEs and their role in plant defense induction, our knowledge of other HAEs in plant-herbivore interactions are limited. In this study, we demonstrate that "hemolymph" from tobacco hornworm (*Manduca sexta*) also induce ROS and Ca^{2+} signaling cascade and thereby acts as an HAE. Using a dye-based imaging technique, our study showed that the application of crude *M. sexta* hemolymph potently increased reactive oxygen species (ROS) production in isolated tomato protoplasts. The addition of antioxidant NAC (N-acetyl-L-cysteine) antagonized hemolymph-induced ROS generation, indicating that *M. sexta* hemolymph is a ROS inducer in isolated protoplasts. Furthermore, incubating the protoplasts with a Calcium (Ca^{2+}) chelator, BAPTA-AM efficiently abolished the hemolymph-induced ROS production, suggesting possible crosstalk between Ca^{2+} and ROS signaling. Interestingly, the application of crude *M. sexta* hemolymph dramatically increased Ca^{2+} in tomato protoplasts.

Also, hemolymph-mediated ROS and Ca^{2+} increase was inhibited in the absence of extracellular Ca^{2+}. Taken together, our study demonstrates that "hemolymph" from *M. sexta* can directly modulate intracellular ROS and Ca^{2+} production and possibly regulate defenses against insect herbivores by acting as an HAE.

Introduction

Plants and insects have co-evolved together for millions of years. More than half of the insects are phytophagous that causes substantial yield losses; thus, adversely impacting agriculture (Oerke et al., 2006). Plants have developed defensive strategies to avoid or resist impending damage caused by these insects. These include physical defenses such as trichomes, spines, and chemical defenses such as volatile organic compounds to attract natural enemies or predators (De Moraes et al., 1998; Kariyat et al., 2012a, 2013, 2017, 2019; Turlings et al., 2018). These insect-specific signals can lead to physiological and biochemical changes in the wounded tissue through a series of interconnected signaling pathways (Green and Ryan, 1972; Reymond et al., 2004; Wu and Baldwin et al., 2009).

Plant defenses are initiated after the perception of an attack through different stimuli such as feeding, crawling, biting, gnawing, sucking, to name a few (Felton and Tumlinson, 2008; Peiffer et al., 2009; Hilfiker et al., 2014; Ray et al., 2015). Insects not only cause mechanical damage but also deposit insect saliva, regurgitant, or frass (Felton and Tumlinson, 2008; Ray et al., 2015). These herbivore specific molecules contain components that induce plant defense responses and are known as herbivore-associated elicitors (HAEs) (Bonaventure et al., 2011; Mohanta et al., 2012; Shinya et al., 2016). HAEs have been known to induce defense signals via intracellular factors such as Ca^{2+} and ROS (Maffei et al., 2004; Wu & Baldwin, 2009; Bonaventure, 2012).

Ca^{2+} remains a versatile signaling molecule that propagates immediately after HAEs encounters the plasma membrane. Ca^{2+} waves have been known to induce long-distance signaling upon herbivore attack (Gilroy et al., 2018; Shao et al., 2020). Previous studies have shown that Ca^{2+} can modulate ROS generation (Mazars et al., 2010; Gilroy et al., 2016). ROS can either act as a friend or foe depending upon their concentrations inside the cell (Camejo et al., 2016). Lower concentrations of ROS act as the second messenger, which contributes to plant defense signal transduction (Talaat, 2019). The initiation of the oxidative burst is often associated with Ca^{2+} propagation and membrane potential fluctuations (Zebelo and Mafei, 2012). Studies have shown that HAEs such as herbivore oral secretion (OS), ovipositional fluids, saliva play an essential role in inducing plant defenses (Alborn 1997; Musser et al., 2002; Felton and Tumlinson 2008; Schäfer et al. 2011; Tian et al., 2012; Louis et al., 2013). Our recent study showed that *Manduca sexta* (Lepidoptera; Sphingidae) OS induced a transient increase of ROS generation in tomato (*Solanum lycopersicum*) protoplasts after 140 s of exposure (Gandhi et al., 2020). A study by Fatouros et al. (2008) found an elicitor known as benzyl cyanide present in the ovipositional fluids of mated female, cabbageworm (*Pieris rapae*; Lepidoptera) induced a plant defense response after 72 hours of exposure to the wounded region. Fall armyworm (*Spodoptera frugiperda;* Lepidoptera) frass has also been shown to induce the expression of Maize protease inhibitor, a wound response protein after herbivory on maize plants (Ray et al., 2015). Bittner et al. (2017) documented the ROS generation upon egg deposition on pine (*Pinus Sylvestris*) by the herbivorous sawfly (*Diprion pini*; Hymenoptera). Histochemical staining of the pine needles indicated the presence of hydrogen peroxide in the tissue that was close to 1- and 3-day-old sawfly eggs. However, the mechanistic link between HAEs, ROS, Ca^{2+} and, ultimately, defense gene expression needs more investigation.

Despite the existing HAEs and their role in plant defense induction, our knowledge of other herbivore generated HAEs in plant-herbivore interactions is still limited. Hemolymph is the extracellular fluid or blood of insects. It has a high concentration of amino acids up to 200 mM, water, which makes 20-50% of its volume and contains a high concentration of potassium, magnesium, and organic anions low concentration of sodium and chloride. Hemolymph contains several pigments such as β-carotene, riboflavin, biliverdin, hemocyanin and cells called hemocytes. Its pH varies from 6.4-6.8 for most insects (Kanost 2009). , The movement of insects on the leaves, is hampered by the presence of trichomes and which can lead to the release of hemolymph. We speculate that the hemolymph might act as a cue to initiate plant signaling cascade. This study investigates the effect of *M. sexta* hemolymph on Ca^{2+} and ROS generation in isolated protoplasts of host plants such as tomato (*S. lycopersicum*) and silverleaf nightshade (*Solanum elaeagnifolium*) and non-host plants such as sorghum (*Sorghum bicolor*) and wild gourd (*Cucurbita pepo spp. texana*). We used a ROS sensing dye "CM-H_2DCFDA" and Ca^{2+} sensing dye "Oregon Green® 488 BAPTA-AM" based imaging approach on isolated protoplasts. Our results showed a drastic transient increase in intracellular ROS and Ca^{2+} production upon application of hemolymph and extracellular Ca^{2+} is relevant in the initiation of hemolymph mediated ROS and Ca^{2+} signaling. Collectively, our study provided evidence that the *M. sexta* hemolymph is a newly discovered HAE, possibly capable of inducing intracellular defense signaling.

Materials and Methods

Plant Material

F1 tomato hybrid seeds (*Solanum lycopersicum*; Variety Valley Girl) were purchased from Johnny's Selected Seeds, Maine, USA and were grown in Sunshine professional growing mix (Sun Gro Horticulture Canada Ltd., MA, USA) in plastic trays (51.435 × 25.4 cm). The seedlings were transplanted into bigger pots (15 cm diameter) after 2- 4 leaf stage and kept in a growth chamber at 25˚C and 65% Relative Humidity. Diluted Organic fish emulsion fertilizer (NPK 5:1:1, Alaska Fish Fertilizer, Pennington Seed, Inc., Madison, GA, USA) was applied twice a week to meet the nutrient needs of the plant. Seeds of silverleaf nightshade (*Solanum elaeagnifolium*) were collected from various native populations in the lower Rio Grande Valley, Texas, USA. Wild gourd (*Cucurbita pepo* spp. *texana*) seeds were obtained from a lab population propagated at The Pennsylvania State University, USA. Seeds of sorghum (*Sorghum bicolor*) (Super Sugar Sudex variety, Gayland ward Seed, USA) and other plants were sown in plastic trays (51.435 × 25.4 cm) with Sunshine professional growing mix. The trays were kept in an incubator (Sheldon Manufacturing, INC.) maintained at 25°C with a photoperiod of 16h day/ 8 h night cycle. The plants were watered regularly and maintained in a growth chamber free of insects.

Hemolymph collection

M. sexta larvae were reared on a wheat-germ based artificial diet (Carolina Biological, Burlington, North Carolina, USA). Hemolymph was collected from the 4th instar larvae by making an incision just below its last proleg. The released hemolymph was collected in eppendorf tubes and immediately stored in -80 ºC until further use.

Protoplast Isolation

Protoplasts were isolated from 4 different plant species by the modified protocol of Zhai et al. (2009) and Nanjareddy et al. (2016). Protoplasts were isolated from 2-5 weeks old young terminal leaves of tomato, wild gourd, sorghum, and silverleaf nightshade plants. 0.5 grams of the leaves were cut with fresh razor blades in 4 ml of the TVL solution (0.3 M Sorbitol and 50 mm $CaCl_2$). The sliced leaves were transferred into a beaker containing 10 ml of enzyme solution that was preheated at 55º C. Vacuum was applied for infiltration of the enzyme solution (0.5 M sucrose, 10 mM MES-KOH [pH 5.7], 20 mM $CaCl_2$, 40 mM KCl, 0.9% macerozyme and 1.5 % cellulase) for 10-15 minutes followed by incubation of the plant tissues at 35 rpm for 12-14 hours. The digested mixture was filtered through a cheese cloth pre-wet in W5 solution (0.1% (w/v) glucose, 0.08% (w/v) KCl, 0.9% (w/v) NaCl, 1.84% (w/v) $CaCl_2$, 2 mM MES-KOH pH 5.7) and the filtrate was centrifuged at 100 g for 7 minutes. The supernatant was removed, and the pellet was dissolved in 1 ml of W5 solution.

ROS Measurements

Intracellular ROS levels in isolated protoplasts upon application of hemolymph were measured as per the methods described by Gandhi et al. (2020). Briefly, ROS levels were measured by loading the protoplasts with a 2 µM ROS-imaging dye, CM-H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) (Fisher Scientific, USA) for one hour. Fluorescent intensity of dye-loaded protoplasts was quantified by using Olympus IX 71 inverted microscope attached with PTI easy-ratio pro system (Photon Technology International, Inc. New Jersey, USA) and SCMOS camera. Crude hemolymph, the extracellular blood from *M. sexta* was applied after 100 seconds of ROS imaging. A change in fluorescence of protoplasts were

recorded with Easy Ratio Pro software from Horiba with an excitation wavelength at 494 nm and emission wavelength at 520 nm. *M. sexta* hemolymph (crude) and other chemicals such as NAC (1 mM) were dropped into the protoplast sample during the live measurements to test their effect on the intracellular ROS generation. Isolated protoplasts were preincubated with 1.5 μM of BAPTA-AM (1,2-bis(o-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid) (Invitrogen™ Molecular Probes™) for 1 hour prior to the ROS measurement in order to test the effect of blocking Ca^{2+} on ROS generation.

Ca^{2+} Measurement

Cytosolic Ca^{2+} concentration was measured as previously described by Granados et al. (1997) where Ca^{2+} sensing dye Fluo-3/AM was used to measure the effect of pectic elicitor on Ca^{2+} signaling in bean leaf (*Phaseolus vulgaris*) protoplasts. Here we used a potent Ca^{2+} binding dye, Oregon green 488 BAPTA-AM (AAT Bioquest, USA), with a modified protocol. Briefly, protoplasts were incubated with Oregon Green 488 dye (2μM) for one hour in the dark at room temperature. A small drop of dye-loaded protoplasts was placed on the stage of Olympus IX 71 inverted microscope attached with PTI easy-ratio pro system (Photon Technology International, Inc. New Jersey, USA) and SCMOS camera. Ca^{2+} measurements were performed as per manufacturer's instructions. Changes in the Ca^{2+} fluorescence intensity were observed upon the application of crude hemolymph.

For zero Ca^{2+} experiments, protoplasts were dissolved in EGTA (10 mM) (Ethylene Glycol-bis (beta-aminoethyl ether) -N, N, N′, N′-Tetra acetic Acid) containing Ca^{2+} free W5 solution. Ca^{2+} and ROS imaging were performed with the above described protocol.

Data Analysis and Presentation

Analysis of ROS and Ca^{2+} imaging data was done by using EasyRatioPro (Horiba Scientific software). Further analyses were performed with Excel (Microsoft, Redmond, WA, USA) and Igor Pro v8.0 (Wavemetrics, Lake Oswego, OR, USA). Origin Pro v2020 (Originlab, Northampton, MA, USA) and Adobe Illustrator v24.1 (Adobe, San Jose, CA, USA) were used to prepare the figures. Averaged data are presented as means± SEM (N = number of protoplasts from 3-5 independent measurements). To compare two groups such as basal ROS levels and ROS levels from hemolymph application, we used the non-parametric Mann-Whitney U test. Non-parametric tests were used since data failed to meet normality assumptions after transformations.

