Gene Regulation in Plant Herbivory Defense: Effect of Insect Mechanical Wounding and Chemical Oral Secretion Factors

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Table of Contents

1 INTRODUCTION 1

1.1 Plant herbivory defense 1

1.1.1 Plant perception of insect herbivory, the triggers: Mechanical wounding and chemical elicitors 1

1.1.2 Signaling: Receptors and pathways 2

1.1.3 Gene regulation caused by insect herbivory 5

1.1.4 Plant defense strategies on insect feeding 7

1.2 Differential gene expression analysis 8

1.2.1 Principles of DNA microarray 9

1.2.2 Bioinformatics tools for microarray data analysis 11

1.2.3 Reverse transcription real time polymerase chain reaction (RT-qPCR) 12

1.3 Mimicking insect feeding both mechanically and chemically 15

1.4 Study Systems 16

1.4.1 *Arabidopsis thaliana - Plutella xylostella* 16

1.4.2 *Phaseolus lunatus - Spodoptera littoralis* 17

2 AIMS OF THIS STUDY 21

3 METHODS AND MATERIALS 23

3.1 Global analysis of microarray data on leaf genes of *Arabidopsis thaliana* treated with MecWorm and *Plutella xylostella* 23

3.1.1 Plant and insect materials 23

3.1.2 Plant treatments 23

3.1.3 Microarray preparation 24

3.1.4 1-Step comparative real-time PCR 25

3.2 Turning MecWorm into SpitWorm: robotic mechanical wounding with simultaneous application of salivary components 27

3.2.1 Plant and insect materials 27

3.2.2 SpitWorm 28

3.2.3 Collection of insect oral secretion 28

3.2.4 Insect foregut volume determination 28

3.2.5 Wounding size determination 28

3.2.6 Optimization of the injection volume of fluorescence solution 29

3.2.7 Fluorescence quantification 29

3.2.8 Analysis of headspace volatiles 30

3.2.9 1-Step comparative real-time PCR 31

4 RESULTS AND DISCUSSION 33
4.1 Global analysis of microarray data on leaf genes of *Arabidopsis thaliana* treated with MecWorm and *Plutella xylostella*..33
4.1.1 Overview of numbers of regulated genes 33
4.1.2 Overview of pathway regulations: Roles of mechanical wounding and chemical factors in both local and systemic samples...................................37
4.1.3 Significant pathways analysis 42
4.1.4 Cell compartment level of regulation: chloroplast, cell wall and nucleus 46
4.1.5 Compensatory energy system of chloroplast 54
4.1.6 Growth-defense tradeoff, signaling and resource communication between local and systemic leaves 55
4.1.7 Quantitative RT-PCR...58
4.2 Turning MecWorm into SpitWorm: robotic mechanical wounding with simultaneous application of salivary components 61
4.2.1 Wounding sizes of leaves fed by *Spodoptera. littoralis* 62
4.2.2 Determination of insect foregut volume..64
4.2.3 Calculation of spit amount left by insect onto plant wounds per bite 65
4.2.4 Oral secretion delivery rate of SpitWorm 66
4.2.5 Fluorescence imaging 66
4.2.6 Quantification of fluorescence on the leaf wounded site left by insect and SpitWorm 68
4.2.7 Volatile organic compounds induction..69
4.2.8 Comparative Quantitative real-time PCR 71
4.3 Mechanical wounding and chemical factors - MecWorm and SpitWorm 72
5 SUMMARY 75
6 ZUSAMMENFASSUNG..77
7 REFERENCES..81
8 APPENDIX 98
9 CURRICULUM VITAE 108
10 DECLARATION OF INDEPENDENT ASSIGNMENT 109
11 ACKNOWLEDGEMENTS...110

1 INTRODUCTION

1.1 Plant herbivory defense

As the basic and biggest food and energy producer for almost all living organisms on our planet, plants suffer biotic and abiotic threats from the environment. In nature, herbivorous insects are one of the major predators on them, especially on higher plants. Despite their physical immobility, plants have survived and propagated for hundreds of millions of years. During this long time, they have coevolved with herbivorous insects and developed strategies to fend, repel and annihilate their insect enemies (Mithöfer and Boland 2016). Plant defenses against herbivores have aroused ardent and tremendous interests and researches with profound achievements, especially in the recent 30 years (Heil 2014). These studies have deciphered that the feeding of insects can initiate a serial of defense events *in planta* such as signaling, secondary metabolites production and volatile emissions, which lead to or result from a shuffling expression of thousands of genes (Ehlting *et al.* 2008). The mechanism of plant defense strategies involves four main steps: perception of herbivory signals, signal transduction, gene regulation and defense implementation.

1.1.1 Plant perception of insect herbivory, the triggers: Mechanical wounding and chemical elicitors

Plants are thought to have evolved the ability to perceive herbivory associated molecular patterns (HAMPs) (Felton and Tumlinson 2008, Mithöfer and Boland 2008) but our understanding of these patterns is still in the very early stages. To date, HAMPs can be classified into two categories: (1) chemical elicitors derived from herbivore oral secretions or oviposition fluids; and (2) those that originate from the specific patterns of wounding, i.e. the mechanical damage and the resulting elicitors from plants. This second category is also called damage associated molecular patterns (DAMPs). Only both aspects together are able to induce the full spectrum of plant defenses (Bricchi *et al.* 2010).

Oral secretions (OS) from feeding insects can contain herbivore-specific compounds with elicitor properties. There are several types of elicitors discovered in insect OS. These elicitors include small molecular size fatty acid - amino acid conjugates (FACs) (Pohnert *et al.* 1999, Halitschke *et al.* 2001, Spiteller and Boland 2003a, Spiteller *et al.* 2004); inceptins (Schmelz *et al.* 2006, Schmelz *et al.* 2007); caeliferins (Alborn *et al.* 2007) and volicitin (Alborn *et al.* 1997); as well as glucose oxidase (GOX) (Coulthard

et al. 1942, Wong *et al.* 2008). These elicitors were reported to induce signaling pathways, biosynthesis of phytohormones and volatile emissions. However, compared with the vast diversity of herbivores that attack plants, the known herbivore-derived elicitors are relatively few. The molecular mechanism of plant perception of these elicitors needs further study (Wu and Baldwin 2010).

For a period of time, mechanical wounding with single or a few cuts or scratches with different wounding tools was used as a control or in combination with OS elicitors to study the defense inducing roles of chemical elicitors from OS, which was effective in inducing plant defense responses such as volatile emission and JA burst (Halitschke *et al.* 2001). However, using lima bean (*Phaseolus lunatus*) as a model system, mechanical wounding alone was not observed to cause induced volatile emission. Only with continuous mechanical wounding similar to a feeding insect, the emission of a blend of volatiles showing mostly quantitative differences on comparison with herbivore (*Spodoptera littoralis*) induced volatiles, was elicited (Mithöfer *et al.* 2005, Bricchi *et al.* 2010). Now that using lima bean as a model system mechanical wounding alone can induce plant herbivory response, it is possible that mechanical wounding itself plays important roles in plant defense induction.

1.1.2 Signaling: Receptors and pathways

1.1.2.1 Onsite signaling

Emerging evidence indicates that many high-affinity receptors for insect herbivores are located in the plant cell plasma membrane (Maffei *et al.* 2012). One of the earliest cellular responses to herbivory is membrane depolarization (Maffei *et al.* 2004, Zebelo and Maffei 2015), accompanying the elevation of cytosolic calcium (Lecourieux *et al.* 2006, Vadassery *et al.* 2009, Reddy *et al.* 2011, Kanchiswamy and Maffei 2015), ion efflux/influx (Bricchi *et al.* 2013), mitogen-activated protein kinase (MAPK) activation and protein phosphorylation (Meldau *et al.* 2009, Arimura and Maffei 2010, Arimura *et al.* 2011), the activation of NADPH oxidase, and the production of reactive oxygen (ROS) and nitrogen (RNS) species (Miller and Mittler 2006, Bricchi *et al.* 2010, Arimura *et al.* 2011, Marino *et al.* 2012). These cascades lead to a rise in the production of the phytohormones jasmonic acid (JA) (Engelberth *et al.* 2001, Koo and Howe 2009, Radhika *et al.* 2010), salicylic acid (SA) (Zipfel 2009, Consales *et al.* 2012), and ethylene (ET) (Arimura *et al.* 2009, Onkokesung *et al.* 2010, Diaz 2011, Scala *et al.* 2013). SA, JA and ET are recognized as key players in

the regulation of the signaling pathways involved (Howe 2004, Lorenzo *et al.* 2004, Pozo *et al.* 2004, Grant and Lamb 2006, von Dahl and Baldwin 2007). Other signaling molecules, including abscisic acid (ABA) (Mauch-Mani and Mauch 2005), brassinosteroids (Nakashita *et al.* 2003), cytokinins (Dervinis *et al.* 2010, Schäfer *et al.* 2015), auxin (Navarro *et al.* 2006, Wang *et al.* 2007) and R genes (Kaloshian 2004, Goggin 2007), have also been implicated in plant defense, but their significance is less well studied.

There are cross-talks among the major phytohormone signaling pathways. JA and ET activate expression of sets of genes that largely overlap with each other, but are mostly distinct from those induced by SA (Thaler *et al.* 2010). In general, JA and ET responses promote resistance to pests as well as some necrotrophic plant pathogens, but do not play a large role in defense to biotrophic pathogens. Several lines of evidence suggest that there is negative cross-talk between the jasmonate and salicylate response pathways (Engelberth *et al.* 2001, Farmer *et al.* 2003, Lorenzo *et al.* 2003, Berrocal-Lobo and Molina 2004, Cipollini *et al.* 2004, Leon-Reyes *et al.* 2009).

1.1.2.2 Systemic signaling

Wounding and defense signalings are not only restricted at the local wounding site or the wounded leaves (Herde *et al.* 1996, Baldwin *et al.* 1997, Rakwal *et al.* 2002, Koziolek *et al.* 2004). The systemic nature of many wound-induced responses provides an attractive opportunity to study intercellular signaling pathways that operate over long distances within the plant (Howe 2004). The production of wound-inducible proteinase inhibitors (PIs) in tomato and other solanaceous species has been widely used as a model system to study systemic wound signaling and its role in plant defense against insects (Green and Ryan 1972, Schilmiller and Howe 2005). In tomato, systemin was thought to be released by wounded leaves to convey wounding or herbivory alert to systemic leaves (Mcgurl *et al.* 1992, Schaller and Ryan 1996). However, systemin was later proven not to be the mobile signal and not necessary for induction of systemic responses. Instead, the production of JA in damaged leaves and the perception of JA in distal leaves are necessary for inducing systemic responses (Li *et al.* 2002).