Results

M. sexta hemolymph induced ROS generation in tomato and silverleaf nightshade protoplasts

M. sexta has coevolved with the Solanaceae family, which can potentially recognize the *M. sexta* derived secretions leading to plant defense signaling. Our previous study showed that *M. sexta* oral secretions induced ROS generation in tomato protoplasts. To evaluate whether hemolymph could induce a similar response, we isolated protoplasts from host plants such as tomato and silverleaf nightshade. As shown in fig. 1A, B, E the application of hemolymph produced a transient, increase in ROS production in isolated tomato protoplasts (basal: 0.015±0.001; hemolymph:0.191±0.015, $P<0.0001$; non-parametric Mann-Whitney test) (Fig. 1A, B E; N=86) and silver leaf protoplast (basal: 0.021±0.003; hemolymph: 0.093±0.007, $P<0.0001$; non-parametric Mann-Whitney test) (Fig. 1C, D, E; N=117). However, the intensity of ROS

generation in silverleaf was lower in comparison to tomato. These results suggest that *M. sexta* hemolymph is capable of inducing ROS in both tomato and silver leaf protoplasts.

M. sexta hemolymph induced ROS generation in wild gourd protoplasts but not in sorghum protoplasts

To unravel whether the non-host plant would initiate a similar ROS response, we isolated protoplasts from wild gourd and sorghum. Interestingly, we found that there was a significant increase in ROS production in wild gourd plants (basal: 0.089±0.016; hemolymph:0.216±0.027, P=0.0078; non-parametric Mann-Whitney test) (Fig. 2A,B,E; N=85) while *M. sexta* hemolymph failed to induce ROS generation in protoplasts isolated from sorghum plants (basal: 0.025±0.003; hemolymph:0.046±0.010, P=0.1222; non-parametric Mann-Whitney test) (Fig. 2C,D,E; N=69). These results indicate a differential sensitivity of *M. sexta* hemolymph-mediated ROS production in non-host plants. Although *M.* sexta does not feed on wild gourd, our data shows that the plants can perceive the elicitors present in its hemolymph.

M. sexta hemolymph-mediated ROS generation was abolished in the presence of antioxidant, NAC

To further confirm the observation that *M. sexta* hemolymph can lead to ROS generation, we used a membrane-permeable antioxidant, NAC, the *N*-acetyl derivative of the natural amino acid L- cysteine. Our result showed that *M. sexta* hemolymph mediated ROS generation was inhibited in NAC preincubated tomato protoplasts (basal: 0.022±0.004;hemolymph:0.034±0.009, P=0.1505; non-parametric Mann-Whitney test) (Fig. 3A,C; N=121). These results suggest that hemolymph is ROS inducer in tomato protoplasts and NAC can quench the hemolymph-mediated ROS.

M. sexta hemolymph-mediated ROS generation was suppressed in the presence of Ca^{2+} chelator, BAPTA-AM

Previous reports have shown that insect feeding triggers the increase in cytosolic Ca^{2+} levels and this Ca^{2+} leads to ROS generation (Maffei et al., 2007). To examine this crosstalk, we used BAPTA-AM, a known membrane-permeable Ca^{2+} chelator. We found that the hemolymph-mediated ROS production was abolished in BAPTA-AM preincubated tomato protoplasts. (basal: 0.032±0.003; hemolymph:0.049±0.009, P=0.2912; non-parametric Mann-Whitney test) (Fig. 3B, C; N=107). This finding suggests that Ca^{2+} is required for hemolymph-mediated ROS generation as chelating the Ca^{2+} with BAPTA-AM effectively diminished the increase in ROS levels.

M. sexta hemolymph induced Ca^{2+} elevation in tomato and sorghum protoplasts

Based on the result shown in Fig. 3B, C, Ca^{2+} plays a role in hemolymph-mediated ROS generation; we hypothesized that hemolymph could influence intracellular Ca^{2+} in isolated protoplasts. To test this hypothesis, we performed Ca^{2+} imaging on the protoplasts and tested the effect of hemolymph on intracellular Ca^{2+} levels. As shown in Fig. 4, the application of crude hemolymph to oregon green 488 BAPTA-AM dye incubated tomato protoplasts showed a rapid Ca^{2+} spike (basal: 0.021±0.003; hemolymph:0.916±0.061, P<0.0001; non-parametric Mann-Whitney test) (Fig. 4A,C; N=133). Based on our earlier observation, hemolymph was able to induce ROS signals in the wild gourd. However, the kinetics of this signal was different than that of tomato. To test whether hemolymph could induce Ca^{2+} in sorghum, we performed Ca^{2+} imaging on isolated sorghum protoplasts. Our result shows that *M. sexta* hemolymph induced a transient Ca^{2+} elevation (basal: 0.25±0.036; hemolymph:1.308±0.039, P<0.0001; non-parametric

Mann-Whitney test) (Fig. 4B,C; N=91). These results suggest that the *M. sexta* hemolymph is a Ca^{2+} inducer in protoplasts isolated from the host and non-host plants.

Extracellular Ca^{2+} is essential for inducing hemolymph-mediated ROS and Ca^{2+} generation in tomato protoplasts

In order to investigate the involvement of extracellular Ca^{2+} in eliciting hemolymph-mediated Ca^{2+} and ROS increase in the protoplasts, we used Ca^{2+} free-EGTA extracellular solution. Interestingly, hemolymph-mediated increase in Ca^{2+} was diminished in the tomato protoplasts (basal: 0.086±0.010; hemolymph:0.093±0.006, P= 0.0811; non-parametric Mann-Whitney test) (Fig. 5A, B; N=133). Furthermore, we found that hemolymph-mediated ROS production was also inhibited in the tomato protoplasts (basal: 0.072±0.007; hemolymph:0.075±0.008, P=0.3295 non-parametric Mann-Whitney test) (Fig. 5C,5D; N=87). This finding suggests that extracellular Ca^{2+} is relevant for the hemolymph-mediated increase in cytosolic Ca^{2+} and ROS production in isolated tomato protoplasts.

Antioxidant NAC abolished the hemolymph mediated Ca^{2+} increase in tomato protoplasts

As shown in Fig. 3, antioxidant NAC inhibited the hemolymph-induced ROS generation. To test whether Ca^{2+} will be generated in the absence of ROS, we performed Ca^{2+} imaging on NAC preincubated tomato protoplasts. Interestingly, our result showed that the *M. sexta* mediated Ca^{2+} increase was abolished in NAC treated tomato protoplasts (basal: 0.007±0.0009; hemolymph:0.008±0.001, P= 0.1995; non-parametric Mann-Whitney test) (Fig. 6A, B; N=128). These results indicate a feedback loop by which ROS modulates Ca^{2+} generation in the tomato protoplasts and validates our earlier observation (Fig. 3B) of the crosstalk between Ca^{2+} and ROS.

Discussion

Our study has shown for the first time that hemolymph is an HAE and is capable of inducing ROS and Ca^{2+} in isolated tomato protoplasts. The ability of the plant to combat insect herbivores relies upon successful recognition of factors present in insect-derived chemicals such as OS, ovipositional fluids, frass etc. When insects feed on plants, they come across trichomes, which act as a barrier, deterring their movement. Non-glandular trichomes contain sharp spikes and are sometime silicified; thus, hampering the herbivore feeding (Lanning and Eleuterius, 1985). A previous study has documented that the ingestion of stellate trichomes by *M. sexta* caterpillars can damage their peritrophic membrane that covers the gut epithelium (Kariyat et al., 2017). This can lead to the release of hemolymph. It is possible that the secreted hemolymph might act as a cue for the plants initiating the signaling cascade and augments plant defense response. Elicitor recognition is followed by activation of signaling cascade involving molecules such as ROS and Ca^{2+} which are a hallmark of plant defense responses. Although considerable research focusing on the role of ROS as critical components in plant defense signal transduction has started to emerge, but our understanding of these pathways is still limited. Real-time measurement of ROS has been possible using fluorescent probes that are highly sensitive and easy to load into the plant cells (Swanson et al., 2011). In this study, we showed that *M. sexta* hemolymph elicited a transient increase in ROS levels in protoplasts of different plant species. This finding is in line with our previous study, where we observed an increase in ROS production upon *M. sexta* OS application (Gandhi et al., 2020), suggesting that both the *M. sexta*-derived secretions act as HAE and induce plant signaling cascade.

Insects vary in their feeding behaviors as well as in the extent of specialization to their host plant (Bandoly et al., 2016). On that basis, herbivores can be divided into generalists and specialists. Generalist herbivores can feed on a wide range of host plants, whereas specialists have a restricted host range and can feed on a single genus. Defense responses to HAEs can differ among specific plant-insect associations (Bonaventure et al., 2011). *M. sexta* is specialized in solanaceous plant species and some solanaceous weeds such as silverleaf nightshade (*Solanum elaeagnifolium*), groundcherry (*Physalis* spp.) and horsnettle (*Solanum carolinense*) are also its preferred hosts (Capinera et al., 2001). Our results showed that hemolymph induced ROS in protoplasts isolated from host plants, (tomato and silverleaf). However, when the *M. sexta* hemolymph was applied on the protoplasts isolated from non-host plants (wild gourd and sorghum), the increase in ROS levels was observed only in the wild gourd protoplasts, but not in sorghum protoplasts. It can be also be assumed that the ROS response is potentially specific to dicots i.e. tomato and wild gourd but cannot be observed in sorghum, which is a monocot species. However, the effect of *M. sexta* hemolymph on ROS generation needs to be investigated in other non-host plants species as well.

Ca^{2+} represents one of the major intracellular ions that plays an important role in biotic and abiotic stress responses (Hetherington and Brownlee, 2004; Pandey et al., 2004; Dodd et al., 2010; Yuan et al., 2014). The concentration of Ca^{2+} in the cytosol under resting conditions is very low (0.0001 mM), whereas it is high in the apoplast or the organelles such as vacuole, mitochondria, and ER (1mM). It is now well established that insect feeding triggers the increase in cytosolic Ca^{2+} levels through the presence of various ion channels (Maffei et al., 2007; Toyota et al., 2018; Shao et al., 2020) and it is one of the early events in plant signaling cascade that occurs upon herbivore feeding. HAEs have been known to affect intracellular Ca^{2+} level in the

herbivore damaged plants. Our study showed an increase in Ca^{2+} levels in tomato and sorghum protoplasts upon the application of *M. sexta* hemolymph. This observation is in line with the previous studies on the role of HAEs in the induction of Ca^{2+} propagation. However, this hemolymph- mediated Ca^{2+} change in sorghum could not translate into ROS signal, possibly because of evolutionary divergence. A study by Maffei et al. (2004) examined the changes in Ca^{2+} levels in lima bean leaves upon insect herbivory by *Spodoptera littoralis*. Another study by Zebelo et al. (2012) also showed Ca^{2+} and ROS production upon herbivory and OS obtained from untreated *S. littoralis* and *S. littoralis* without the ventral eversible gland (VEG).

Previous studies have reported the interrelationship between ROS and Ca^{2+} generation when the plants encounter biotic and abiotic stress conditions (Pei et al., 2000; Sagi and Fluhr, 2001; Sanders et al., 2002; Yang and Poovaiah, 2002; Hancock et al., 2002; Foreman et al., 2003; Maffei et al., 2006). Our results showed that BAPTA-AM suppressed the hemolymph-mediated ROS increase in tomato protoplasts. This evidence confirms that intracellular Ca^{2+} is necessary for the ROS production. A similar observation was reported in our previous study where ROS generation was attenuated upon application of *M. sexta* OS in BAPTA-AM preincubated tomato protoplasts. This observation suggests that there exists a mechanistic link between ROS and Ca^{2+} that underlie the signaling responses in plants upon insect attack. Hemolymph-mediated Ca^{2+} release may affect the mitochondria, which is the source of ROS production, and blocking Ca^{2+} by BAPTA-AM might inhibit mitochondrial activity and thus suppressing ROS production (Gandhi et al., 2020).

Previous work showed that the treatment of parsley cells (*Petroselinum crispum*) with the oligopeptide elicitor, Pep-13 derived from fungus, *Phytophtora sojae* triggered an elevation in

cytoplasmic free Ca^{2+} as well as oxidative burst. However, removal of extracellular Ca^{2+} by the chelating agent, BAPTA inhibited the Pep-13 mediated Ca^{2+} as well as ROS increase. This indicates that Ca^{2+} as well as ROS generation, are dependent on external Ca^{2+} (Blume et al., 2000). Our findings corroborate previous observation as we also found that the removal of the extracellular Ca^{2+} in the solution prevented the Ca^{2+} increase in tomato protoplasts upon *M. sexta* hemolymph treatment. This can be potentially due to the blockage of the entry of Ca^{2+} from the apoplast to the cytosol through the plasma membrane Ca^{2+} channels, which further inhibits release of Ca^{2+} from intracellular stores and suppresses both intracellular Ca^{2+} and ROS production.

To verify whether hemolymph-mediated Ca^{2+} production is dependent on intracellular ROS, we used NAC, a membrane-permeable antioxidant (Joo et al., 2001). Our results showed that NAC treatment inhibited the hemolymph- induced Ca^{2+} generation. From this observation, we could speculate that ROS might lead to Ca^{2+} release via ROS-activated Ca^{2+} channels, indicating that Ca^{2+} and ROS signaling involves a cellular feedback loop with ROS amplifying the Ca^{2+} signal and vice-versa. A previous study by Demidchik et al. (2006) have reported hyperpolarization- activated Ca^{2+} conductance upon application of 10 mM H_2O_2 in the elongation zone of epidermal protoplasts. Another study by Takeda et al. (2008) showed that ROS produced by RHD 2 NADPH oxidase activated hyperpolarization- activated Ca^{2+} channels and caused the influx of Ca^{2+} into the cells in *Arabidopsis thaliana.* Interestingly, Ca^{2+} also activated the oxidases that lead to ROS generation suggesting that there exists a positive feedback loop between ROS and Ca^{2+}.

Figures

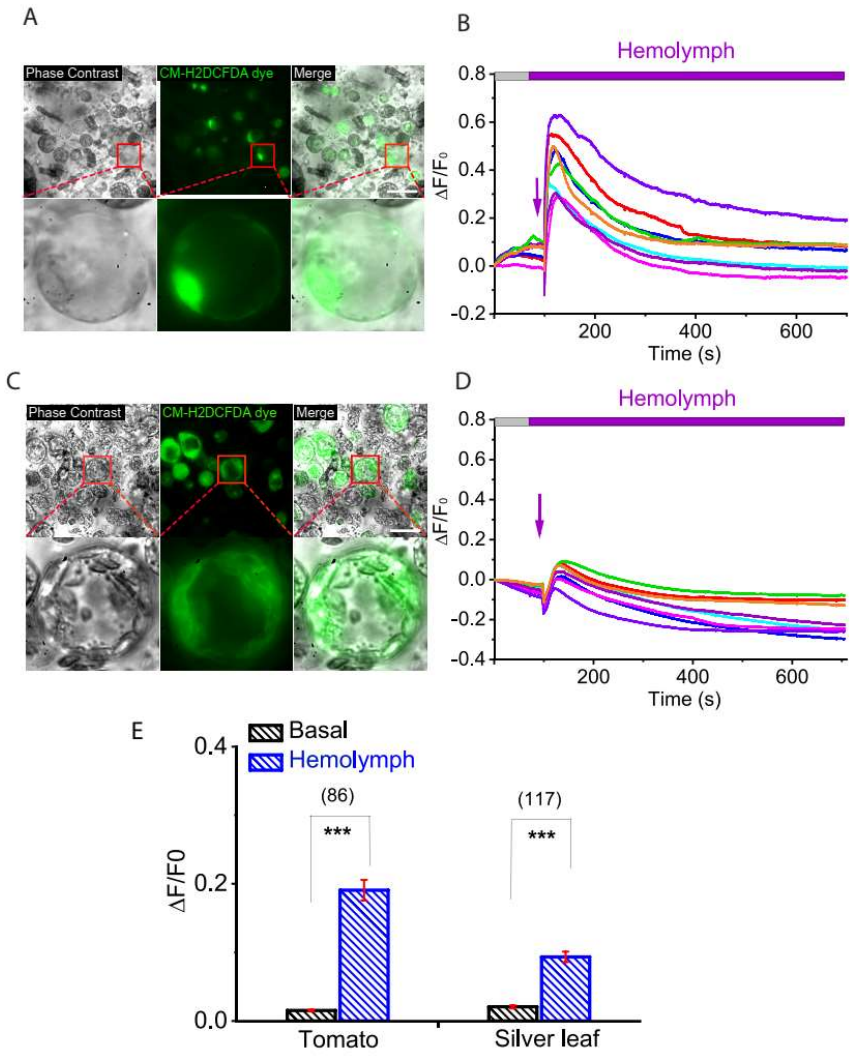

Figure 1- Effect of *M. sexta* hemolymph on ROS generation in tomato (*Solanum lycopersicum*) and silverleaf nightshade protoplasts (*Solanum elaeagnifolium*). (A) Representative phase-contrast image of tomato protoplasts at 100x magnification. Isolated protoplasts were loaded with ROS-sensing dye, CM-H_2DCFDA (middle). (B) Representative ROS imaging of tomato protoplast upon application of crude *M. sexta* hemolymph. (C) Representative phase-contrast and CM-H_2DCFDA dye loaded silverleaf protoplasts at 100x magnification. (D) Representative ROS imaging of silverleaf protoplasts upon application of *M. sexta* hemolymph. (E) Bar graph analysis of data shown in (B) and (D) illustrating the maximum ROS generation after *M. sexta* hemolymph application. The number of protoplasts (N) from 3-5 independent measurements is depicted in parentheses in E.

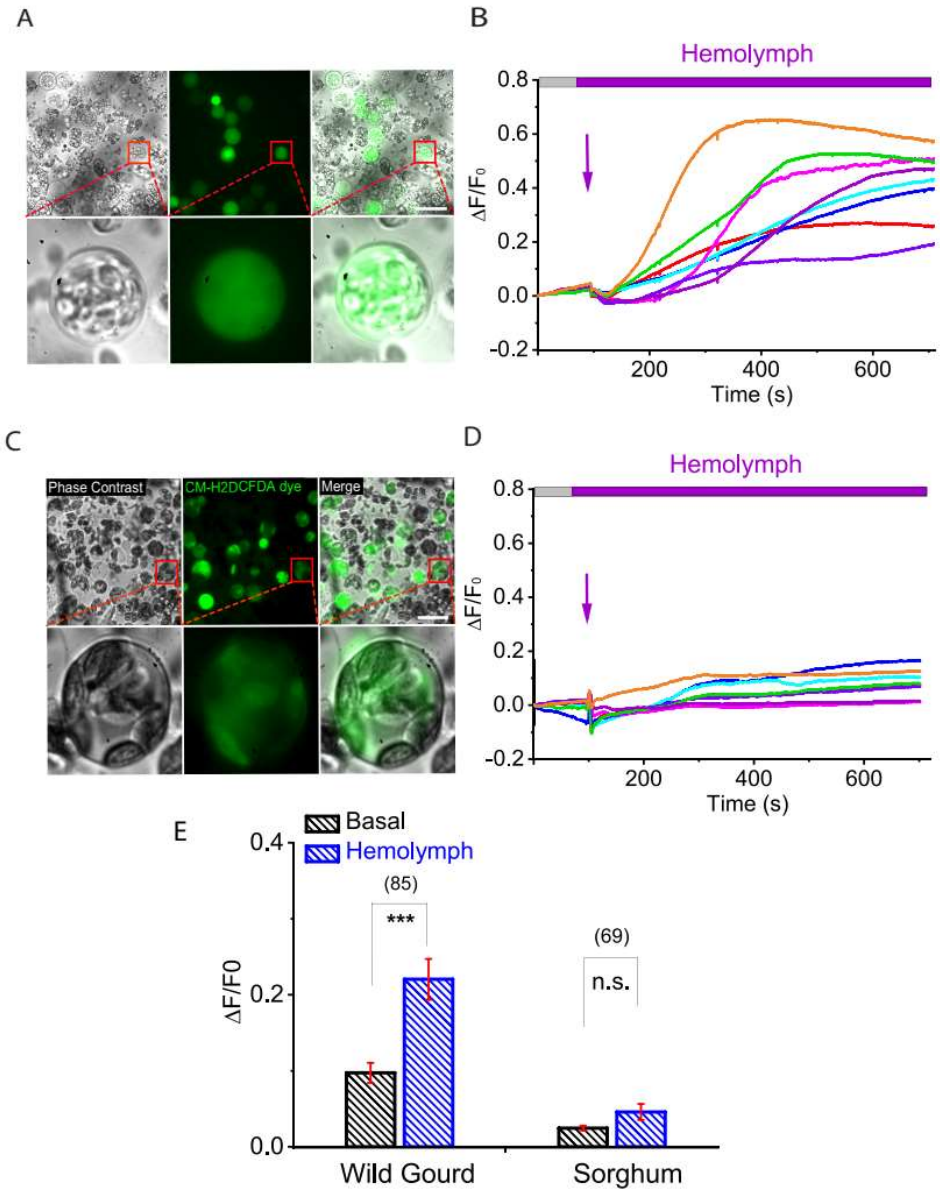

Figure 2- Effect of *M. sexta* hemolymph on ROS elevation in protoplasts isolated from wild gourd (*Curcubita pepo spp. texana*) and sorghum (*Sorghum bicolor*). (A) Representative Phase contrast image of wild gourd protoplasts at 100x magnification. Isolated protoplasts are loaded with ROS-sensing dye, CM-H_2DCFDA (middle) (B) Representative ROS imaging of wild gourd protoplasts upon application of *M. sexta* hemolymph. (C) Representative Phase contrast image of sorghum protoplasts at 100x magnification. (D) Representative ROS imaging of sorghum protoplasts upon application of *M. sexta* hemolymph. (E) Bar graph analysis of data shown in (B) &(D) depicting the maximum ROS generation upon *M. sexta* hemolymph application. The number of protoplasts (N) from 3-5 independent measurements is depicted in parentheses in E.

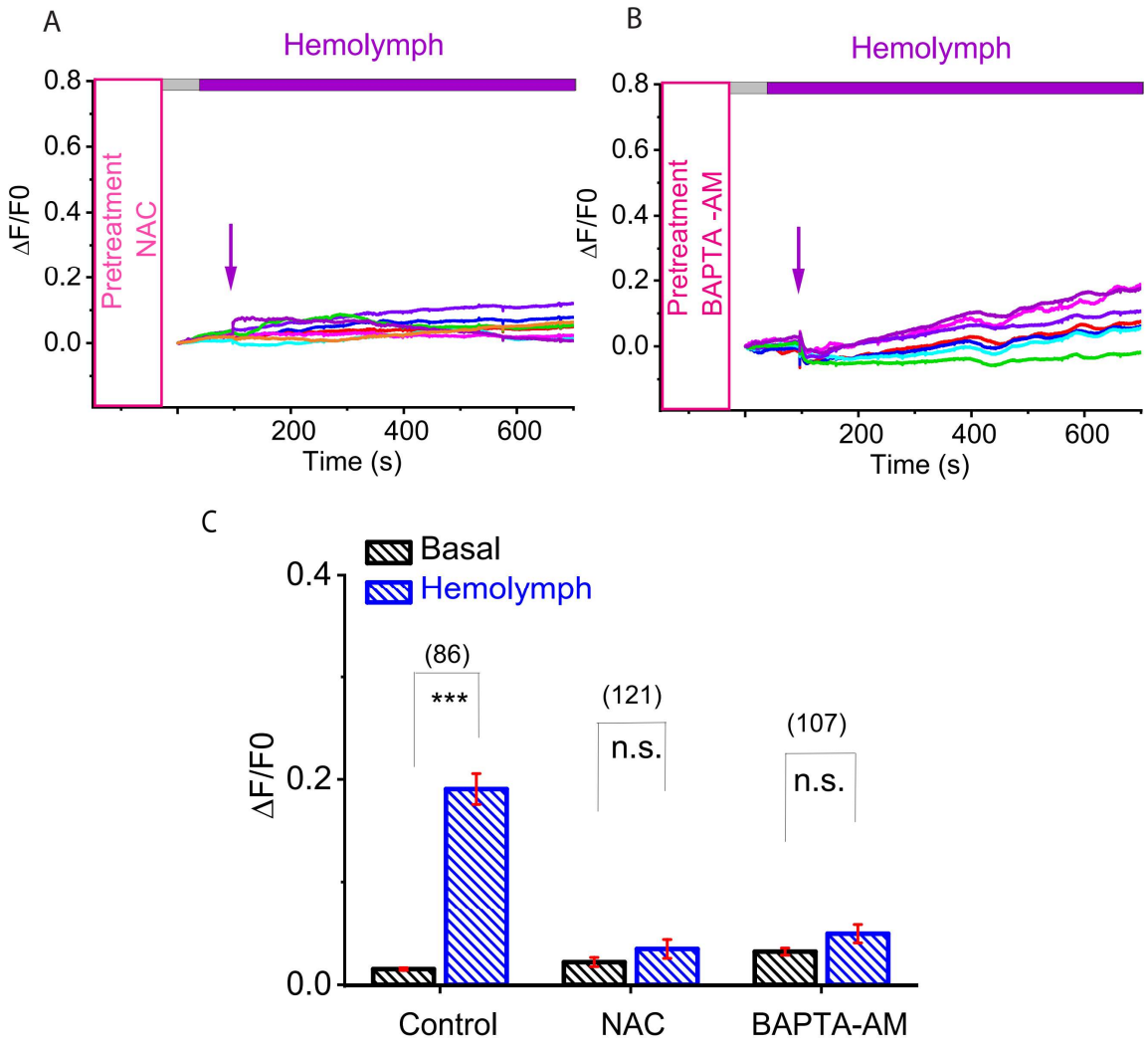

Figure 3: Effect of antioxidant NAC and Ca^{2+} chelator BAPTA-AM on hemolymph-mediated ROS generation in tomato (*S. lycoperscium)* protoplasts. (A) Representative ROS imaging of tomato protoplasts preincubated with (A) NAC (B) BAPTA-AM, with the application of the *M. sexta* hemolymph. (C) The bar graph analysis of data from Fig. 1B, E and Fig. 3A, B) illustrates the maximum ROS generation after applying *M. sexta* hemolymph. The number of protoplasts (N) from 3-5 independent measurements is provided in parentheses in C. Control was the tomato protoplasts without preincubation with NAC and BAPTA-AM. The number of protoplasts (N) from 3-5 independent measurements is depicted in parentheses in C.