The roles of mechanical wounding and chemical elicitors in systemic signaling have also been studied. Several kinds of chemical (Leon *et al.* 2001) and physical (Stankovic *et al.* 1998) signals and even their combination (Malone 1996) induced by

local wounding have been implicated. A number of studies have indicated that the vascular system is involved in the transportation of these mobile signals (Jones *et al.* 1993, Orians *et al.* 2000). Electric and hydraulic signals were proposed to play a role as well (Wildon *et al.* 1992, Malone *et al.* 1994, Stankovic and Davies 1997, Mousavi *et al.* 2013). Recently published results show that mechanical wounding of the midrib of *Arabidopsis thaliana* plant leaves causes systemic Ca^{2+} responses. These responses can be suppressed by the presence of insect oral secretions (Kiep *et al.* 2015). From these results it can be concluded that systemic signaling can be induced by mechanical wounding alone and chemical elicitors can have a regulating effect to the induction.

1.1.2.3 Damaged-self recognition signaling

The concept of 'damaged-self recognition' is based on the observation of a general principle underlying animal feeding or microbial infection on plant tissues (Dicke and Sabelis 1988, Devaiah *et al.* 2009, Heil 2014). All of these feeding activities result in the disruption and disintegration of plant cells. "*Most elicitors of plant herbivory defenses represent, or contain, parts of plant-derived molecules that are degraded, digested or localized outside their original cell compartment. To the plant, these elicitors indicate the 'damaged self'. Such elicitors are released from disrupted cells and are probably perceived by receptors that monitor the extracellular chemistry. Thus, the information on the 'damaged self' is transported into the inner compartments of intact and metabolically active cells, which react through metabolic responses such as the synthesis of systemic signals and defense compounds*" (Heil 2009).

There have been a number of studies showing that both the mechanical damaging alone and applying damaged leaf extract or 'leaf juice' to damaged plant parts can induce defense responses of plant (Green and Ryan 1972, Ryan 1974, Turlings *et al.* 1993, Mattiacci *et al.* 1995). These studies proved that the damaging and damaged motifs of the plant itself can induce the herbivore defense signaling, independently from elicitors from herbivores or pathogens. Therefore, it was highlighted that 'the damaged plant cell itself' is actually a source of elicitors that are common to all types of herbivory. These plant-derived elicitors were called 'Damaged-self associated molecular patterns' (DAMPs)(Heil and Land 2014).

Further studies showed that flame wounding or applying leaf extract or solutions of sucrose or ATP to slightly wounded lima bean (*P. lunatus*) leaves induced the secre-

tion of extrafloral nectar, an indirect defense mechanism. Treatments inducing extrafloral nectar secretion also enhanced endogenous concentrations of the defense hormone jasmonic acid. Endogenous JA was also induced by mechanically damaging leaves of lima bean, *Arabidopsis thaliana*, maize, strawberry, sesame and tomato. In lima bean, tomato and sesame, the application of leaf extract further increased endogenous JA content, indicating that damaged-self recognition is taxonomically widely distributed. Transcriptomic patterns obtained with untargeted 454 pyrosequencing of lima bean in response to flame wounding or the application of leaf extract or JA were highly similar to each other, but differed from the response to mere mechanical damage. It was concluded that the amount or concentration of damaged-self signals can quantitatively determine the intensity of the wound response and that the full damaged-self response requires the disruption of many cells (Heil 2009, Heil *et al.* 2012).

1.1.3 Gene regulation caused by insect herbivory

Plant defense activities and strategies are conducted through gene expression. When the insect is feeding, signals are transmitted to the nucleus of the plant cell; they trigger the regulation of gene expression. Thus, accompanying the various defense activities of plant, there is a high number of genes which are up- or down-regulated, or even switched on or shut off. The reshufflings of gene expression have certainly become a hotspot for research on plant defense against insect herbivory.

Research on gene regulation of plants defenses against chewing insects covers mainly three aspects: a) global analysis of gene regulation (van Verk *et al.* 2009, Rehrig *et al.* 2011, Artico *et al.* 2014); b) analysis of specific defense gene groups or pathways (e.g. JA responsive genes, mechanical wounding responsive genes, calcium signaling genes, gene regulators, etc.) (Staswick *et al.* 2002, Browse and Howe 2008, Mondego *et al.* 2011, Porth *et al.* 2011, VanDoorn *et al.* 2011, Bricchi *et al.* 2013); and c) candidate genes determination for special physiological process or disease study (Laluk and Mengiste 2011, Nakata *et al.* 2013).

On the level of plant defense triggering, responsive genes to insects' feeding are distinct from those to wounding in both numbers and identities (Appel *et al.* 2014). For the plant it is crucial to distinguish between wound- and herbivore-specific components of insect attack as well as between attack from different feeding guilds such as

chewing and piercing-sucking herbivores to mount appropriate plant defense responses (Heidel-Fischer *et al.* 2014).

On the level of plant defense strategies, there has been a lot of success in identifying regulated genes. These genes are involved in signaling pathways (Ankala *et al.* 2013, Kovalchuk *et al.* 2015, Schäfer *et al.* 2015, Thireault *et al.* 2015), secondary metabolism (Mewis *et al.* 2012, Gaquerel *et al.* 2014, Seidl-Adams *et al.* 2015), herbivory associated molecular patterns (HAMPs) and elicitors (Lawrence *et al.* 2008, Suzuki *et al.* 2012), resource allocations (Philippe *et al.* 2010) or growth environment (Casteel *et al.* 2008).

It has been proved in downstream studies that when plant is fed by herbivory insect, structures and functions of cell compartments, such as cell wall (Sasidharan *et al.* 2011) and chloroplast (Macedo *et al.* 2006, Tang *et al.* 2006, Nabity *et al.* 2013), are influenced (Aldea *et al.* 2006). A plant cell is a comprehensive facility that composes of communication and collaboration of all organelles. When plant suffers insect herbivory, it is the healthy cells surrounding the damaged cells or even the systemic cells who conduct the defense responses. How do these healthy cells deal with the damage signal from the damaged parts? Which compartments need immediate recovery and which need give priority to defense production? How is this collaboration regulated at gene expression level? Up to now the understanding of regulation of related genes and pathways at the comprehensive compartment level is limited.

With the development of new technologies, especially the next-generation sequencing (NGS), it is possible to get the expression information of the whole genome in a very efficient way. Since all the ‘life information’ of an organism is ‘coded’ in the genome, the ideal scenario would be that all the regulated genes representing specific pathways, organs, or tissues could be clearly grouped and perfectly represent the physiological performances of the responsive parts. However, there are both inner and outer plant factors which break this perfection: on one hand, plant genomes are relatively huge, so are the number of genes, the networking of genes is complicated, morphological variance of each plant and our knowledge in annotating the genes and pathways is limited compared to the information that is obtained; on the other hand, the mechanism of expression of each gene is delicate, even very little variation of temperature, light condition, nutrition condition, water condition or other growth factors can cause a variation of gene expression.

Nevertheless, with the help of powerful statistics, computer analysis tools, and the development of our knowledge, in combination with the downstream researches (e.g. proteomics and metabolomics), gene expression information can be used to do global analysis for general perspectives and as proofs and inspirations for downstream studies.

1.1.4 Plant defense strategies on insect feeding

When plants are under insect attack, some species produce defensive traits that affect the insect preferences, such as host plant selection and feeding behavior. Others affect the insect performances, such as growth rate and development. These traits of plants include morphological features for physical defense and the production of compounds for chemical defense. Physical defense elements include thorns, trichomes, cuticles, wax, resins and extrafloral nectaries (EFN). Chemical defense elements include secondary metabolites such as glucosinolates, cyanogenic glucosides, alkaloids, phenolics, and protease inhibitors (PIs) which can function as toxins, repellents, or antidigestives.

These defense features of plants are also categorized as constitutive defense and induced defense. Constitutive defenses are physical and chemical defensive traits that plants have regardless of the presence of herbivores; in contrast, inducible defenses are mounted only after plants are attacked by herbivores.

Another system of categorizing plant defense strategies is direct and indirect defense. Direct defense are the toxins, repellents, antidigestive compounds, etc., that directly and negatively affect herbivore growth, reproduction, or fecundity. Indirect defense is e.g. associated with the production of volatile compounds that betray the location of feeding herbivores to their predators, or substances that reward (e.g., extrafloral nectar) and thus nourish organisms that provide carnivore services for the plant and thereby reduce their herbivore loads (Wu and Baldwin 2010).

While enhanced secondary metabolism undoubtedly exerts an influence on primary metabolism, accumulating evidence indicates that rather than stimulating photosynthesis insect herbivory reduces photosynthetic carbon fixation (Wasternack *et al.* 1998, Gassmann and Futuyma 2005, Halitschke *et al.* 2011, Kerchev *et al.* 2012). A direct influence from lower carbon fixation is resource allocation within damaged plant. The existing publications suggest that induced defenses evolved be-

cause they have lower resource allocation costs than constitutive resistance traits (Karban *et al.* 1997, Baldwin 1998, Howe and Jander 2008). However, herbivory defense and recovery are very costly processes for plants. They result in lower growth rate, later and slower fruit and seed production, decreased flowering and of course, lower biomass (Strauss *et al.* 2002). Hence plants have developed optimal resource allocation patterns for herbivory defense before, during and after herbivory (Ito and Sakai 2009, Siemens *et al.* 2010).

Resource allocation is a whole plant mission. It is important for the plant to have an efficient and ecological communication and transportation system. Therefore, it is not only the local damaged leaves which are conducting herbivory defense. Systemic plant leaf defense is also critical and important. It has been shown that Ca^{2+} signaling (Arimura and Maffei 2010, Kanchiswamy and Maffei 2015, Kiep *et al.* 2015), cytokinin signaling (Schäfer *et al.* 2015), cadmium accumulation (Plaza *et al.* 2015), ROS pathway and JA signaling (Maffei *et al.* 2012), and salicylic acid pathway (Hettenhausen *et al.* 2014) are involved.

1.2 Differential gene expression analysis

During the last years several methods have been developed to study gene regulation of organisms under certain conditions such as diseases, biotic or abiotic stresses and different life periods, or to compare gene expressions between different systems, e.g. genes, pathways, cells, organs, tissues or whole genomes. All of these methods are based on the basic hybridization of nucleic acids, or the polymerase chain reaction (PCR). For example, for detecting all possible differences or mutations in all DNA or RNA sequences, direct sequencing of the genome can be used; for locating and detecting specific genes on chromosomes, there is fluorescence in situ hybridization (FISH); for comparing gene expression quantitatively, most commonly used methods are real-time PCR by comparing the relative abundance of specific genes after proportional amplification of the cDNA, and DNA microarray by high throughput quantification of the genome or specific cDNAs.

With the development of new technologies, especially the next-generation sequencing (NGS), it is possible to get the expression information of the whole genome in a very efficient way. Next-generation high-throughput DNA sequencing techniques were selected by Nature Methods as the method of the year in 2007 (Editorial 2008, Ansorge 2009, Hurd and Nelson 2009). Due to the decline of price in the recent years, more

and more researchers are using RNA sequencing (RNA-Seq) technology instead of microarray as genome-wide association mapping method (Hurd and Nelson 2009, Zhao *et al.* 2014, Sarwat and Yamdagni 2016). RNA-Seq has become an alternative to microarrays and not only provides sequence information, but also measures quantitative and qualitative transcriptional differences (Heidel-Fischer *et al.* 2014). However, for high-throughput gene expression analysis of an already well-studied system such as *A. thaliana*, with the main purpose of gene relative expression level instead of sequence information, microarray with the well-established analysis tools and relative lower costs is still widely used.

Nevertheless, with the help of powerful statistics, computer analysis tools, and the development of our knowledge, in combination with the downstream researches (e.g. proteomics and metabolics), gene expression information can be used to do global analysis for general perspectives and as proofs for downstream studies. Despite of the imperfection of technology, high-throughput gene expression analysis remains to be a powerful tool, and will continue to be so in the future (Liang *et al.* 2004, Ullman *et al.* 2013).

1.2.1 Principles of DNA microarray

The DNA microarray technology started to appear during the second half of the 1990s (Schena *et al.* 1995), and has historically evolved from the initial experimental reports published in the mid-1970s (Galau *et al.* 1974, Klein *et al.* 1974, Lauer *et al.* 1977) which indicated that labeled nucleic acids could be used to monitor the expression of nucleic acid molecules attached to a solid support. Representing techniques are Southern blot (Southern 1975) and Northern blot (Alwine *et al.* 1977). However, it was not until 1995 that the first article describing the application of DNA microarray technology to expression analysis was published by Patrick Brown and his colleagues at Stanford University (Schena *et al.* 1995).

DNA microarray is an orderly arrangement of thousands of identified sequenced genes fixed on an impermeable solid support, usually glass, silicon chips, nylon membrane or microscopic polystyrene beads. Each identified sequenced gene corresponds to a fragment of single-stranded genomic DNA, cDNA, PCR product or chemically synthesized oligonucleotide of up to 70 nucleotides (70-mers). Each spot or bead attaches thousands of copies of the same fragment to insure the full hybridization of complementary targets from the samples. A single DNA microarray slide/chip may

contain up to thousands of spots representing part of or the entire genome of an organism, depending on the topic being worked on.

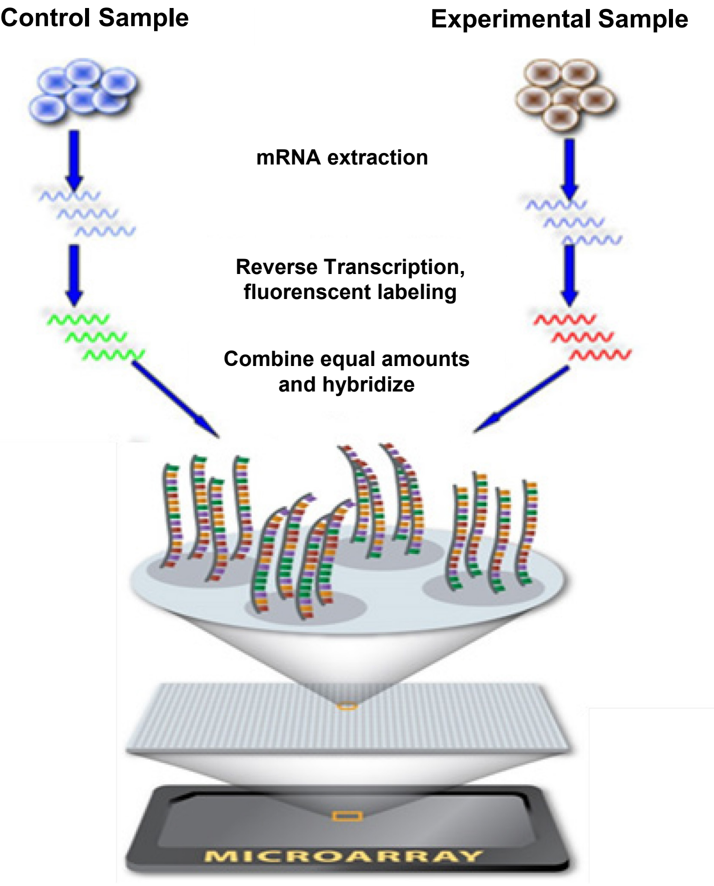

Figure 1. Basic structure of a two color cDNA microarray chip. Modified from pictures obtained online (Grigoryev 2011, Genetic Science Learning Center 2013).

If relative expression of two samples is to be examined, each labeled with a different dye (See Figure 1, red for experiment, green for control), the resulting image is analyzed by calculating the ratio of the intensity of two dyes. If a gene is over-expressed in the experimental sample, then more sample-cDNA than control-cDNA will hybridize to the spot representing expressed gene. In turn, the spot will fluoresce red with greater intensity than it will fluoresce green. The red-to-green fluorescence ratio thus indicates which gene is up or down-regulated in the appropriate sample. A single microarray experiment can provide information on the expression of thousands of genes, pathways, virtually the entire genome, to compare expression patterns between any two states.

1.2.2 Bioinformatics tools for microarray data analysis

After microarray hybridization, the fluorescence signals are normally measured by intensity. These intensities are then background-corrected to remove the effects of non-specific binding or spatial heterogeneity; log2 transformed to avoid disadvantage of treating up- and down-regulated genes differently; and normalized to the control to exclude the biological biases. For each step of data processing, there are different statistical methods developed depending on the design, the purpose and sample size of the experiments (Finkelstein *et al.* 2002, Quackenbush 2002, Ritchie *et al.* 2007).

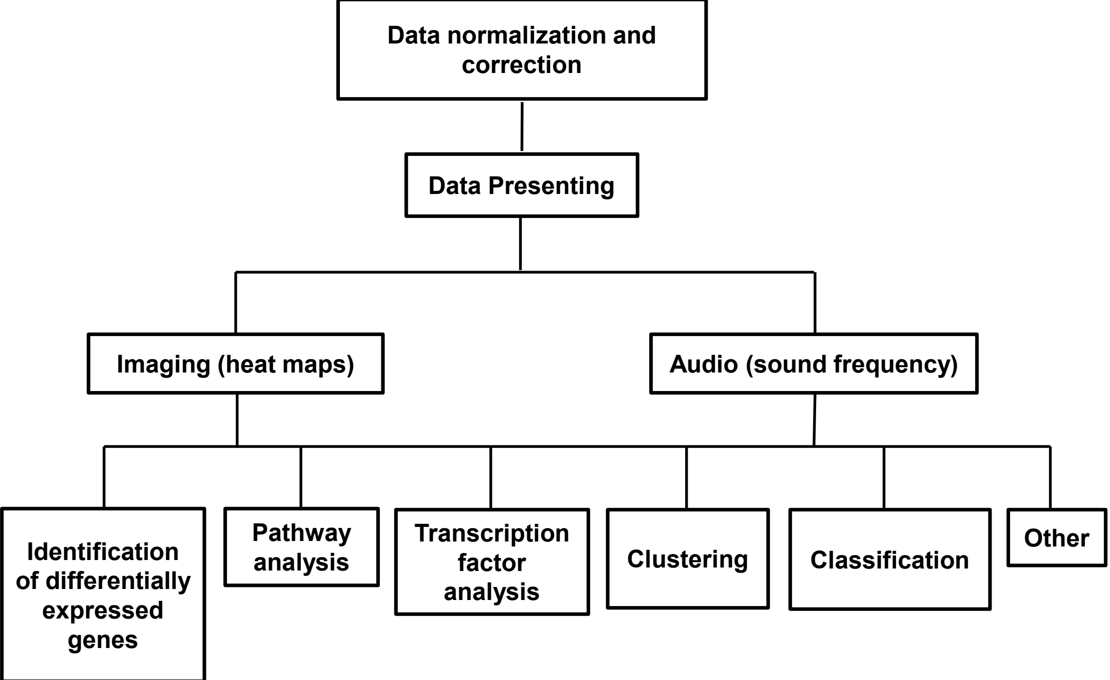

Figure 2. Bioinformatics data mining of microarray results.

After statistical normalization, the data are generally processed with different tools (softwares, statistic packages, databases, etc.) for analysis and visualization (Cleveland 1979, Kerr *et al.* 2000, Finkelstein *et al.* 2002, Huang *et al.* 2009b, a). The most common way of presenting the microarray data is done with heat maps showing up- or down-regulated genes in colors with different intensities. Recently a method was published that it is possible to present the expression data as audio signals, or musical frequencies (Staege 2015).

Microarray data are powerful information sources for many analysis purposes (Figure 2). For example, gene clustering to reveal biologically meaningful patterns from big set of expression data (Svrakic *et al.* 2003); gene identification to highlight and identify significantly regulated genes as candidate genes (Dekkers *et al.* 2012, Zhao *et al.*

2015); pathway and network analysis to examine how are the pathways regulated in a specific experiment (Genoud and Metraux 1999, Guffanti 2002, Yue and Reisdorf 2005); transcription factor analysis (Pritsker *et al.* 2004, Chowdhary *et al.* 2010); classification to predict or assign certain genes to groups or classes (Golub *et al.* 1999, Quackenbush 2001); as well as other analysis such as Gene Set Enrichment Analysis (GSEA) by focusing on gene sets, i.e. groups of genes that share common biological function, chromosomal location, or regulation (Subramanian *et al.* 2005); and transcription profiling to profile an organism or cell at all life stages under different conditions (Zhou *et al.* 2015, Zhu *et al.* 2015) .

1.2.3 Reverse transcription real time polymerase chain reaction (RT-qPCR)

Reverse transcription real-time PCR (RT-qPCR) [1] (Bustin *et al.* 2009) is an advanced form of the polymerase chain reaction (PCR). First, it uses RNA instead of DNA template, which means it starts with the step of reverse transcription (RT PCR) to convert RNA to cDNA. Second, it records the product of each PCR reaction cycle by fluorescence excitation or quenching technique. Third, it enables the relative quantification of specific gene expression through comparing the fluorescence amount of target genes and control genes.

RT-qPCR has become one of the cornerstones in molecular biology, in that it enables the quantification of gene expression in a precise and fast way. It is widely used to quantify the expression of specific candidate genes and to verify the results from other comparative genomic or proteomic analysis. Nowadays, with the development of the technique, it is also possible to do absolute RT-qPCR to determine the exact copy number of the target gene even within a single cell or genome (Tellinghuisen and Spiess 2015).