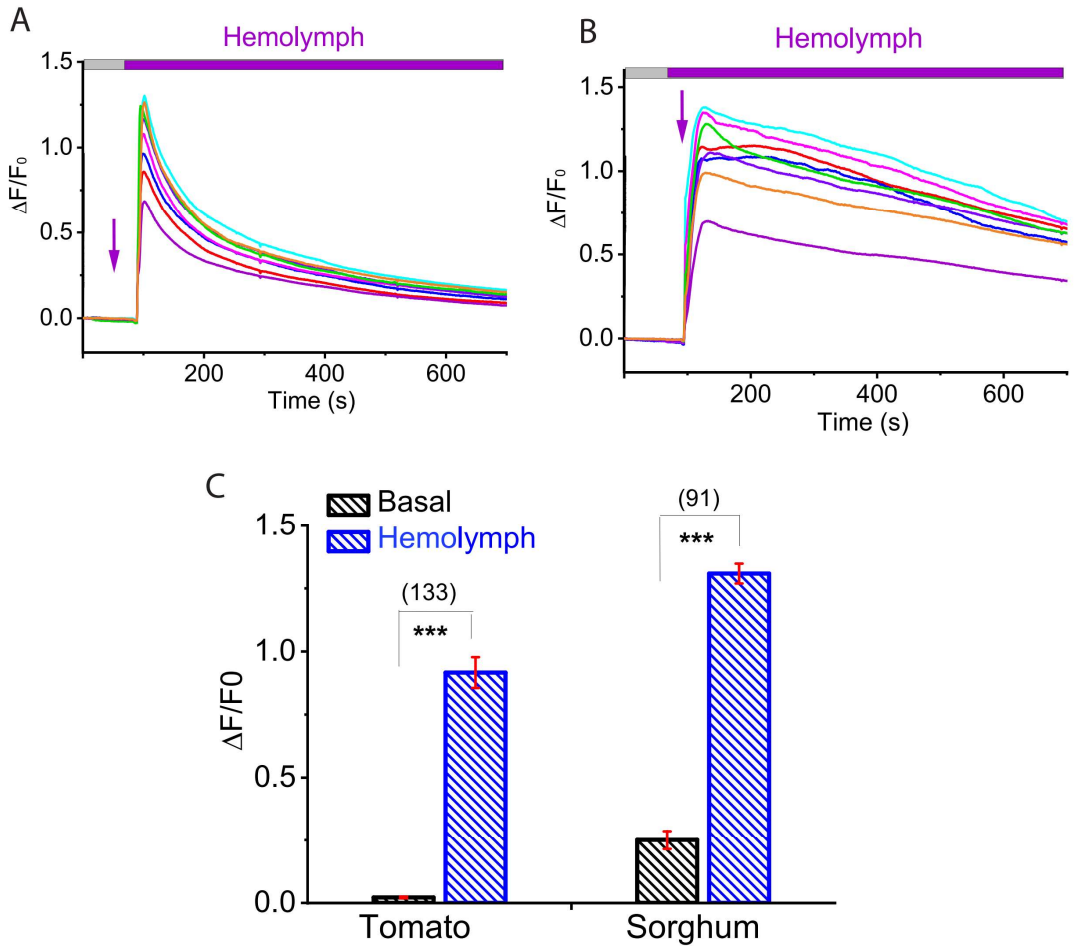

Figure 4: Effect of *M. sexta* hemolymph on Ca^{2+} elevation in tomato (*S. lycoperscium*) and sorghum (S. *bicolor*) protoplasts. (A**).** Representative Ca^{2+} imaging of tomato protoplasts upon application of *M. sexta* hemolymph. (B) Representative Ca^{2+} imaging of sorghum protoplasts upon application of *M. sexta* hemolymph (C) Bar graph analysis of data shown in (A) and (B) depicting the maximum Ca^{2+} generation after *M. sexta* hemolymph application. The number of protoplasts (N) from 3-5 independent measurements is depicted in parentheses in E.

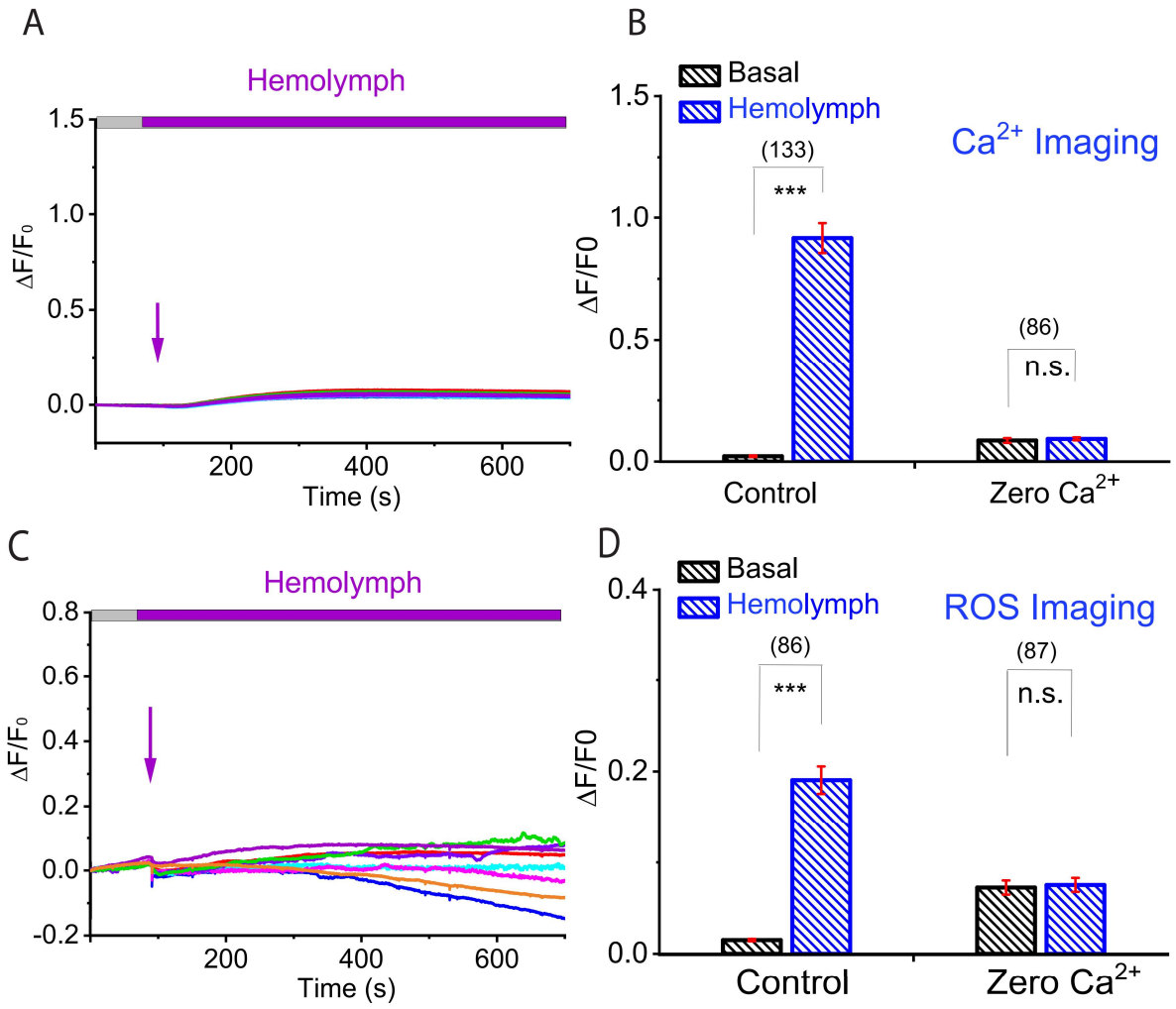

Figure 5: Effect of EGTA on Ca^{2+} and ROS generation in isolated tomato (*S. lycoperscium*) protoplasts. (A) Representative Ca^{2+} imaging of tomato protoplasts in zero- Ca^{2+} solution upon application of *M. sexta* hemolymph**.** (B) Bar graph analysis of data shown in (Fig. 4A, C) illustrating the maximum Ca^{2+} generation after *M. sexta* hemolymph application. (C) Representative ROS imaging of tomato protoplasts in zero- Ca^{2+} solution upon application of *M. sexta* hemolymph. (D) Bar graph comparing the maximum ROS production in tomato protoplasts upon application of *M. sexta* hemolymph from data shown in Fig. 1(B, C). The number of protoplasts (N) from 3-5 independent measurements is depicted in parentheses in E.

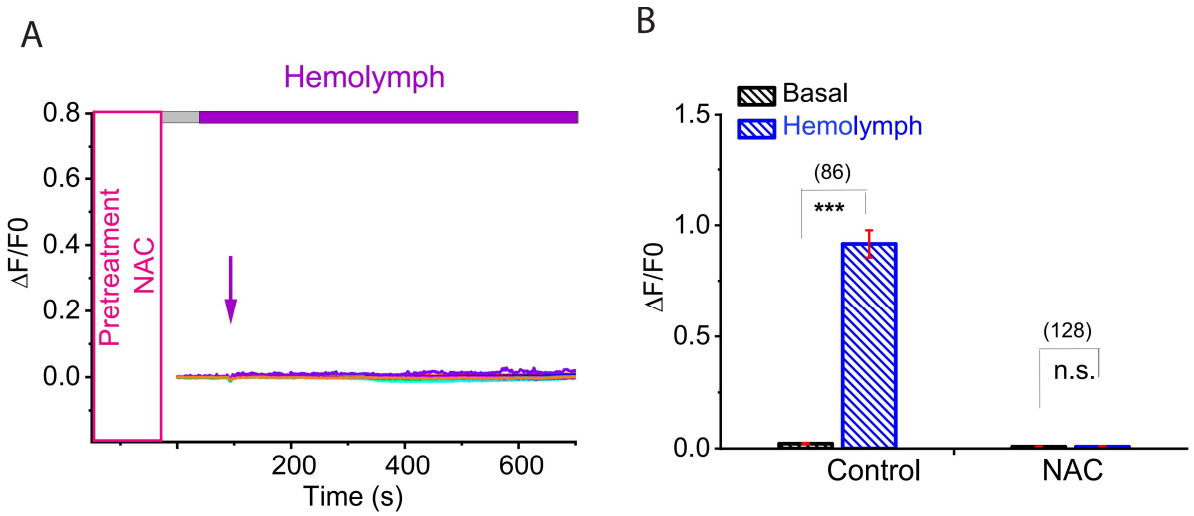

Figure 6: Effect of NAC on Ca^{2+} generation in tomato (*S. lycoperscium*) protoplasts. (A) Representative Ca^{2+} imaging of isolated tomato protoplasts preincubated with NAC, upon the application of the *M. sexta* hemolymph. (B) Bar graph analysis of data shown in Fig. 4A, C and Fig. 6A illustrating the maximum Ca^{2+} generation after *M. sexta* hemolymph application.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Recent years have witnessed immense progress in the research related to the discovery of early defense signaling components but studies on the molecular identification and characterization of these molecules that are involved in herbivore-mediated signaling are still scanty. However, with the advent of advanced molecular imaging, biochemical, and genetic techniques, it has become possible to unravel the signaling pathways employed by plants for responding to the herbivore attack. Plants can transmit information about the herbivore attack to the unstressed parts of the plant through trio of signaling molecules that includes V_m, ROS, and Ca^{2+}. Herbivory causes perturbations in the membrane potential in plants which is followed by fast electrical signals and hence activation of signal transduction pathways. ROS and Ca^{2+} act as an important signaling molecules in plant defense response against herbivores. Future direction will focus on understanding the molecular mechanism of V_m, ROS, Ca^{2+} and involvement of plant ion channels in the long-distance signaling cascade. Transforming the plants with biosensors such as reduction-oxidation sensitive green fluorescent proteins (Ro-GFP) localized at the plasma membrane and tonoplast can provide insights into ROS changes at the cellular level during herbivore attack. Moreover, generating mutant plants lacking a specific ion channel is a

promising strategy for unveiling its role in defense response against herbivores. Although circumstantial evidence suggests the role that membrane potential, ROS, and Ca^{2+} play in long-range signaling in plants, it will be tempting to elucidate the spatial and temporal relationships between them. There is a need to identify and characterize the elicitor binding sites at the molecular level. Elicitors could be used to generate biopesticides by spraying in the field as these can prime the plants against future herbivore attacks. Such studies will unravel the complex regulatory networks that modulate the plant defenses and will establish links that exist between different pathways.

Our *Manduca sexta* OS mediated ROS modulation research revealed that *M. sexta* OS is a ROS elicitor and possibly regulates defenses against insect herbivores. Remarkably, the OS effect was dependent on the larval diet of *M. sexta,* while PF OS induced ROS, and the DF OS failed to generate ROS, indicating a potential evolutionary divergence of induced resistance in plants. We speculate that the variation of primary and secondary species-specific metabolites plays a major role in OS composition, and it is also plausible to expect that OS components of generalist vs specialist and chewing vs sucking mouthparts could also covary with their host plants. This study also reported two chemicals, 1) membrane-permeable ROS "tbH_2O_2" and 2) antioxidant "NAC," which could be efficiently employed in dissecting the role of intracellular ROS in plants-herbivore interaction research, a novel cell biology approach in plant-herbivore studies.

Furthermore, our study identified that *M. sexta* OS induced ROS production was Ca^{2+}-dependent, suggesting crosstalk between Ca^{2+} and ROS signaling pathway. Collectively, these data indicate that herbivore-associated elicitor (HAE) increased ROS production, which could be

a key starting player in the plant defense line up. Future direction involves identifying individual components of *M. sexta* OS responsible for the ROS generation. To address this, we would like to do HPLC purification and mass spectroscopy analysis of the *M. sexta* OS. Furthermore, we would like to test the effect of *M. sexta* OS on tomato plasma membrane ion channels via patch-clamp measurement. Finally, we would like to test the isolated potent ROS elicitor component in the field for the development of novel crop protection strategies.

Our *M. sexta* hemolymph-mediated ROS and Ca^{2+} research depicted that hemolymph from *M. sexta* can directly modulate intracellular ROS and Ca^{2+} production and possibly regulate defenses against insect herbivores by acting as an HAE. The addition of antioxidant NAC antagonized hemolymph-induced ROS generation, indicating that *M. sexta* hemolymph is a ROS inducer in isolated protoplasts. Furthermore, incubating the protoplasts with Ca^{2+} chelator, BAPTA-AM efficiently abolished the hemolymph-induced ROS production, suggesting possible crosstalk between Ca^{2+} and ROS signaling. Collectively, this data suggests that hemolymph is a newly discovered HAE that has the potential to trigger signaling cascade in plants. Future direction involves identifying individual components of *M. sexta* hemolymph responsible for the ROS and Ca^{2+} generation by mass spectroscopy and HPLC purification. Knowledge of these components that trigger specific defense responses can help to develop potential elicitors that could have implications in crop protection.

REFERENCES

Abdul, K., Lina, M. S., & Richard B. J. (2018). "Emerging Roles of the Membrane Potential: Action Beyond the Action Potential." *Frontiers in Physiology*, 9

Acevedo, F. E., Loren, J. R. V., Seung, H. C., Swayamjit, R., & Gary, W. F. (2015). "Cues from Chewing Insects - the Intersection of DAMPs, HAMPs, MAMPs and Effectors." *Current Opinion in Plant* Biology, 26: 80-86.

Agrawal, A., Lau, J., & Hambäck, P. (2006). Community heterogeneity and the evolution of interactions between plants and insect herbivores. *The Quarterly Review of Biology, 81*(4), 349-376. doi:10.1086/511529

Agrawal, A. A., Mark, F., Reinhard, J., Juha-Pekka, S., Jessica, B. G., Amy, E. F., & Jed, P. S. (2009). "Phylogenetic Ecology of Leaf Surface Traits in the Milkweeds (*Asclepias* Spp.): Chemistry, Ecophysiology, and Insect Behavior." *The New Phytologist*, 183 (3): 848-867.

Agrawal, A. A., Hastings, A. P., Johnson, M. T., Maron, J. L., & Salminen, J. (2012). Insect Herbivores Drive Real-Time Ecological and Evolutionary Change in Plant Populations. *Science, 338*(6103), 113-116. doi:10.1126/science.1225977

Alborn, H.T. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science*, 276:945-994.

Annika, E. H., Taryn, L. B. (2016). Long-distance plant signaling pathways in response to multiple stressors: the gap in knowledge. *Journal of Experimental Botany*, 67(7): 2063-2079.

Arimura, G. I., Kenji, M., & Junji, T. (2009). "Chemical and Molecular Ecology of Herbivore-Induced Plant Volatiles: Proximate Factors and Their Ultimate Functions." *Plant & Cell Physiology* 50 (5): 911-923.

Arimura, G. I., Kost, C., Boland, W. (2005). Herbivore-induced, indirect plant defences. Biochimica et Biophysica Acta (BBA). *Molecular and Cell Biology of Lipids*, 1734: 91-111.

Arimura, G. I., Maffei, M. E. (2010) Calcium and secondary CPK signaling in plants in response to herbivore attack. *Biochemical and Biophysical Research Communications*, *400*: 455-460.

Arimura, G.I., Ozawa, R., Maffei, M. E. (2011). Recent Advances in Plant Early Signaling in Response to Herbivory. *International Journal of Molecular Sciences*, 12: 3723-3739.

Armstrong, F., Leung, J., Grabov, A., Brearley, J., Giraudat, J., Blatt, M. R. (1995). Sensitivity to ABA of guard cell K^+ channels is suppressed by abi 1-1, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. *Proceedings of the National Academy of Sciences*, USA 92: 9520-9524.

Atkinson, N.J., & Urwin, P.E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*, 63(10): 3523-3543. doi:10.1093/jxb/ers100

Baek, D., Nam, J., koo, Y.D. *et al.* (2004). Bax-induced cell death of *Arabidopsis*is meditated through reactive oxygen-dependent and -independent processes. *Plant Molecular Biology*, 56: 15-27.

Baldwin, I. T., & Catherine, A. P. (1999). "The Eco-Physiological Complexity of Plant Responses to Insect Herbivores." *Planta*, 208(2): 137-145.

Bandoly, M., Grichnik, R., Hilker, M., Steppuhn, A. (2016). Priming of anti-herbivore defence in Nicotiana attenuata by insect oviposition: herbivore-specific effects. *Plant, Cell & Environment* 39: 848-859.

Batistič, O., & Jörg, K. (2012). "Analysis of Calcium Signaling Pathways in Plants." *Biochimica Et Biophysica Actaz* 1820 (8): 1283-1293.

Baxter, A., Mittler, R., & Suzuki, N. (2014). "ROS as Key Players in Plant Stress Signalling." *Journal of Experimental Botany*, 65 (5): 1229-1240.

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transducation in plants. *Current Science*, 89: 1113-1121.

Bittner, N., Trauer-Kizilelma, U. & Hilker, M. (2017). Early plant defence against insect attack: involvement of reactive oxygen species in plant responses to insect egg deposition. *Planta*, 245: 993-1007.

Blume, B., Nürnberger, T., Nass, N., Scheel, D. (2000). Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell.* 12(8):1425-1440. doi:10.1105/tpc.12.8.1425

Bolwell, G. P., Bindschedler, L. V., Blee, K. A., Butt, V. S., Davies, D. R., Gardner, S. L., Gerrish, C., Minibayeva, F. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany*, *53*: 1367-1376.

Bonaventure, G. (2012). Perception of insect feeding by plants. *Plant Biology*, *14*: 872-880.

Bouché, N., Ayelet, Y., Wayne, A. S., & Hillel, F. (2005). "Plant-Specific Calmodulin-Binding Proteins." *Annual Review of Plant Biology*, 56: 435-466.

Boudsocq, M., & Jen, S. (2013). "CDPKs in Immune and Stress Signaling." *Trends in Plant Science* 18 (1): 30-40.

Bricchi, I., Leitner, M., Foti, M., Mithöfer, A., Boland, W., Maffei, M. E. (2010). Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: early signaling and volatile emission in Lima bean (*Phaseolus lunatus* L.). *Planta*, *232*: 719-729.

Bricchi I, Bertea CM, Occhipinti A, Paponov IA, Maffei ME. Dynamics of membrane potential variation and gene expression induced by *Spodoptera littoralis, Myzus persicae,* and *Pseudomonas syringae* in *Arabidopsis*. *Plos one*. 2012 ;7(10):e46673. DOI: 10.1371/journal.pone.0046673.

Bricchi, I., Occhipinti, A., Bertea, C.M., et al. (2013). Separation of early and late responses to herbivory in Arabidopsis by changing plasmodesmal function. *The Plant Journal*, 73: 14-25.

Chen, C., (2005). Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Research*, *33*.

Cheng, S. H., Matthew, R., Willmann, H.C., & Jen, S. (2002). "Calcium Signaling through Protein Kinases. The *Arabidopsis* Calcium-Dependent Protein Kinase Gene Family." *Plant Physiology*, 129 (2): 469-485.

Choi, W.G., Richard, H., Sarah, J., Swanson, S. H. K., & Simon, G. (2016). "Rapid, Long-Distance Electrical and Calcium Signaling in Plants." *Annual Review of Plant Biology*, 67 (1): 287-307.

Choudhury, F. K., Rivero, R. M., Blumwald, E., Mittler, R. (2016). Reactive oxygen species, abiotic stress and stress combination. *The Plant Journal*, 90: 856-867.

Choudhury, S., Panda, P., Sahoo, L., Panda, S. K. (2013). Reactive oxygen species signaling in plants under abiotic stress. *Plant Signaling & Behavior*, *8*.

Chung, S. H., Rosa, C., Scully, E. D., Peiffer, M., Tooker, J. F., Hoover, K., Luthe, D. S., & Felton, G. W. (2013). Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences*, 110: 15728-15733.

Cole, K. S., & Curtis, H.J. (1938). Electrical impedance of *Nitella* during activity. *Journal Genetic Physiol*ogy, 22:37-64.

Davenport, R. (2002). Glutamate receptors in plants. *Annals of Botany*, 90: 549-557.

Davies, D. R. (2006). Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. *Journal of Experimental Botany*, 57: 1817-1827.

Day, I. S., Vaka, S., Reddy, G., & Reddy, A. S. N. (2002). "Analysis of EF-Hand-Containing Proteins in Arabidopsis." *Genome Biology*, 3 (10): RESEARCH0056. \

Demidchik, V., Adobea, E. P., & Tester, V. (2004). Glutamate activates cation currents in the plasma membrane of *Arabidopsis* root cells. *Planta,* 219: 167-175.

Demidchik V, Shabala SN, Davies JM. Spatial variation in H_2O_2 response of *Arabidopsis thaliana* root epidermal Ca^{2+} flux and plasma membrane Ca^{2+} channels. *Plant J*. 2007;49(3):377-386. doi:10.1111/j.1365-313X.2006.02971.x

De Moraes, C.M., Lewis, W.J. Paré, P.W., Alborn, H.T. & Tumlinson, J.H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393: 570-573.

Dennison, K. L., & Spalding, E. P. (2000). Glutamate-gated calcium fluxes in *Arabidopsis*. *Plant Physiology*, 124: 1511-1514. doi:10.1104/pp.124. 4.1511

Dicke, M., Sabelis, M.W., Takabayashi, J. *et al.* Plant strategies of manipulating predator prey interactions through allelochemicals: Prospects for application in pest control. *J Chem Ecol* **16,** 3091–3118 (1990). https://doi.org/10.1007/BF00979614

Dingledine, R., Borges, K., Bowie, D., & Traynelis, S. F. (1999). The glutamate receptor ion channels. *Pharmacological Reviews*, 51: 7-61.

Dodd, A. N., Jörg, K., & Dale, S. (2010). "The Language of Calcium Signaling." *Annual Review of Plant Biology*, 61: 593-620.

Doss, R. P., James, E., Oliver, W. M., Proebsting, S. W., Potter, S. K., Stephen, L. C., et al. (2000). "Bruchins: Insect-Derived Plant Regulators That Stimulate Neoplasm Formation." *Proceedings of the National Academy of Sciences*, 97 (11): 6218-6223.

Drerup, M. M., Schlücking, K., Hashimoto, K., Manishankar, P., Steinhorst, L., Kuchitsu, K., Kudla, J. (2013). The Calcineurin B-Like Calcium Sensors CBL1 and CBL9 Together with Their Interacting Protein Kinase CIPK26 Regulate the *Arabidopsis* NADPH Oxidase RBOHF. Molecular *Plant*, 6: 559-569.

Duszyn, M., Brygida, Ś., Adriana, S. J., & Krzysztof, J. (2019). "Cyclic Nucleotide Gated Channels (CNGCs) in Plant Signalling- Current Knowledge and Perspectives." *Journal of Plant Physiology*, 241: 153035.

Edel, K. H., Elodie, M., Colin, B., Jörg, K., & Alistair, M. H. (2017). "The Evolution of Calcium-Based Signalling in Plants." *Current Biology*, 27 (13): 667-679.