There are different ways of fluorescence labelling for qPCR. Two most commonly used are shown in Figure 3. On Figure 3-A there is a probe (short nucleotide, very high-specifically bonded to the target sequence, with a usual length of 20- 30-mers) with a reporter fluorescent 5´-end and a quencher at 3´-end. Fluorescence is not emit-

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[1] qPCR has many names, i.e. qPCR, real-time PCR, qRT-PCR, real-time qPCR or real-time qRT-PCR. Bustin and others proposed in the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines that abbreviation qPCR should be used for quantitative real-time PCR and that RT-qPCR should be used for reverse transcription-qPCR since applying the abbreviation RT-PCR to qPCR causes confusion and is inconsistent with its use for conventional (legacy) reverse transcription-PCR.

ted as long as reporter and quencher are linked to the same probe. When the two are dislocated (when the probe is dissolved away by 5´-exonuclease activity of TaqDNA polymerase during PCR elongation), reporter molecule freely emits the fluorescence that can be detected. The method shown in Figure 3-B uses an intercalating fluorophore which directly illuminate the newly synthesized double-stranded DNAs (dsDNAs). Special intercalating dyes are used to strongly increase emission of fluorescence whenever they intercalate into a dsDNA. This method doesn't need fluorescent probes.

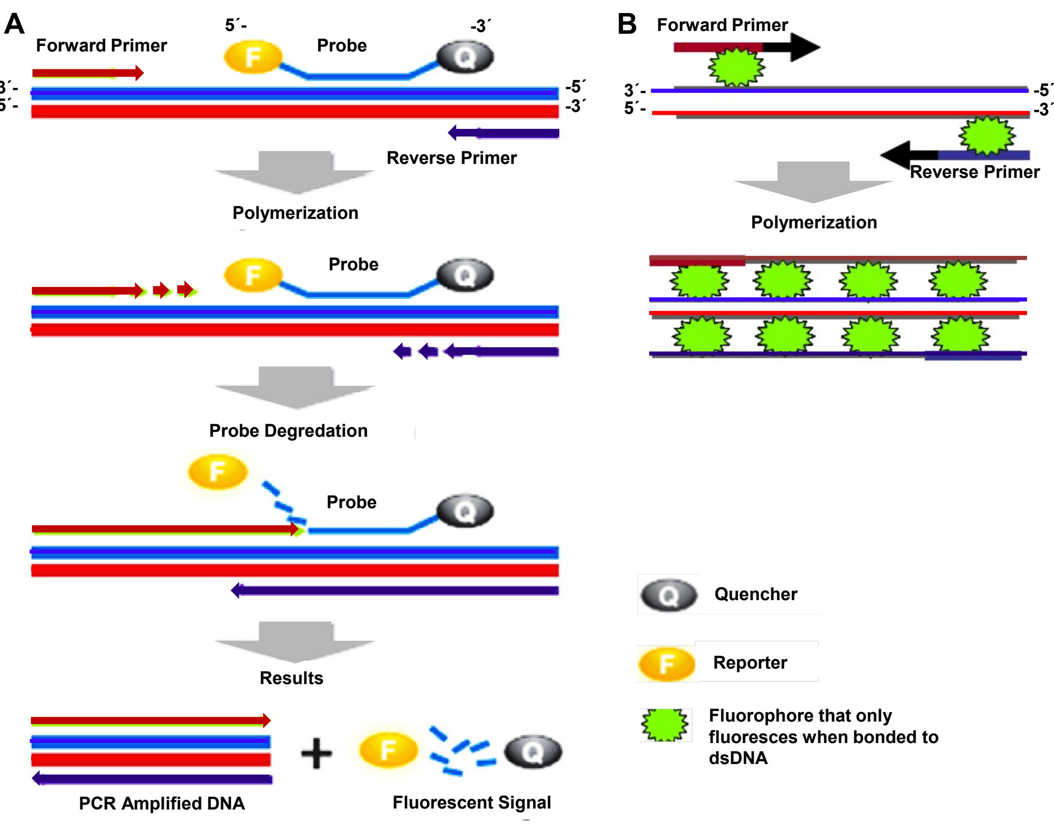
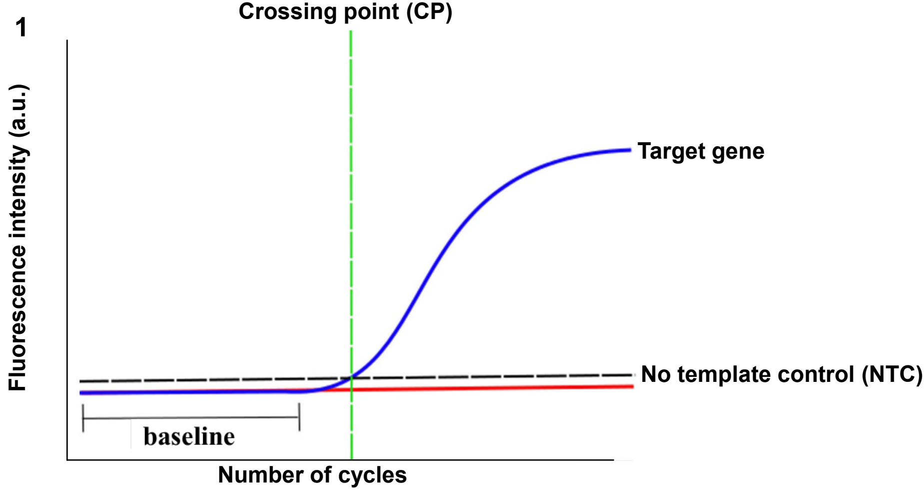


Figure 3. Two common fluorescence labelling methods of qPCR. Modified from picture obtained online (Medicinal Genomics 2015).

In qPCR, the intensity of the fluorescence emitted during amplification is directly proportional to the amount of amplified DNA. Fluorescence is monitored during the whole PCR process (along all 30 to 45 cycles). The higher the initial number of DNA molecules in the sample, the faster fluorescence will increase during the PCR cycles. In other words, if a sample contains more targets, fluorescence will be detected in earlier cycles. Initially, fluorescence remains at background levels, and increases in

fluorescence are not detectable (Figure 4-1) even though product accumulates exponentially.



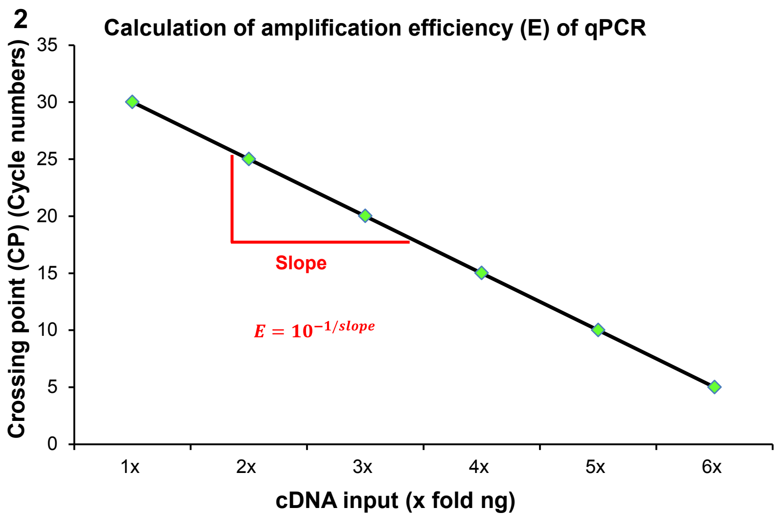


Figure 4. Two variants in calculation of relative expression of genes from qPCR. 1. Detection of crossing point (*CP*), 2. Determination of amplification efficiency (*E*). Pictures modified from webpages (Pfaffl *et al.* 2002, Porterfield 2015).

Eventually, enough amplified product accumulates to yield a detectable fluorescence signal. The cycle in which fluorescence can be detected is termed as quantitation cycle (*Cq*), threshold cycle (*Ct*) or crossing point (*CP*) (Figure 4-1) and is the basic result of qPCR: lower *CP* values mean higher initial copy numbers of the target. The amplification efficiency (*E*) of a gene is calculated based on the slope of the standard curve of the amplification of a specific gene (*CP* versus different DNA amounts). $E = 10^{-1/slope}$ (Figure 4-2).

The mostly used qPCR relative expression ratio calculating formula is as follows:

$$relative\ expression = \frac{E_{target}^{\overline{CP}_{target\ control} - \overline{CP}_{target\ sample}}}{E_{ref}^{\overline{CP}_{ref\ control} - \overline{CP}_{ref\ sample}}}$$

$$= \frac{E_{target}^{\Delta CP_{target}}}{E_{ref}^{\Delta CP_{ref}}}$$

E_{target} is the real-time PCR efficiency of a target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript, reference gene is normally a housekeeping gene from the same system and has a stable expression level under all conditions ΔCP_{target} is the *CP* deviation of the target gene transcript; ΔCP_{ref} is the *CP* deviation of reference gene transcript (Pfaffl 2001).

The second model available, the "delta-delta *CP* method" for comparing relative expression results between treatments in real-time PCR is presented by PE Applied Biosystems (Perkin Elmer, Forster City, CA, USA). The model presumes the optimal and identical real-time amplification efficiencies of target and reference gene of $E = 2$. "Delta-delta *CP* method" is only applicable for a quick estimation of the relative expression ratio. For such a quick estimation the relative expression of a gene can be shortened:

$$relative\ expression = \frac{E_{target}^{\Delta CP_{target}}}{E_{ref}^{\Delta CP_{ref}}} = 2^{(\Delta CP_{target} - \Delta CP_{ref})} = 2^{-(\Delta CP_{ref} - \Delta CP_{target})}$$

$$= 2^{-\Delta\Delta CP}$$

1.3 Mimicking insect feeding both mechanically and chemically

Feeding herbivores cause extensive and continuous mechanical wounding to plants along with introduction of oral secretions (OS). To study the individual contributions of these two factors separately, mechanical wounding of insect feeding was originally mimicked with different tools, including razor blades (Schmelz *et al.* 2003, Lawrence and Novak 2004, Angelini *et al.* 2008), pattern wheels (Major and Constabel 2006, Skibbe *et al.* 2008, VanDoorn *et al.* 2010), forceps (Vogel *et al.* 2007, Botelho *et al.* 2008, Hind *et al.* 2011), paper punches (Raghava *et al.* 2010) and needles (Zhou *et al.*

2009). All of these studies clearly revealed local defense responses but no induced volatile emission. Only after applying continuous mechanical wounding similar to a feeding insect by a robotic system (MecWorm), a clear defense response was observed, i.e. the emission of a blend of volatiles showing mostly quantitative differences on comparison with herbivore (*S. littoralis*) elicited volatiles (Mithöfer *et al.* 2005, Bricchi *et al.* 2010).

Methods to study insect OS or insect elicitors include mainly applying OS or OS-derived components to mechanical wounding to mimic insect feeding and examine plant defense response. Musser *et.al* (Musser *et al.* 2006) used two different methods to suppress the production of OS of caterpillars during feeding by either surgically removing the labial salivary gland or cauterizing the insect's spinneret. In spite of different wounding or OS application methods, the OS amounts were normally 1-20 µL (with dilution factors varying from 1:1 to 1:5) on a wounding area varying from a few scratches or puncture rows up to 2% of the total leaf (Skibbe *et al.* 2008, Hummel *et al.* 2009, VanDoorn *et al.* 2010). In all of these previous attempts only estimations for the chosen dilutions of OS and wounding areas were given. Major and Constabel (Major and Constabel 2006) used a range of oral secretion dilutions including 1:1, 1:5, 1:20, 1:60, and 1:180 (v/v) with water over 100 puncture holes and decided to use 1:5 for further experiments because the target gene *PtdTI3* reached a maximum induction at that point. However, one can calculate that these amounts were often more than thousand times higher than the real amount left behind by the insect, which is not more than a few picoliters (Rossignol and Spielman 1982).