Elzbieta, K., Kazimierz, T. (2000). Ways of Ion Channel Gating in Plant Cells. *Annals of Botany*, 86(3): 449-469.

Erb, M., & Reymond, P. (2019). Molecular Interactions Between Plants and Insect Herbivores. *Annual Review of Plant Biology*,70: 527-557.

Fatouros, N. E, Broekgaarden, C., Bukovinszkine, G., et al. (2008). Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proceedings of the National Academy of Sciences of the USA*, 105: 10033-10038.

Felton, G. W, & Tumlinson, J. H. (2008). Plant-insect dialogs: complex interactions at the plant-insect interface. *Current Opinion Plant Biol*ogy, 11(4):457-463. doi:10.1016/j.pbi.2008.07.001

Felton, G. W., Chung, S. H., Hernandez, M. G. E., Louis, J., Peiffer, M., Tian, D. (2014). Herbivore Oral Secretions are the First Line of Protection Against Plant-induced Defences. *Annual Plant Reviews online*, 37-76.

Feng, Z., Rongsong, Liu., & Donald, L. D. (2008). "Plant–Herbivore Interactions Mediated by Plant Toxicity." *Theoretical Population Biology*, 73 (3): 449-59.

Fichman, Y., Miller, G., Mittler, R. (2019). Whole-Plant Live Imaging of Reactive Oxygen Species. *Molecular Plant*, 12: 1203-1210.

Forde, B. G., & Lea, P. J. (2007). Glutamate in plants: metabolism, regulation, and signalling. *Journal of Experimental Botany*, 58: 2339- 2358

Forde, B.G., Roberts, M. R. (2014). Glutamate receptor-like channels in plants: a role as amino acid sensors in plant defence? F1000 *Prime Reports*, 6: 37.

Foreman, J., Demidchik, V., Bothwell, J. H. F., Mylona, P., Miedema, H., Torres, M. A., Linstead, P., Costa, S., Brownlee, C., Jones, J. D. G. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature*, 422: 442-446.

Gaffey, C. T., Mullins, L. J. (1958). Ion fluxes during the action potential in Chara. *Journal Physiology*, 144:505-524.

Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferrière, N., Thibaud, J. B., & Sentenac, H. (1998). Identification and disruption of a plant shaker-like outward channel involved in K^+ release into the xylem sap. *Cell*, *94*(5), 647–655.

Gandhi, A.; Kariyat, R.R.; Chappa, C.; Tayal, M.; Sahoo, N. Tobacco Hornworm (*Manduca sexta*) Oral Secretion Elicits Reactive Oxygen Species in Isolated Tomato Protoplasts. *Int. J. Mol*. Sci. 2020, 21, 8297.

Gilroy, S., Białasek, M., Suzuki, N., et al. (2016). ROS, Calcium, and Electric Signals: Key Mediators of Rapid Systemic Signaling in Plants. *Plant Physiology*, 171(3):1606-1615. doi:10.1104/pp.16.00434

Gilroy, S., Nobuhiro, S., Gad, M., Won-Gyu, C., Masatsugu, T., Amith, R. D., & Ron, M. (2014). "A Tidal Wave of Signals: Calcium and ROS at the Forefront of Rapid Systemic Signaling." *Trends in Plant Science*, 19 (10): 623-630.

Gomez-Cabrera, M. C., Domenech, E., Romagnoli, M., Arduini, A., Borras, C., Pallardo, F. V., Sastre, J., Viña, J. (2008). Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *The American Journal of Clinical Nutrition*, 87: 142-149.

Görlach, A., Bertram, K., Hudecova, S., Krizanova, O. (2015). Calcium and ROS: A mutual interplay. *Redox Biology*, 6: 260-271.

Granados ME, Soriano E, Saavedra Molina A (1997) Use of pluronic acid F-127 with Fluo-3/AM probe to determine intracellular calcium changes elicited in bean protoplasts. *Phytochem Anal* 8: 204-20

Green, T. R., Ryan, C. A. (1972). Wound-Induced Proteinase Inhibitor in Plant Leaves: A Possible Defense Mechanism against Insects. *Science*.175(4023):776-777.

Halitschke, R., Ursula, S., Georg, P., Wilhelm, B., & Ian, T. B. (2001). "Molecular Interactions between the Specialist Herbivore *Manduca Sexta* (Lepidoptera, Sphingidae) and Its Natural Host *Nicotiana Attenuata* . III. Fatty Acid-Amino Acid Conjugates in Herbivore Oral Secretions Are Necessary and Sufficient for Herbivore-Specific Plant Responses." *Plant Physiology*, 125 (2): 711-717.

Halliwell, B., Gutteridge, J. M. C. (2015). Free radicals in biology and medicine; Oxford University Press: Oxford,

Hamann, T. (2015). "The Plant Cell Wall Integrity Maintenance Mechanism – A Case Study of a Cell Wall Plasma Membrane Signaling Network." *Phytochemistry*, 112:100-109.

Hancock, J. T., Desikan, R., Clarke, A., Hurst, R. D., & Neill, S. (2002). Cell signalling following plant/pathogen interactions involves the generation of reactive oxygen and reactive nitrogen species. *Plant Physiology and Biochemistry*, 40(6-8): 611-617

Handley, R., Barbara, E., and Jon, Å. (2005). "Variation in Trichome Density and Resistance against a Specialist Herbivore in Natural Populations of *Arabidopsis Thaliana*." *Ecological Entomology,* 30 : 284-292.

He, P., Shan, L., Sheen, J. (2007). The use of protoplasts to study innate immune responses. *Methods Molecular Biology,* 354: 1-9.

Hetherington, A. M., Brownlee, C. (2004). The generation of Ca^{2+} signals in plants. *Annual Review Plant Biology*, 55:401-427.

Hilfiker, O., Groux, R., Bruessow, F., Kiefer, K., Zeier, J., Reymond, P. (2014). Insect eggs induce a systemic acquired resistance in *Arabidopsis*. *Plant Journal*, 80: 1085-1094.

Hille, B. (1992). Ionic Channels of Excitable Membranes. 2nd ed., Sinauer Associates, Sunderland, MA.

Hille, B., Julia, B., Donner, F., Babcock, T. N., & Duk-Su, K. (1999). "Stimulation of Exocytosis without a Calcium Signal." *The Journal of Physiology*, 520(1): 23-31.

Hilker, M., & Meiners, T. (2006). Early herbivore alert: insect eggs induce plant defense. *Journal of chemical ecology*, *32*(7), 1379–1397. https://doi.org/10.1007/s10886-006-9057-4

Hodgkin, A. L, Huxley, A. F. (1952). The components of membrane conductance in the giant axon of Loligo. *Journal Physiology*, 116:473-496.

Holdaway-Clarke, T. L., Feijo, J. A., Hackett, G.R., Kunkel, J.G., & Hepler, P. K. (1997). Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *The Plant Cell*, 9: 1999-2010.

Howe, G. A., Jander, G. (2008). Plant Immunity to Insect Herbivores. *Annual Review of Plant Biology*, 59: 41-66.

Imbiscuso, G., Antonio, T., Massimo, M., & Simone, B. (2009). "Herbivory Induces a ROS Burst and the Release of Volatile Organic Compounds in the Fern *Pteris Vittata* L." *Journal of Plant Interactions*, 4 (1): 15-22.

Jacks, T. J., & Davidonis, G. H. (1996). "Superoxide, Hydrogen Peroxide, and the Respiratory Burst of Fungally Infected Plant Cells." *Molecular and Cellular Biochemistry,* 158 (1): 77-79.

Jammes, F., Heng-Cheng, H., Florent, V., Roxane, B., & June, M. K. (2011). "Calcium-permeable Channels in Plant Cells." *The FEBS Journal*, 278 (22): 4262-4276.

Janků, M., Lenka, L., & Marek, P. (2019). "On the Origin and Fate of Reactive Oxygen Species in Plant Cell Compartments." *Antioxidants* 8 (4).

Jeworutzki, E., Roelfsema, M. R. G., Anschütz, U., Krol, E., Elzenga, J. T. M., Felix, G., Boller, T., Hedrich, R., Becker, D. (2010). Early signaling through the *Arabidopsis* pattern recognition receptors FLS2 and EFR involves Ca^{2+} -associated opening of plasma membrane anion channels. *The Plant Journal*, 6: 367-378.

Jha, S. K., Sharma, M., & Pandey, G. K. (2016). Role of Cyclic Nucleotide Gated Channels in Stress Management in Plants. *Current genomics*, *17*(4), 315-329.

Johansson, I., Larsson, C., Ek, B., Kjellbom, P. (1996). The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^2 and apoplastic water potential. *The Plant Cell*, 8: 1181-1191.

Johnson, M. T. J. (2011). "Evolutionary Ecology of Plant Defences against Herbivores." *Functional Ecology*, 25 (2): 305-311.

Joo, J. H., Bae, Y. S., & Lee, J. S. (2001). Role of auxin-induced reactive oxygen species in root gravitropism. *Plant physiology*, 126(3), 1055-1060.

Kanchiswamy, C. N., Hirotaka, T., Stefano, Q., Massimo, E., Maffei, S. B., Cinzia, B., Simon, A. Z., et al. (2010). "Regulation of Arabidopsis Defense Responses against *Spodoptera Littoralis* by CPK-Mediated Calcium Signaling." *BMC Plant Biology*, 10 (1): 97.

Kang, J., Mehta, S., Turano, F. J. (2004). The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss. *Plant and Cell Physiology*, 45:1380-1389.

Kang, J., Turano, F. J. (2003). The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, USA 100: 6872-6877.

Kanost, Michael R. (2009). "Hemolymph." In Encyclopedia of Insects, *Elsevier*. 446-449. Kant, M. R., Jonckheere, W., Knegt, B., Lemos, F., Liu, J., Schimmel, B. C. J., Villarroel, C. A.,

Ataide, L. M. S., Dermauw, W., Glas, J. J., Egas, M., Janssen, A., Leeuwen, T. V., Schuurink, R. C., Sabelis, M. W., Alba, J. M. (2015). Mechanisms and ecological consequences of plant defence induction and suppression in herbivore communities. *Annals of Botany*, 115: 1015-1051.

Karban, R. (2010). The ecology and evolution of induced resistance against herbivores. *Functional Ecology*, 25: 339-347.

Kariyat, R. R., Mauck, K. E., Moraes, C. M. D., Stephenson, A. G., Mescher, M. C. (2012a). Inbreeding alters volatile signalling phenotypes and influences tri-trophic interactions in horsenettle (*Solanum carolinense* L.). *Ecology Letters*, 15: 301-309.

Kariyat, R. R., J. M. Mena-Alí, B. Forry, M. C. Mescher, C. M. De Moraes, and A. G. Stephenson. 2012b. Inbreeding, herbivory, and the transcriptome of *Solanum carolinense* L. *Entomologia Experimentalis et Applicata* 144: 134–144.

Kariyat, R. R., Balogh, C. M., Moraski, R. P., Moraes, C. M. D., Mescher, M. C., Stephenson, A. G. Constitutive and herbivore-induced structural defenses are compromised by inbreeding in *Solanum carolinense* (Solanaceae). *American Journal of Botany*. (2013); 100: 1014-1021

Kariyat, R. R., Jason, D. S., Andrew, G. S., Consuelo, M. D. M., and Mark, C. Mescher. (2017). "Non-Glandular Trichomes of Solanum Carolinense Deter Feeding by *Manduca Sexta* Caterpillars and Cause Damage to the Gut Peritrophic Matrix." *Proceedings of the Royal Society B: Biological Sciences* 284 (1849): 20162323.

Kariyat, R.R., Raya, C.E., Chavana, J. et al. Feeding on glandular and non-glandular leaf trichomes negatively affect growth and development in tobacco hornworm (*Manduca sexta*) caterpillars. Arthropod-Plant Interactions 13, 321–333 (2019). https://doi.org/10.1007/s11829-019-09678-z

Katsuhara, M., & Tazawa, M. (1992). Calcium-regulated channels and their bearing on physiological activities in Characean cells. *Philosophical Transactions of the Royal Society of London*, 338: 19-29.

Kaur, J., & Kariyat, R. (2020). Role of Trichomes in Plant Stress Biology. *Evolutionary Ecology of Plant-Herbivore Interaction***,** 15-35.

Kerchev, P. I., Fenton, B., Foyer, C. H., Hancock, R. D. (2011). Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. *Plant, Cell & Environment*, *35*: 441-453.

Kessler, A., & Baldwin, I. T. (2001). Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, 291(5511):2141-2144. doi:10.1126/science.291.5511.2141

Kiep, V., Jyothilakshmi, V., Justus, L. (2015). Maaß, Wilhelm Boland, Edgar Peiter, and Axel Mithöfer. "Systemic Cytosolic Ca^{2+} Elevation Is Activated upon Wounding and Herbivory in *Arabidopsis*." *New Phytologist* 207 (4): 996-1004.

Kim, J., Tooker, J. F., Luthe, D. S., Moraes, C. M., & Felton, G. W. (2012). Insect Eggs Can Enhance Wound Response in Plants: A Study System of Tomato *Solanum lycopersicum* L. and *Helicoverpa zea* Boddie. *PLoS ONE, 7*(5). doi:10.1371/journal.pone.0037420

Kim, J., & Felton, G. W. (2013). Priming of antiherbivore defensive responses in plants. *Insect science*, *20*(3), 273–285. https://doi.org/10.1111/j.1744-7917.2012.01584.x

Kimura, S., Kaya, H., Kawarazaki, T., Hiraoka, G., Senzaki, E., Michikawa, M., Kuchitsu, K.(2012). Protein phosphorylation is a prerequisite for the Ca^{2+} -dependent activation of *Arabidopsis* NADPH oxidases and may function as a trigger for the positive feedback regulation of Ca^{2+} and reactive oxygen species. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1823*: 398-405.

Köhler, C., Neuhaus, G. (2000). Characterisation of calmodulin binding to cyclic nucleotide-gated ion channels from *Arabidopsis thaliana*. *FEBS Lett*er, 471(2-3):133-136. doi:10.1016/s0014-5793(00)01383-1

Kristiansen, K. A., Jensen, P. E., Møller, I. M., Schulz, A. (2009). Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H_2DCFDA and confocal laser microscopy. *Physiologia Plantarum*, 136: 369-383.

Kurusu, T., Kazuyuki, K., Masataka, N., Yoshitaka, N., & Hidetoshi, I. (2013). "Plant Mechanosensing and Ca^{2+} Transport." *Trends in Plant Science* 18 (4): 227-233.

Kwaaitaal, M., Huisman, R., Maintz, J., Reinstädler, A., Panstruga, R. (2011). Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochemical Journal*, 440: 355-365.

Kwon, K.C., Verma, D., Jin, S., Singh, N. D., Daniell, H. (2013). Release of Proteins from Intact Chloroplasts Induced by Reactive Oxygen Species during Biotic and Abiotic Stress. *PLoS ONE*, *8*.

Lam, H. M., Chiu, J., Hsieh, M. H., Meisel, L., Oliveira, I. C., Shin, M., Coruzzi, G. (1998). Glutamate-receptor genes in plants. *Nature,* 396:125-126.

Lamb, C., Dixon, R. A. (1997). The Oxidative Burst In Plant Disease Resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*, 4: 251-275.

Lanning, F. C., and L. N. Eleuterius. (1985). "Silica and Ash in Tissues of Some Plants Growing in the Coastal Area of Mississippi, USA." *Annals of Botany*, 56 (2): 157-72.

Leng, Q., Mercier, R. W., Hua, B. G., Fromm, H., & Berkowitz, G. A. (2002). Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. *Plant physiology*, *128*(2), 400–410. https://doi.org/10.1104/pp.010832

Li, F., Wang, J., Ma, C., Zhao, Y., Wang, Y., Hasi, A., Qi, Z. (2013). Glutamate receptor-like channel3.3 is involved in mediating glutathione-triggered cytosolic calcium transients, transcriptional changes, and innate immunity responses in *Arabidopsis*. *Plant Physiology*, 162: 1497-1509.

Liao, C., Zheng, Y., Guo, Y. (2017). MYB30 transcription factor regulates oxidative and heat stress responses through ANNEXIN-mediated cytosolic calcium signaling in *Arabidopsis*. *New Phytologist*, *216*: 163-177.

Little, D., Caroline, G. D., Friederike, B., & Philippe, R. (2007). "Oviposition by Pierid Butterflies Triggers Defense Responses in *Arabidopsis*." *Plant Physiology*, 143 (2): 784-800.

Louis, J., Peiffer, M., Ray, S., Luthe, D. S., Felton, G. W. (2013). Host-specific salivary elicitor(s) of European corn borer induce defenses in tomato and maize. *New Phytology,* 199:66-73.

Luan, S. (2009). "The CBL-CIPK Network in Plant Calcium Signaling." *Trends in Plant Science* 14 (1): 37-42.

Luo, S., Zhang, X., Wang, J., Jiao, C., Chen, Y., Shen, Y. (2017). Plant ion channels and transporters in herbivory-induced signalling. *Functional Plant Biology,* 45: 111-131.

Maffei, M. E., Mithöfer, A., Arimura, G. I., Uchtenhagen, H., Bossi, S., Bertea, C. M., Cucuzza, L. S., Novero, M., Volpe, V., Quadro, S., Boland, W. (2006). Effects of Feeding *Spodoptera littoralis* on Lima Bean Leaves. III. Membrane Depolarization and Involvement of Hydrogen Peroxide. *Plant Physiology*, 140:1022-1035.

Maffei, M. E., Mithöfer, A., Boland, W. (2007). Insects feeding on plants: Rapid signals and responses preceding the induction of phytochemical release. *Phytochemistry*, 68: 2946-2959.

Maffei, M., Bossi, S., Spiteller, D., Mithöfer, A., Boland, W. (2004). Effects of Feeding *Spodoptera littoralis* on Lima Bean Leaves. I. Membrane Potentials, Intracellular Calcium Variations, Oral Secretions, and Regurgitate Components. *Plant Physiology*, 134:1752-1762.

Mahajan, S., Tuteja, N. (2007). Calcium signaling network in plants. *Plant signaling and behavior,* 2:79-85.

Maischak, H., Grigoriev, P.A., Vogel, H., Boland, W., Mithöfer, A. (2007). Oral secretions from herbivorous lepidopteran larvae exhibit ion channel-forming activities. *FEBS Letters*, *581*: 898-904.

Manzoor H, Kelloniemi J, Chiltz A, Wendehenne D, Pugin A, Poinssot B, Garcia-Brugger A. (2013). Involvement of the glutamate receptor AtGLR3.3 in plant defense signaling and resistance to *Hyaloperonospora arabidopsidis*. *The Plant Journal,* 76: 466-480.

McAinsh, M.R., Hetherington, A. M. (1998). Encoding specificity in Ca^{2+} signalling system. *Trends in Plant Science,* 3: 32-,36.

McCormack, E., & Janet, B. (2003). "Calmodulins and Related Potential Calcium Sensors of *Arabidopsis*." *New Phytologist* 159 (3): 585-598.

Meena, M. K., Ramgopal, P., Deepthi, K., Keerthi, D., Yogesh, P., Michael, R., Mathew, M. K., Wilhelm, B., Axel, M., & Jyothilakshmi, V. (2019). "The Ca^{2+} Channel CNGC19 Regulates *Arabidopsis* Defense Against *Spodoptera* Herbivory." *The Plant Cell*, 31 (7): 1539-1562.

Michard, E., Lima, P. T., Borges, F., et al. (2011). Glutamate receptor-like genes form Ca^{2+} channels in pollen tubes and are regulated by pistil d-serine. *Science,* 332: 434-437.

Miller, G., & Ron, M., (2006). "Could Heat Shock Transcription Factors Function as Hydrogen Peroxide Sensors in Plants?" *Annals of Botany* 98 (2): 279-288.

Miller, G., Karen, S., Rachel, T, Diego, C., Miguel, A., Torres, V. S., Jeffery, L., Dangl., & Ron, M. (2009). "The Plant NADPH Oxidase RBOHD Mediates Rapid Systemic Signaling in Response to Diverse Stimuli." *Science Signaling,* 2 (84): ra45.

Miller, G., Shulaev, V., Mittler, R. (2008). Reactive oxygen signaling and abiotic stress. *Physiologia Plantarum,* (133): 481-489.

Miller, N. D, Durham, Brooks, T. L., Assadi, A. H., Spalding, E. P. (2010). Detection of a gravitropism phenotype in glutamate receptor-like 3.3 mutants of *Arabidopsis thaliana* using machine vision and computation. *Genetics*, 186:585-593.

Mithöfer, A., Boland, W. (2008). Recognition of herbivory-associated molecular patterns. *Plant Physiol*ogy, 146(3):825-831. doi:10.1104/pp.107.113118

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., Gollery, M., Shulaev, V., Breusegem, F. V. (2011). ROS signaling: the new wave? *Trends in Plant Science,* 16:300-309.

Mohanta, T. K., Occhipinti, A., Atsbaha Zebelo, S., Foti, M., Fliegmann, J., Bossi, S., Maffei, M.E., Bertea, C.M. (2012). *Ginkgo biloba* responds to herbivory by activating early signaling and direct defenses. *PLoS ONE* , *7*, e32822.

Molassiotis, A., Fotopoulos, V. (2011). Oxidative and nitrosative signaling in plants. *Plant Signaling & Behavior*, 6: 210-214.

Moloi, M. J., & Amie, J. V. W. (2006). "The Reactive Oxygen Species Are Involved in Resistance Responses of Wheat to the Russian Wheat Aphid." *Journal of Plant Physiology,* 163 (11): 1118-25.

Monshausen, G. B., & Elizabeth, S. H. (2013). "A Force of Nature: Molecular Mechanisms of Mechanoperception in Plants." *Journal of Experimental Botany,* 64 (15): 4663-4680.

Moraes, C. M., Lewis, W. J., Paré, P. W., Alborn, H. T., & Tumlinson, J. H. (1998). Herbivore-infested plants selectively attract parasitoids. *Nature, 393*(6685), 570-573. doi:10.1038/31219

Moore, C.A., Bowen, H. C., Scrase-Field, S., Knight, M. R., White, P. J. (2002). The deposition of suberin lamellae determines the magnitude of cytosolic Ca^{2+} elevations in root endodermal cells subjected to cooling. *Plant Journal*, 30: 457-465.

Mousavi, S. A., Chauvin, A., Pascaud, F., Kellenberger, S., Farmer, E. E. (2013). GLUTAMATE RECEPTOR-LIKE genes mediate leaf-to-leaf wound signalling. *Nature,* 500: 422-426.

Muday, G. K., & Heather, B. H. (2018). "Nervous System-like Signaling in Plant Defense." *Science*, 361 (6407): 1068-1069.

Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J. B., Felton, G. W. (2002). Herbivory: caterpillar saliva beats plant defences. *Nature,* 416:599-600.

Nanjareddy, K., Arthikala, M. K., Blanco, L., Arellano, E. S., & Lara, M. (2016). Protoplast isolation, transient transformation of leaf mesophyll protoplasts and improved Agrobacterium-mediated leaf disc infiltration of *Phaseolus vulgaris*: tools for rapid gene expression analysis. *BMC Biotechnology,* 16:53. doi: 10.1186/s12896-016-0283-8

Noctor, G., Reichheld, J. P., Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Seminars in Cell & Developmental Biology,* 80: 3-12.

Oerke, E.C. (2006). Crop losses to pests. *The Journal of Agricultural Science,* 144: 31-43.

Ogasawara, Y., Hidetaka, K., Goro, H., Fumiaki, Y., Sachie, K., Yasuhiro, K., Haruka, H., et al. (2008). "Synergistic Activation of the *Arabidopsis* NADPH Oxidase AtrbohD by Ca2+ and Phosphorylation." *The Journal of Biological Chemistry*, 283 (14): 8885-8892.

Oparka, M., Walczak, J., Malinska, D., Oppen, L. M. V., Szczepanowska, J., Koopman, W. J., Wieckowski, M. R. (2016). Quantifying ROS levels using CM-H_2DCFDA and HyPer. *Methods*, 109:3-11.

Orozco-Cardenas, M., & Clarence, A. R. (1999). "Hydrogen Peroxide Is Generated Systemically in Plant Leaves by Wounding and Systemin via the Octadecanoid Pathway." *Proceedings of the National Academy of Sciences of the United States of America,* 96 (11): 6553-6557.

Pandey, G. K., Cheong, Y. H., Kim, K. N., Gran, J. J, Li, L., Hung, W. et al. (2004). The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. *Plant Cell*, 16:1912-1924.

Pei, Z. M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G. J., Grill, E., Schroeder, J. I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature*, 406: 731-734.

Peiffer, M., Tooker, J. F., Luthe, D. S., Felton, G. W. (2009). Plants on early alert: glandular trichomes as sensors for insect herbivores. *New Phytology,* 184: 644-656.

Portman, S. L., Felton, G. W., Kariyat, R. R., Marden, J. H. (2020). Host plant defense produces species-specific alterations to flight muscle protein structure and flight-related fitness traits of two armyworms. *The Journal of Experimental Biology*, *223*.

Portman, S. L., Kariyat, R. R., Johnston, M. A., Stephenson, A. G., Marden, J. H. (2015). Inbreeding compromises host plant defense gene expression and improves herbivore survival. *Plant Signaling & Behavior*, 10.