From the former studies it is clear that different amounts of OS applied to mechanical wounding result in different gene regulations. Quantitative effects of insect OS introduced to plant wounding area indicate that it is important to quantify the delivery ability of OS from insect to plant, i.e. how much OS is delivered per bite by insect. To mimic the insect feeding precisely, a method to study the role of OS and OS elicitors both quantitatively and qualitatively is needed (Pare *et al.* 2005, Delphia *et al.* 2006).

1.4 Study Systems

1.4.1 *Arabidopsis thaliana - Plutella xylostella*

Arabidopsis thaliana (Figure 5-1) is a member of the mustard family (Cruciferae or Brassicaceae) with a broad natural distribution throughout Europe, Asia, and North

America (Meinke *et al.* 1998). In the lab, *A. thaliana* may be grown in Petri dishes, pots, or hydroponics, under fluorescent lights or in a greenhouse.

With the self-pollinating nature and an entire lifecycle of six weeks, *A. thaliana* is a popular model organism in plant biology and genetics (Meyerowitz and Pruitt 1985). For a complex multicellular eukaryote, *A. thaliana* has a relatively small genome of approximately 135 mega base pairs (Mbp), which has been completely sequenced in 2000 (Kaul *et al.* 2000). This makes *A. thaliana* an important model system for identifying genes and determining their functions. In plant-insect interaction, *A. thaliana* is widely used, especially in secondary metabolites and phytohormone signaling pathway research (Cipollini *et al.* 2004, Mewis *et al.* 2005, Truman *et al.* 2007, De Geyter *et al.* 2012). Hence, *A. thaliana* was used in this study to investigate the gene regulation in plant leaves after insect herbivory.

Plutella xylostella or the diamondback moth (Lepidoptera: Yponomeutidae) (Figure 5-2) is probably of European origin but is now found throughout the Americas and in Europe, Southeast Asia, Australia, and New Zealand. Total development time from the egg to pupal stage averages 25 to 30 days. Diamondback moth attacks only plants in the family Brassicaceae. *P. xylostella* has enemies at several life stages. Large larvae, prepupae, and pupae are often killed by the parasitoids *Microplitis plutellae* (Muesbeck) (Hymenoptera: Braconidae), *Diadegma insulare* (Cresson) (Hymenoptera: Ichneumonidae), and *Diadromus subtilicornis* (Gravenhorst) (Hymenoptera: Ichneumonidae) (Verkerk and Wright 1996, Herrick *et al.* 2008), all specific on *P. xylostella*. The relatively short life cycle, the feeding specificity and presence of specific higher trophic level make *P. xylostella* a suitable system in study both direct and indirect defense of plants to herbivores.

1.4.2 *Phaseolus lunatus - Spodoptera littoralis*

Phaseolus lunatus (Figure 5-3), butter bean or lima bean, is an herbaceous plant in the Fabaceae (legume or bean family) native to Central and South America, now cultivated in warm, semi-tropical regions throughout the world. *P. lunatus* is perennial, but generally grown as annual. It has erect bush forms, which grow to around 1 m tall and twining forms, up to 4 m long. Plants have trifoliate compound leaves with oval leaflets, each up to 9 cm long. The white to yellow flowers, which occur in loose, open unbranched clusters (racemes) develop into broad, flat pods up to 9 cm long.

Lima bean has long history in plant-herbivore interaction research (Larson 1926, Liu *et al.* 1989, Bouwmeester *et al.* 1999, Maffei *et al.* 2004, Tabata and Yasuda 2011, Boggia *et al.* 2015), due to its significantly induced VOCs after herbivore feeding and JA application (Dicke *et al.* 1993, Hopke *et al.* 1994, Dicke *et al.* 1999). It has also been used in indirect defense research due to the increased production of extrafloral nectar (EFN) (Heil 2004, Radhika *et al.* 2008). Accompanying the fast developing NGS technology, transcriptome sequencing of *P. lunatus* has also been done (Li *et al.* 2015) a whole genome sequencing is ongoing (pers. commun. A. Mithöfer).

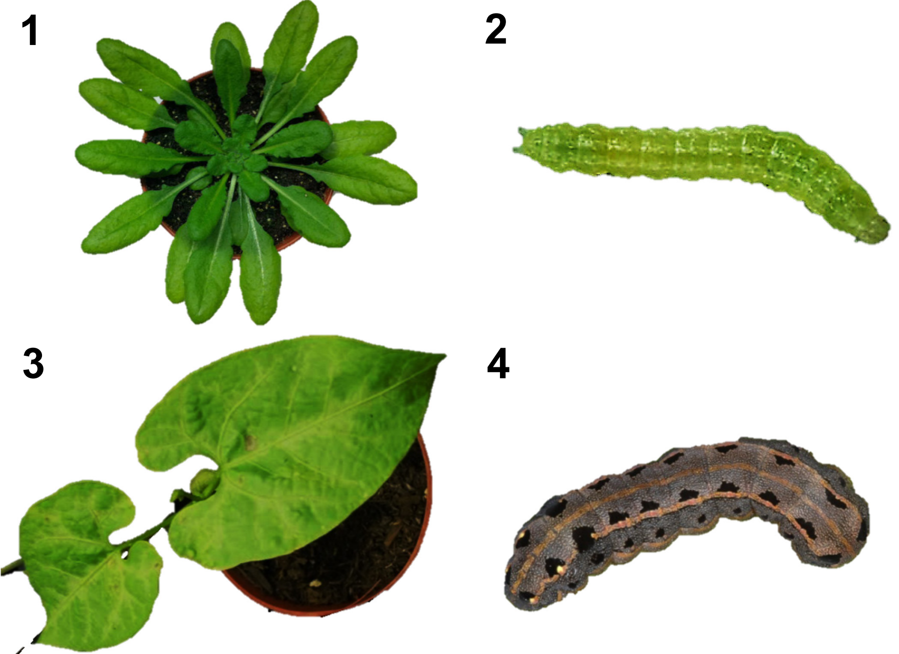


Figure 5. Study systems. **1**. *Arabidopsis thaliana*, ecotype: Columbia; age: 22 days after germination; **2**. *Plutella xylostella*, Diamondback moth; Lepidoptera: Plutellidae; G-88 strain, 4th instar; **3**. *Phaseolus lunatus*, lima bean, 12 to 16 days old seedlings showing two fully developed primary leaves; **4**. *Spodoptera littoralis*, Lepidoptera, Noctuidae, age 4th instar.

Due to the physical feature of having broad even leaves, lima bean is used in insect mechanical wounding study (Maffei *et al.* 2004, Mithöfer *et al.* 2005). Therefore it is suitable for this work, especially for the wounding size measurement and OS trail observation (Felton *et al.* 2014) and quantification.

Spodoptera littoralis (Figure 5-4), the African Cotton Leafworm or Egyptian Cotton Leafworm also known as the Mediterranean Brocade (Lepidoptera, Noctuidae), is a generalist. The host range of *S. littoralis* covers over 40 families, containing at least 87 species of economic importance (Salama *et al.* 1970). *S. littoralis* is polyphagous, adult moths are up to 20 mm long with a wingspan of approximately 40 mm; fully

developed larvae are 35 to 45 mm long; their color varies from grey to reddish or yellowish; eggs are laid in batches covered with orange-brown hairs. Life cycle lasts averagely 25 days (Delvare and Rasplus 1994). Moths lay most of their egg masses (20-1000 eggs) on the lower surface of younger leaves or upper parts of the plant (Khalifa 1982). On cotton, the first three larval instars feed mainly on the lower surface of the leaves, whereas later instars feed on both sides of the leaf. Third- and fourth-instars rest on the plant and remain stationary unless overcrowded.

As a chewing insect and a feeding generalist, *S. littoralis* has a relatively large salivary gland which contains elicitors that trigger plant herbivory defenses (Schulze *et al.* 2006, Haring *et al.* 2008, Kim *et al.* 2011, Bonaventure 2012, 2014, Felton *et al.* 2014). Hence it has become widely used in mechanical wounding and chemical elicitor research in plant herbivory defense (Spiteller and Boland 2003b, Maffei *et al.* 2004, Mithöfer *et al.* 2005, Maffei *et al.* 2006, Arimura *et al.* 2008).

2 AIMS OF THIS STUDY

Plant herbivory defense studies have covered all the levels: Genetics, transcriptomics, proteomics, metabolomics, physiology and mechanics. However, all the defense responses are triggered by one event: insect feeding. This event involves two aspects: mechanical wounding and chemical elicitors. Since it was proved that continuous mechanical wounding (MecWorm) is able to induce plant volatile emission defense, mechanical wounding itself can play an important role in inducing damage signaling and defense gene expression. It is then becoming interesting for me to use MecWorm as a mean of mechanical wounding mimicking tool, to compare the gene regulation after MecWorm wounding and insect wounding, both locally and systemically. By employing global microarray analysis, it should be possible to obtain general information on the gene expression level, i.e. which pathways, genes, cell compartments and networks are influenced by mechanical wounding or chemical elicitors. The question is, how they are influenced, and then if they are influenced by the two factors individually or collaboratively. By this, it might be possible to understand and to clarify the specific roles of the two feeding aspects of insect. To further verify the results from microarray analysis and to more precisely mimic insect feeding, the next step should be adding chemical elicitors to MecWorm, i.e. to further develop a "SpitWorm". The new SpitWorm should be able to combine mechanical wounding and insect oral secretion (or OS dilutions) or elicitor compounds to study their specific roles.

With these questions and purposes, the concrete aims of this study are summarized below:

- Study plant gene regulation on insect feeding through microarray analysis
    1. to understand how the mechanical wounding and chemical factor from insect oral secretion trigger gene regulation;
    2. to find out which pathways are influenced by mechanical wounding and which are influenced by chemical factors;
    3. to investigate how plant cell compartments and energy flow react and adjust themselves to herbivory;
    4. to see how is the resource trade-off between plant growth and defense, and between local and systemic signaling;

5. to find a method of distinguishing between the effects of the mechanical wounding and influence of oral secretion on gene expression level;
6. to analyze whole genome microarray data of plant from different levels (gene, pathway, cell organelle and between damaged leaves and systemic leaves) and conditions (time points and treatments);
7. to verify microarray data by RT-qPCR gene expression.

- Develop the robotic insect mimicking system MecWorm into 'SpitWorm'

1. to determine an optimal solution of OS delivered to SpitWorm;
2. to establish a way to visualize and to quantify the OS coming from the insect foregut and the OS left on the wounding size;
3. to compare the feeding effect or defense reactions of plant against SpitWorm and insect, to verify that SpitWorm can mimic insect feeding both mechanically and chemically, more precise than former studies.