Price, P., Bouton, C.E., Gross, P., McPheron, B., Thompson, J., & Weis, A.E. (1980). Interactions Among Three Trophic Levels: Influence of Plants on Interactions Between Insect Herbivores and Natural Enemies. *Annual Review of Ecology, Evolution, and Systematics, 11*, 41-65

Qiu, Yongjian, Jing Xi, Liqun Du, Jeffrey C. Suttle, and B. W. Poovaiah. 2012. "Coupling Calcium/Calmodulin-Mediated Signaling and Herbivore-Induced Plant Response through Calmodulin-Binding Transcription Factor AtSR1/CAMTA3." *Plant Molecular Biology* 79 (1): 89-99.

Rae, M. G., Martin, D. J., Collingridge, G. L., Irving, A. J. (2000). Role of Ca^{2+} Stores in Metabotropicl-Glutamate Receptor-Mediated Supralinear Ca^{2+} Signaling in Rat Hippocampal Neurons. *The Journal of Neuroscience*, 20: 8628-8636.

Reddy, A. S. N. (2001). Calcium: silver bullet in signalling. *Plant Science*, 160: 381-404.

Reddy, A. S., Ali, G. S., Celesnik, H., Day, I. S.(2011). Coping with Stresses: Roles of Calcium- and Calcium/Calmodulin-Regulated Gene Expression. *The Plant Cell*, 23: 2010-2032.

Reymond P, Bodenhausen N, Van Poecke RM, Krishnamurthy V, Dicke M, Farmer EE (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16 3132–3147

Rejeb, I., Pastor, V., Mauch-Mani, B. (2014). Plant Responses to Simultaneous Biotic and Abiotic Stress: Molecular Mechanisms. *Plants*, *3*: 458-475.

Sagi, M., Fluhr, R, (2001). Superoxide production by plant homologues of the gp91phox NADPH oxidase: modulation of activity by calcium and by tobacco mosaic virus infection. *Plant Physiology,* 126: 1281-1290.

Sanders, D., Pelloux, J., Brownlee, C., Harper, J. F. (2002). Calcium at the crossroads of signaling. *Plant Cell.* 14:S401-17. doi: 10.1105/tpc.002899. PMID: 12045291; PMCID: PMC151269.

Schäfer, M., Fischer, C., Meldau, S., Seebald, E., Oelmüller, R., Baldwin, I. T. (2011). Lipase Activity in Insect Oral Secretions Mediates Defense Responses in *Arabidopsis*. *Plant Physiology*, *156*: 1520-1534.

Schmelz, E. A. (2015). Impacts of insect oral secretions on defoliation-induced plant defense. *Current Opinion in Insect Science*, *9*: 7-15.

Shao, Q., Gao, Q., Lhamo, D., Zhang, H., Luan, S. (2020). Two glutamate- and pH-regulated Ca^{2+} channels are required for systemic wound signaling in *Arabidopsis*. *Science Signal*, 13(640):eaba1453. doi:10.1126/scisignal.aba1453

Sharma, P., Ambuj, B. J., Rama, S. D., Mohammad, P. (2012). "Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions." *Journal of Botany*, 1-26.

Sharma, S., Kooner, R., Arora, R. (2017). Insect Pests and Crop Losses. In *Breeding Insect Resistant Crops for Sustainable Agriculture*, Eds.; Springer Singapore: Singapore, 2017; pp. 45.

Shin, R., Berg, R. H., Schachtman, D. P. (2005). Reactive Oxygen Species and Root Hairs in *Arabidopsis* Root Response to Nitrogen, Phosphorus and Potassium Deficiency. *Plant and Cell Physiology*, 46: 1350-1357.

Shin, R., Schachtman, D. P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proceedings of the National Academy of Sciences*, 101: 8827-8832.

Shinya, T., Yuko, H., Yoshitake, D., John, T., Christeller, K. O., Naoto, S., Ivan, G. (2016). "Modulation of Plant Defense Responses to Herbivores by Simultaneous Recognition of Different Herbivore-Associated Elicitors in Rice." *Scientific Reports*, 6 (1): 1-13.

Steffens, B., Steffen-Heins, A., Sauter, M. (2013). Reactive oxygen species mediate growth and death in submerged plants. *Frontiers in Plant Science*, 4.

Stotz, H. U., Kroymann, J., & Mitchell-Olds, T. (1999). Plant-insect interactions. *Current Opinion in Plant Biology, 2*(4), 268-272. doi:10.1016/s1369-5266(99)80048-x

Suh, S. J, Park J, Lee Y. (1998). Possible involvement of phospholipase A2 in light signal transduction of guard cells of *Commelina communis*. *Physiologia Plantarum*,104: 306-310

Suzuki, N, Gad, M, Jorge, T. M., Vladimir, S., Miguel, A. C. T., Ron, M. (2011). "Respiratory Burst Oxidases: The Engines of ROS Signaling." *Current Opinion in Plant Biology,* 14 (6): 691-699.

Swanson, S. J., Choi, W. G., Chanoca, A., Gilroy, S. (2011). In Vivo Imaging of Ca^{2+} , pH, and Reactive Oxygen Species Using Fluorescent Probes in Plants. *Annual Review of Plant Biology*, 62: 273-297.

Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K., Dolan, L. (2008). Local Positive Feedback Regulation Determines Cell Shape in Root Hair Cells. *Science*, 319: 1241-1244.

Talaat, N.B.(2019).. Role of Reactive Oxygen Species Signaling in Plant Growth and Development. Reactive Oxygen, Nitrogen and Sulfur Species in Plants. John Wiley & Sons, Ltd, 225-266.

Tan, S., Sagara, Y., Liu, Y., Maher, P., Schubert, D. (1998). The Regulation of Reactive Oxygen Species Production during Programmed Cell Death. *Journal of Cell Biology*, 141: 1423-1432.

Tayal, M., Somavat, P., Rodriguez, I., Thomas, T., Christoffersen, B., Kariyat, R. (2020a). Polyphenol-Rich Purple Corn Pericarp Extract Adversely Impacts Herbivore Growth and Development. *Insects*, 11: 98.

Tayal, M., Somavat, P., Rodriguez, I., Martinez, L., Kariyat, R. (2020b). Cascading effects of polyphenol-rich purple corn pericarp extract on pupal, adult, and offspring of tobacco hornworm (*Manduca sexta* L.). *Communicative & Integrative Biology*, 13: 43-53.

Teardo, E., Carraretto, L., De Bortol,i S., Costa, A., Behera, S., Wagner, R., Lo Schiavo, F., Formentin, E., Szabo, I. (2015). Alternative splicing-mediated targeting of the *Arabidopsis* GLUTAMATE RECEPTOR3.5 to mitochondria affects organelle morphology. *Plant Physiology*, 167: 216-227.

Teardo, E., Formentin, E., Segalla, A., Giacometti, G. M., Marin, O., Zanetti, M., Lo Schiavo, F., Zoratti, M., Szabò, I. (2011). Dual localization of plant glutamate receptor AtGLR3.4 to plastids and plasma membrane. *Biochimica et Biophysica Acta*, 1807: 359-367.

Tena, G, Marie, B, & Jen, S. (2011). "Protein Kinase Signaling Networks in Plant Innate Immunity." *Current Opinion in Plant Biology*, 14 (5): 519-529.

Thordal-Christensen, H., Ziguo, Z., Yangdou, W., & David, B. C. (1997). "Subcellular Localization of H_2O_2 in Plants. H_2O_2 Accumulation in Papillae and Hypersensitive Response during the Barley—Powdery Mildew Interaction." *The Plant Journal*, 11 (6): 1187-1194.

Tian, D., Peiffer, M., Shoemaker, E., Tooker, J. F., Haubruge, E., Francis, F., Luthe, D. S, Felton, G. W. (2012). Salivary glucose oxidase from caterpillars mediates the induction of rapid and delayed-induced defenses in the tomato plant. *PLoS One* 7, e36168

Torres, M. A, & Jeffery, L. D. (2005). "Functions of the Respiratory Burst Oxidase in Biotic Interactions, Abiotic Stress and Development." *Current Opinion in Plant Biology,* 8 (4): 397-403.

Toyota, M., Dirk, S., Sawai-Toyota, S., Wang, J., Tong, Z., Abraham, J. K., Gregg, A. H, & Simon, G. (2018). "Glutamate Triggers Long-Distance, Calcium-Based Plant Defense Signaling." *Science*, 361 (6407): 1112-1115.

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., et al. (2010). Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological Reviews,* 62: 405-496.

Turlings, T. C., Erb, M. (2018). Tritrophic Interactions Mediated by Herbivore-Induced Plant Volatiles: Mechanisms, Ecological Relevance, and Application Potential. *Annual Review of Entomology***,** 63:433-452.

Vadassery, J., Michael, R., Bettina, H., Jonathan, G., Wilhelm, B., & Axel, M. (2012). "CML42-Mediated Calcium Signaling Coordinates Responses to *Spodoptera* Herbivory and Abiotic Stresses in Arabidopsis." *Plant Physiology*, 159 (3): 1159-1175.

Van der, M. E. (2015). Herbivorous Insects- A Threat for Crop Production. 103-114.

Vatsa, P., Chiltz, A., Bourque, S., Wendehenne, D., Garcia-Brugger, A., Pugin, A. (2011). Involvement of putative glutamate receptors in plant defence signaling and NO production. *Biochimie,* 93: 2095-2101. doi:10.1016/ j. biochi.2011.04.006

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., Sharma, H. C. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signaling & Behavior*, 7: 1306-1320.

Wojtala, A., Bonora, M., Malinska, D., Pinton, P., Duszynski, J., Wieckowski, M. R. (2014). Methods to Monitor ROS Production by Fluorescence Microscopy and Fluorometry. *Methods in Enzymology Conceptual Background and Bioenergetic/Mitochondrial Aspects of Oncometabolism*, 243-262.

Woolley, J., Stanicka, J., Cotter, T. (2013). Recent advances in reactive oxygen species measurement in biological systems. *Trends in Biochemical Sciences, 38*: 556-565.

Wu, J., Baldwin, I. T. (2009). Herbivory-induced signalling in plants: perception and action. *Plant, Cell & Environment*, 32: 1161-1174.

Wu, J., Lei, W., H, W., & Ian, T. B. (2013). "Narboh D, a Respiratory Burst Oxidase Homolog in *Nicotiana Attenuata*, Is Required for Late Defense Responses after Herbivore Attack." *Journal of Integrative Plant Biology*, 55 (2): 187-198.

Wudick, M. M, Portes, M. T., Michard, E. et al. (2018). CORNICHON sorting and regulation of GLR channels underlies pollen tube Ca^{2+} homeostasis. *Science*, doi: 10.1126/science.aar6464

Yan, Y., Wei, C. L., Zhang, W. R., Cheng, H. P., Liu, J. (2006). Cross-talk between calcium and reactive oxygen species signaling. *Acta Pharmacologica Sinica*, *27*: 821-826.

Yang, T, Poovaiah, B. W. (2002). Hydrogen peroxide homeostasis: activation of plant catalase by calcium/calmodulin. *Proceeding National Academy Science USA,* 99: 4097-4102.

Yang, T., & Poovaiah, B. W. (2003). "Calcium/Calmodulin-Mediated Signal Network in Plants." *Trends in Plant Science,* 8 (10): 505-512.

Young, V. R., Ajami, A. M. (2000). Glutamate: an amino acid of particular distinction. *Journal of Nutrition*, 130: 892S-900S.

Yuan, F., Yang, H. M., Xue, Y., Kong, D. D., Ye, R., Li, C. J. et al. (2014). OSCA1 mediates osmotic-stress-evoked Ca^{2+} increases vital for osmosensing in *Arabidopsis*. *Nature,* 514:367 371.

Zebelo, S. A., Maffei, M. E. (2012). Signal Transduction in Plant–Insect Interactions: From Membrane Potential Variations to Metabolomics. *Plant Electrophysiology*, 143-172.

Zebelo, S. A., Maffei, M. E. (2014). Role of early signalling events in plant-insect interactions. *Journal of Experimental Botany*, 66: 435-448.

Zhai, Z., Jung, H. I., Vatamaniuk, O. K. (2009). Isolation of Protoplasts from Tissues of 14-day-old Seedlings of *Arabidopsis thaliana*. *Journal of Visualized Experiments*,1-3.

Zhao, L., Chen, J., Cheng, D., Sun, J., Liu, Y., Tian, Z. (2009). Biochemical and molecular characterizations of *Sitobion avenae*-induced wheat defense responses. *Crop Protection*, 28: 435-442.

BIOGRAPHICAL SKETCH

Akanksha Gandhi completed her schooling from DAV Senior Secondary Model School, Abohar, Punjab, India in 2013. After that, she joined Punjab Agricultural University, Ludhiana, Punjab where she pursued Bachelor's in Biotechnology (Hons.) in 2017. To fulfil her quest, she decided to join Sahoo lab in the United States where she conducted the research on Plant defense signaling. She completed Master's in Biology from the University of Texas Rio Grande Valley in December 2020. She will start her PhD at International Max Planck Institute of Research Ecology in Jena, Germany. She has strong interest in research and wants to acquire new skills and knowledge. She can be contacted through email- akankshagandhi037@gmail.com