3 METHODS AND MATERIALS

3.1 Global analysis of microarray data on leaf genes of *Arabidopsis thaliana* treated with MecWorm and *Plutella xylostella*

3.1.1 Plant and insect materials

Arabidopsis thaliana seeds (ecotype Columbia) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC). Seeds were sown on a Mini-Tray: vermiculite (3:1) soil mix (Einheitserdenwerk, Fröndenberg, Germany) and stratified for 7 days at 4 °C. Afterwards, plants were moved to ventilated growth rooms with constant air flow and 40% humidity at 23 °C. Plants were grown at a distance of 30 cm from fluorescent light banks with 4 bulbs of cool white and 4 bulbs of wide spectrum lights at a 14 h light/10 h dark photoperiod. Grow domes were removed after 5 days and plants were fertilized once with 1 mL of Scotts Peters Professional Peat Lite Special 20N:10P:20K with trace elements and 1 liter water per flat, added to the bottom of the tray. Approximately 6 days after germination, plants were transferred to individual pots (7.5 x 7.5 cm^2) and grown for 22 days.

Plutella xylostella (Diamondback moth, Lepidoptera: Plutellidae) eggs (G-88 strain) were originally obtained from the New York State Agricultural Experimental Station (Geneva, NY), and a colony was maintained at the MPI in Jena. Larvae were reared on a wheat germ based artificial diet according to published procedures (Shelton *et al.*, 1991) at 27 °C and 16 h light/8 h dark cycles. Herbivory screens were performed with fourth-instar *P. xylostella* larvae.

3.1.2 Plant treatments

All induction experiments were performed 4 weeks post germination. All plants were at a vegetative growth stage and pre-bolting. For each experiment, control plants were included and subjected to the same environmental conditions (except for the respective experimental trigger) as treated plants. Insect herbivory screens were carried out with two larvae per rosette leaf. Mechanical wounding was performed with MecWorm (Mithöfer *et al.*, 2005). Rosette leaves were damaged continuously during experiments, inflicting damage on a leaf area comparable to insect herbivory at the various time points. Details are stated in the particular experiments. Leaf material was immediately frozen in liquid nitrogen and stored at -80 °C. Experiments were conducted for 1, 3, and 9 hours, each with three biological replicates and randomized be-

tween the treatments. Damaged areas of leaves treated by MecWorm for 1 h, 3 h and 9 h are 0.93 cm^2, 1.81 cm^2 and 5.49 cm^2, respectively (see page 62).

3.1.3 Microarray preparation

Leaf material was ground to a fine powder in liquid nitrogen, and total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturers´ protocol. An additional DNAse (Turbo DNAse, Ambion) treatment was included prior to the second purification step to eliminate any contaminating DNA. A second purification step was performed with RNeasy columns (Qiagen, Hilden, Germany) to eliminate contaminating polysaccharides, proteins and the DNAse enzyme. RNA integrity was verified on an Agilent 2100 Bioanalyzer using the RNA Nano chips (Agilent Technologies, Palo Alto, CA). RNA quantity was determined photospectrometrically.

The content on the *Arabidopsis* microarray is derived from the ATH1 v.5 database of The Institute for Genomic Research (TIGR) and from the *Arabidopsis* MPSS (massively parallel signature sequencing) database at the University of Delaware. Nearly 40,000 features (60-mer oligonucleotides) represent full genome coverage of *A. thaliana* (28,500 annotated genes from TIGR) and more than 10,000 unannotated transcripts from University of Delaware (*Arabidopsis* MPSS website)(University of Delaware 2006).

Total RNA was amplified using the Agilent low input linear amplification kit according to the process outlined by the manufacturer (Agilent Technologies). 1-5 µg of amplified target cRNA was labeled with either cy5 or cy3 using the Micromax kit (Perkin Elmer, Boston, MA). The labeled material was passed through Zymo RNA Clean-up Kit-5 columns (Zymo Research Corporation, CA) to remove any unincorporated label and eluted in 15-20 µl of RNAse-free water (Ambion, Austin, TX). Concentration of labeled cRNA and label incorporation was determined by Nanodrop-1000 spectrophotometer analysis. All of the labeling and post labeling procedures were conducted in ozone-free enclosure to ensure the integrity of the label. Labeled material was setup for fragmentation reaction, hybridized overnight in a rotating oven at 60° C in an ozone-free room, followed by washing steps. All conditions were performed according to the Agilent protocol. Arrays were scanned using the Agilent scanner. Agilent's feature extraction software (Version 7.5) was used for extracting array data.

3.1.4 1-Step comparative real-time qPCR

For RNA extraction, *A. thaliana* plants were treated continuously for 9 h with MecWorm or *P. xylostella* larvae, 9 replicates (three biological replicates and three technical replicates) for each treatment. 9 non-wounded plant leaves under the same environment were used as control.

Benzoate-CoA ligase (*BZO1*) is involved in glucosinolates synthesis in *A. thaliana* seeds (Kliebenstein *et al.* 2007), and functions as a cinnamoyl CoA ligase that catalyzes the formation of cinnamoyl CoA to provide the substrate for the pathway(s) by which this phenylpropanoid derivative is converted to benzoic acid (BA) (Lee *et al.* 2012).

Clathrin adaptor complex small chain family protein (*CAP*) performs critical roles in shaping rounded vesicles in the cytoplasm for intracellular trafficking. During mitosis, clathrin binds to the spindle apparatus. The stabilization of kinetochore fibers requires the trimeric structure of clathrin in order to crosslink microtubules (Royle *et al.* 2005).

Pectinesterase family protein (*PE*) plays a role in the modulation of plant cell wall mechanical stability during fruit ripening, cell wall extension during pollen germination and pollen tube growth, abscission, stem elongation, tuber yield and root development (Fries *et al.* 2007), as well as plant-herbivore interaction (von Dahl *et al.* 2006).

Acid phosphatase (*ATACP5*) was found to be related to known purple acid phosphatases, especially to mammal type 5 acid phosphatases. It contains all residues involved in metal ligand binding and resistance to tartrate inhibition and displayed peroxidation activity. In *A. thaliana* transgenic plants *ATACP5* showed responses to phosphate starvation and to ABA and salt stress. It was proposed that AtACP5 protein could be involved in phosphate oxygen species in the metabolism of oxygen species in stressed or senescent parts of the plant (del Pozo *et al.* 1999) and in abiotic stress.

Wounding areas of plant leaves with amount of 80 to 100 mg were collected and then grounded in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's protocol. TURBO^{TM} DNase (Ambion) and RNeasy MinElute Cleanup kit were used to purify the RNA. RNA was then directly applied to 1-step Comparative Quantitative real-time PCR using the Verso^{TM} SYBR Green 1-

Step QRT-PCR Low ROX Kit (ABgene), with an Mx3000P Real-Time PCR system (Stratagene). Primers for RT-qPCR were designed with Primer3plus[1] and analyzed by OligoAnalyzer 3.1[2]. The process was conducted according to the manufacturer's protocol with a 25 µL reaction system, consisting of 0.25 µL Verso Enzyme Mix, 12.5 µL 1-Step qPCR SYBR Mix, 1.25 µL RT Enhancer, 1.75 µL forward and reverse primers (1 µM) each, 2 µL RNA template (25 ng/µL) and 5.5 µL water (PCR grade). The procedure was as follows: 1 cycle of cDNA synthesis for 15 min at 50 ˚C; 1 cycle of Thermo-Start activation for 15 min at 95 ˚C; 40 cycles of Denaturation (15 sec at 95 ˚C), annealing (30 sec at 55 ˚C for *CAP* and *ATACP 5*; 30 sec at 60 ˚C for *PE* and *BZO1*), and extension (30 sec at 72 ˚C), signals being collected at the end of each annealing step. To verify the product with a dissociation curve, an extra cycle with 30 sec at 95 ˚C, 30 sec at 60 ˚C and 30 sec at 95 ˚C was added, signals being collected for the whole cycle. PCR conditions were determined by comparing threshold values, followed by non-template control for each primer pair. Relative RNA levels were normalized with the level of 40S ribosomal protein S18 gene (*RPS18)* and calibrated with control expression amount for each target gene. Three technique replicates and two biologic replicates were used for each sample. Primers for each gene are:

BZO1 (AT1G65880.1)	forward	5'-GTGTTGTGTTCCTACGGTTT-3'
	reverse	5'-CCATCTGTTGATTCTCTGGT-3';
CAP (At1g60970.1)	forward	5'-ATCTTGCTTTTGGATTCTGA-3'
	reverse	5'- CAAAGAAATGGAGATCTTGC-3';
PE (At2g26440.1)	forward	5'-CACATAAGCAACTCCCTCTC-3'
	reverse	5'-CTCACTCGGGTCATACTCAT-3';
ATACP 5 (At3g17790.1)	forward	5'-GTTTTGGGAAACCATGACTA-3'
	reverse	5'-AGCTTTGACGTAAGAGTTGC-3';
RPS18 (AT1G22780.1)	forward	5'-TCAATCAAGGGTATTGGAAG-3'
	reverse	5'-GACGCTCAAGATCATCTCTC-3'.

[1]Primer designing online tool Primer3plus: http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi
[2] Primer designing online tool OligoAnalyzer 3.1: https://eu.idtdna.com/calc/analyzer

3.2 Turning MecWorm into SpitWorm: robotic mechanical wounding with simultaneous application of salivary components

To achieve this goal, following problems need to be solved:

For visualization and quantification of insect OS delivered to the wounded area:

I. The wounding sizes of insect feeding at different time periods so that SpitWorm can reach the same;

II. A method to label the insect OS

III. The optimal amount of labeling compound injected into insect foregut that it leaves strong enough signal for quantification and doesn't influence the feeding activity of insect.

IV. The foregut volume as the OS volume of insect for quantifying labeling signals;

V. To calculate the OS delivery amount by insect to the wounded leaf area per bite.

For setting up SpitWorm:

I. An adjustable and stable delivery speed of insect OS onto SpitWorm;

II. Optimal OS delivery concentration for SpitWorm by comparing the labeling signal quantities left at the wounding site with that by labeled insect feeding for the same time period and same wounding size;

For verifying SpitWorm:

I. Comparison of resulting gene regulation of plant after SpitWorm treatment and insect feeding;

II. Comparison of volatile organic compounds releasing of plants after SpitWorm treatment and insect feeding.

3.2.1 Plant and insect materials

Lima bean *Phaseolus lunatus* L. (Ferry Morse cv. Jackson Wonder Bush) was grown from seed in plastic pots (diameter 5.5 cm) using sterilized potting soil at 23 °C and 60% humidity. For daylight radiation, fluorescent tubes (ca. 270 µE m^{-2} s^{-1}) with a photo phase of 16 h were used. Experiments were conducted with 12 to 16 days old seedlings showing two fully developed primary leaves.

Spodoptera littoralis (Lepidoptera, Noctuidae) eggs (Bayer CropScience AG, Monheim, Germany) were reared on an agar-based artificial diet (500 g white beans powder soaked overnight in 1.2 L water, 9 g ascorbic acid, 9 g parabene, 4 mL formaldehyde (36.5%), and 75 g agar boiled in 1.0 L of H_2O), raised at 22 ˚C to 24 ˚C, 14 h to 16 h photophase, experimental age 3rd to 5th instar.

3.2.2 SpitWorm

A 50 µL syringe was connected to a capillary (fused silica, 0.25 mm i.d., SUPELCO) to the MecWorm (Mithöfer *et al.* 2005) which was directed through the hollow 'biting' needle to a lateral hole close to the needle tip. A syringe pump (Harvard Apparatus PHD 2000) was used to actuate the syringe generating a stable and quantitative delivery of OS (Figure 30, A-b). To determine the lowest amount of OS that could be delivered by the punching needle, ink instead of OS was used.

3.2.3 Collection of insect oral secretion

Regurgitate was collected from larvae by slight squeezing of the animals with tweezers and collection of the discharged enteric liquid with a Gilson Pipetman P20 Variable Volume Pipette (2 to 20 µL).

3.2.4 Insect foregut volume determination

After feeding, insect larvae were suffocated in 75% ethanol solution for 30 seconds. After dissection, the length (h) and width (d) of the foreguts were measured and foregut volume (V_g) was calculated as a cylinder ($V_g = h(d/2)^2$) (Figure 29). 5 replicates were used.

3.2.5 Wounding size determination

To adjust comparable wounding sizes of MecWorm/SpitWorm and insect feeding, wounding sizes generated by feeding larvae were measured at different time periods. Larvae, starved for 12 hours, were fed with lima bean plants for 5 min, 1 h, 3 h, 9 h and 17 h.

The pictures were then printed out on a paper sheet; scaled unit areas and wounding areas were cut off from the same paper and weighed. Wounding sizes were determined dividing wounding area weights by scaled unit area weights. Only treatments with wounding areas away from the leaf edges where used (Figure 6). 4 replicates were used for each treatment and control.

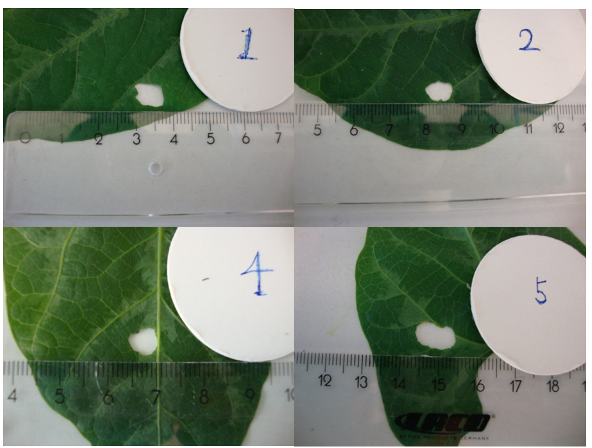


Figure 6. Lima bean (*Phaseolus lunatus*) after being fed by *S. littoralis* for 5 min.

3.2.6 Optimization of the injection volume of fluorescence solution

After being starved for 12 hours, larvae were injected with 1 µL, 5 µL, 10 µL, and 15 µL saturated aqueous Lucifer yellow CH Dipotassium salt solution (Fluka, λ_{Ex} = 428 , λ_{Em} = 536 nm, 1 mg/mL), 4 replicates for each concentration. Then the larvae were fed with lima bean plants for 5 min. As control, 4 larvae without being injected were fed with Lima bean leaves directly after 12 hours starvation. After feeding, pictures of larva with fluorescent foregut were taken under UV light at wavelength λ_{Ex} = 428 nm.

Fluorescence injected larva was then fed on lima bean plants for 5 min, wounding areas were then observed and pictured with a LEICA LMD6000 fluorescence microscope and wounding sizes were calculated.

3.2.7 Fluorescence quantification

To compare the spit amount at wounding areas from insect feeding and SpitWorm treatment, leaves were treated for 5 min by fluorescence dye injected larvae and SpitWorm with fluorescence dye labeled diluted insect OS (5 µL saturated Lucifer yellow solution (1 mg/mL) into 44 µL insect OS (filtered with a 200 µm filter), then diluted with water with dilution factors 1:5, 1:10, 1:30), respectively, 3 replicates for each treatment.

Wounded leaf areas from fluorescence labeled larvae feeding and SpitWorm wounding were cut off and grounded in liquid nitrogen and suspended in 1 mL H_2O for 1 h at 4 °C in dark, centrifuged for 10 min at 12.6 × 1000 rcf. Fluorescence quantification of supernatant was done with FP-750 Spectrofluometer, Jasco. A standard curve with dilutions of saturated Lucifer yellow solution (1 mg/mL) with grounded leaf

supernatant as solvent was generated (0 µL/mL, 0.005 µL/mL, 0.01 µL/mL, 0.015 µL/mL, 0.02 µL/mL, 0.025 µL/mL, 0.03 µL/mL, three replicates).

3.2.8 Analysis of headspace volatiles

For headspace volatiles collection, each control plants and insect treated plants (one larvae per plant) were enclosed in desiccators (2.5 L) (Figure 7). For MecWorm and SpitWorm treatments, the test leaves and the punch head of MecWorm and SpitWorm were enclosed in a cuboid Plexiglas cabinet (approximately 500 mL) (Figure 8). The wounding time and area were 17 h and 7.25 cm^2, respectively. Headspace volatiles emitted by lima bean leaves treated with either larvae or the MecWorm and Spit-Worm were continuously collected for 24 h on charcoal traps (1.5 mg of charcoal) using closed-loop-stripping (CLS) method (Kunert *et al.* 2009).

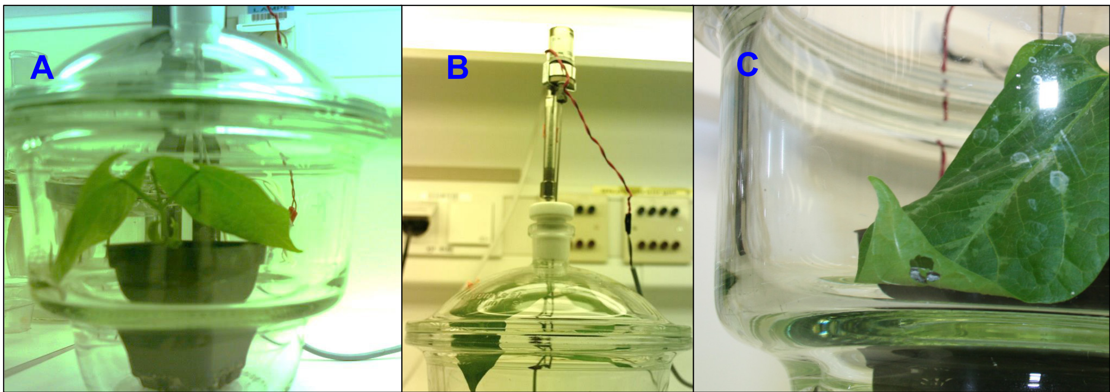


Figure 7. For volatile collection, the control and larvae treatment were enclosed in a glass desiccator with a cap with fitting holes on top for volatile collectors. **A**: Control or insect treatment plant in the desiccator; **B**: Cap of the desiccator with fitting holes connecting to the volatile collector; **C**: Close-up of insect treatment in the desiccator.

All experiments were started at noon around 13:00 pm. Setups were kept at 22 - 24 ^{o}C with a light/dark rhythm of 7 h light, 10 h dark, 7 h light. For all samples after volatile collection, adsorbed compounds were eluted with dichloromethane (2 × 15 µL, supplemented with 1–bromodecan as internal standard (0.27 mM final concentration), adjusted to a final volume of 40 µL with dichloromethane including the internal standard) and directly analyzed by gas chromatography mass spectrometry (GC-MS). The TRACE GC-quadrupole MS system (Thermo Fischer Scientific, Bremen, Germany) was equipped with a fused silica capillary column ZB–5 (15 m × 0.25 mm × 0.25 µm, Zebron, Phenomenex, USA). Helium at 1.5 mL/min served as carrier gas with an injector temperature of 220 ^{o}C running in splitless mode. Separation of the compounds was achieved under programmed temperature conditions (45 ^{o}C for 2 min, then at 10 ^{o}C/min to 200 ^{o}C, then at 30 ^{o}C/min to 280 ^{o}C and kept for 1 min). The MS

was run with a transfer line temperature of 280 °C in EI mode (70 eV), an ion source temperature of 200 °C and a scan range of 35 to 452 amu. 5 replicates for each treatment were analyzed.

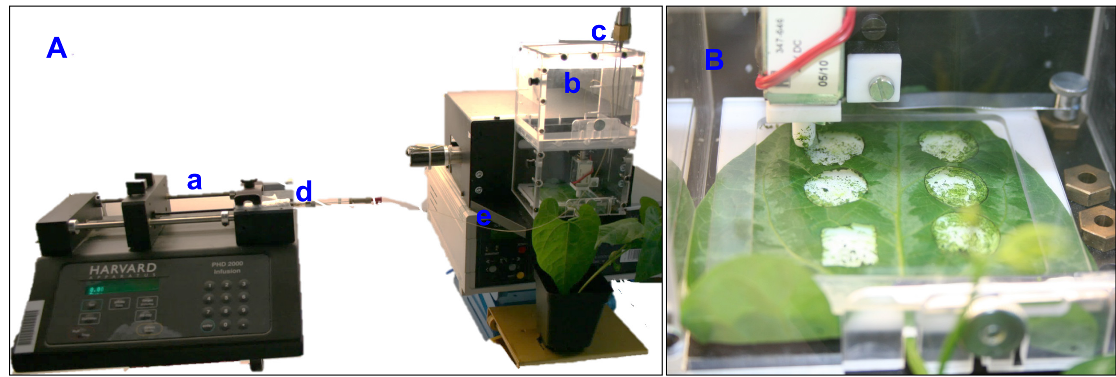


Figure 8. SpitWorm with a cuboid plexiglas cabinet for volatile collection. **A**: SpitWorm. a). automatic syringe pump; b). plexiglas cabinet; c). volatile collector; d). 50 µL syringe; e). glass capillary for delivering OS. **B**: close-up of the biting tip of SpitWorm.

3.2.9 1-Step comparative real-time PCR

For gene expression analysis, plants were treated by insect, MecWorm, and SpitWorm for 1 hour, an untreated plant served as control. Three technical replicates and two biologic replicates were used for each sample.

For RNA extraction, primer design and the reaction system followed the same procedure as the RT-qPCR experiment in microarray validation (see page 25). The reaction procedure was as follows: 1 cycle of cDNA synthesis for 15 min at 50 ˚C; 1 cycle of Thermo-Start activation for 15 min at 95 ˚C; 40 cycles of denaturation (15 sec at 95 ˚C), annealing (30 sec at 55 ˚C for *PR2* and *PAL*; 30 sec at 60 ˚C for *LOX3* and *PR3*), and extension (30 sec at 72 ˚C), signals being collected at the end of each annealing step. Relative RNA levels were normalized with the level of *PACT1* and calibrated with 0 h control expression amount for each target gene.

Primers for each gene are:

LOX3	forward	5'-TGGATGACCGATGAAGAA-3',
	reverse	5'-TGTTGCTATGACGAATGG-3';
PAL	forward	5'-GAAACCTTAGAATCCATCACCA-3',
	reverse	5'-TAGAAGCCAAGCCAGAACC-3';
PR2	forward	5'-AAACTCCTACCCTCCATCACAA-3',
	reverse	5'-CCATCCCTCACCACAACA-3';

PR3	forward	5'-GGCGAGGACAGGATAGCAG-3',
	reverse	5'-TCACAAAGGGAAACACAGATT-3'
PACT1	forward	5'-AGGCTCCTCTTAACCCCAAG -3',
	reverse	5'-GTGGGAGAGCATAACCCTCA-3'.

4 RESULTS AND DISCUSSION

4.1 Global analysis of microarray data on leaf genes of *Arabidopsis thaliana* treated with MecWorm and *Plutella xylostella*

To understand from the gene expression level the defense mechanisms of plants against insects, microarray assays of *A. thaliana* leaf genes, from both local damaged leaves and systemic leaves, under treatments of MecWorm and larvae of *P. xylostella* were conducted and analyzed. *A. thaliana* was chosen because of a well-established and well-studied fully-sequenced genome. *P. xylostella* was chosen because it is a specialist for the plant family Brassicaceae. The microarray was conducted to represent the full genome of *A. thaliana* (28,500 annotated genes from TIGR) and more than 10,000 unannotated transcripts from University of Delaware (*Arabidopsis* MPSS website), here around 20,000 genes were mapped and analyzed in all the treatments with the MapMan software (Thimm *et al.* 2004). Each experiment contained a control (non-treated plant with same growth age and condition as treatments), a plant treatment from MecWorm and a plant treatment from insect larvae with three replicates each. For damaged leaves three time points of treatment were used: 1 h, 3 h, and 9 h. For systemic leaves 2 time points were used and named as: 9 hS and 24 hS.

As for the verification of the microarray data, candidate genes were not chosen according to specific biological or physiological functions, but according to the difference of expression levels in different treatments. The chosen genes showed different expression levels between treatments, with fold changes higher than 10 times between the lowest and highest. These chosen genes are, Benzoate-CoA ligase (*BZO1*, At1g65880.1), which was not regulated by MecWorm but up-regulated by *P. xylostella*; Clathrin adaptor complex small chain family protein (*CAP,* At1g60970.1), which was not regulated by MecWorm but down-regulated by *P. xylostella*; Pectinesterase family protein (*PE,* At2g26440.1), which was down-regulated by MecWorm but up-regulated by *P. xylostella*; and acid phosphatase 5 (*ATACP5*, At3g17790.1), which was up-regulated by MecWorm but down-regulated by *P. xylostella*.

4.1.1 Overview of numbers of regulated genes

There were around 20,000 genes mapped in *Arabidopsis* leaves in each treatment (Figure 9-I). No matter how they were induced (regulated, up or down), the same 20,000 genes were expressed in every treatment. In 9 h damaged leaves (Figure 9-II),

there were 1204 genes induced by *P. xylostella* treatment and 1450 by MecWorm treatment, 734 genes were induced by both *P. xylostella* and MecWorm treatments. In 24 h systemic leaves (24 hS), there were 781 genes induced by *P. xylostella* treatment and 433 genes by MecWorm treatment, 235 genes were induced by both *P. xylostella* and MecWorm treatments (Figure 9-III).

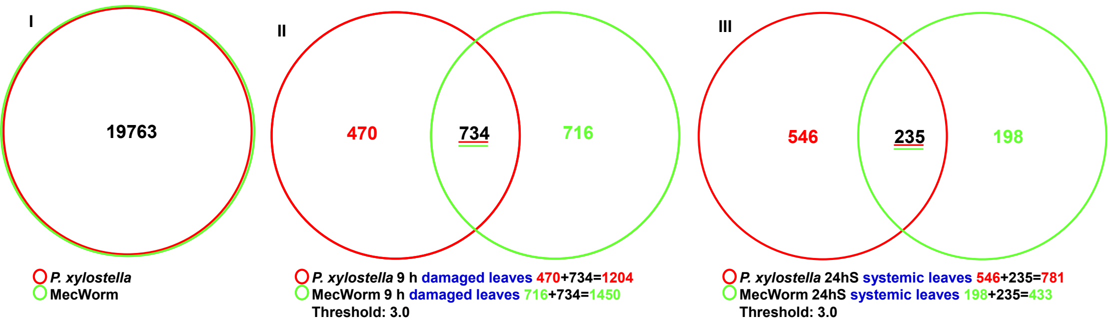


Figure 9. Numbers of induced (regulated) genes in *A. thaliana* leaf treated by *P. xylostella* (red circle) and MecWorm (green circle). Common area of the red and green circles represents genes that are regulated in both treatments. (I): There are in total 19763 genes expressed in all treatments (no threshold); (II): regulated genes in 9 h treatment damaged leaves; and (III): 24 h systemic leaves. Threshold for (II) and (III): 3.0; Software: MapMan.

In order to differentiate the respective roles of mechanical wounding and chemical factors, it is necessary to organize the two circles in Figure 9-II and III into different areas and analyze them one by one. As shown in Figure 10-I and II, area 'a' covers the genes that were regulated in insect treatment but not MecWorm treatment, hence regulation of these genes are chemical factor induced. Area 'b' covers the genes that were induced in MecWorm treatment but not insect treatment, therefore these genes that were induced by mechanical wounding but inhibited by chemical factors.

As shown above in Figure 9, the common area of the two circles represents genes that were regulated by both insect and MecWorm. However, these genes can be divided again into two parts: genes that were not differentially regulated by insect and MecWorm (difference of relative expression fold compare to control lower than 3 times), and genes that were differentially regulated by insect and MecWorm (difference of relative expression fold compare to control higher than 3 times).

In Figure 10, genes without significant regulation difference between insect and MecWorm treatments were marked as area 'c' and genes with significant regulation difference were marked as area 'd'.

As for area 'c', regulations in MecWorm treatment and insect were same, which means that in insect treatments these genes were regulated by mechanical wounding

and chemical factors didn't influence this regulation. Therefore, area 'c' represents genes that were specifically regulated by mechanical wounding.

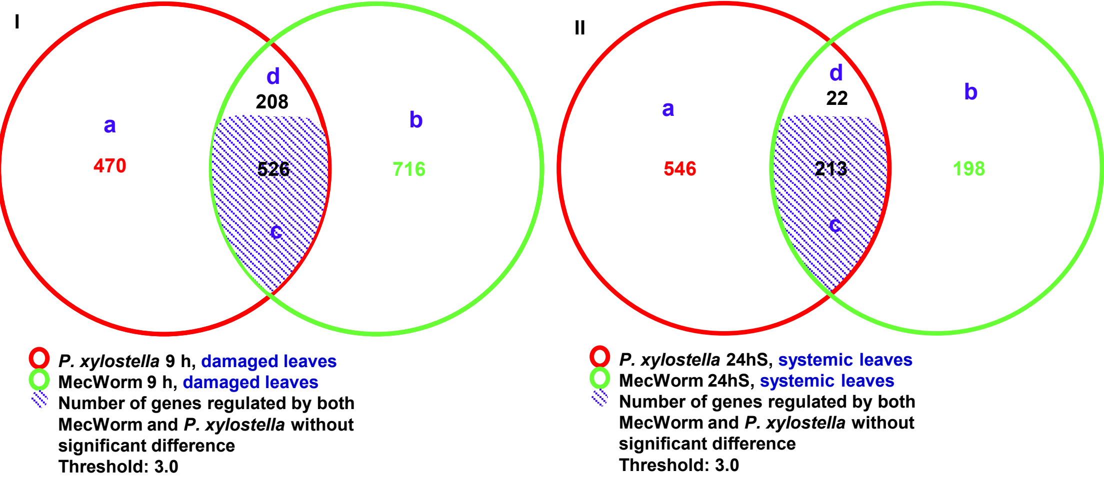


Figure 10. Different parts of gene regulation in *A. thaliana* leaves damaged by *P. xylostella* and MecWorm 9 h local treatments (I) and 24 h systemic leaves (II). Threshold: 3.0; Software: MapMan.

In case of area 'd', regulations of these genes were significantly different between MecWorm and insect treatments, showing that these genes were significantly regulated by both MecWorm and insect. Hence in insect treated plant these genes were co-regulated by MecWorm and chemical elicitors.

To combine the analysis of all the four areas, in insect treated plant leaf, area 'a' plus area 'd' (area 'a + d + (b)') covers all the genes that were regulated by chemical factors and area 'c' plus area 'd' (area 'c + d + (b)') covers all the genes that were regulated by mechanical wounding.

In damaged leaves, the mechanical wounding specific genes are 734 (Figure 10-I, area 'c + d'), which is around 61% of the total insect regulated genes. Chemical factor specific genes are 470+208=678 (Figure 10-I, area 'a + d'), which is around 53% of the total insect regulated genes. From the numbers, one can see that in damaged leaves, mechanical wounding plays a bigger role than chemical factors. Total effect of mechanical wounding and chemical factors are bigger than 100% because of the co-regulated 'd' area. Area 'b' was not included here because in insect treatments the 'b' area was induced by mechanical wounding but inhibited by chemical factors which results in no regulation in the insect treatment.

In 24 hour treatment systemic leaves (24 hS) (Figure 10-II), there are less genes significantly regulated by mechanical wounding (MecWorm, 433, green circle) than

by insect (*P. xylostella,* 781, red circle). The number of mechanical wounding specific genes is 235 (Figure 10-II, area ‘c + d’, 30% of the total insect regulated genes). The number of chemical elicitors regulated genes is 568 (Figure 10-II, area ‘a + d’, 73.9% of the total insect regulated genes). Thus, in systemic leaves, chemical elicitors play a major role in systemic defense induction. Area ‘b’ was not included because of the antagonizing effect between mechanical wounding and chemical factors in insect treated plant.

On the other hand, from the results shown in Figure 10 it can be seen that the insect feeding regulated genes in plants doesn’t fully cover the genes that are induced by pure mechanical wounding (MecWorm treatments). The ‘shifting’ (area ‘b’) of insect induced genes from the mechanical wounding induced genes indicates that the roles of mechanical wounding and chemical factors in regulating plant herbivory defense is not simply accumulative. The chemical factors have a role that can both inhibit some of the mechanical regulated genes and induce new regulation of genes. Gene regulation in plant herbivory defense is the result of a co-operation of mechanical wounding and the chemical factors. The co-operation of these two aspects is analyzed below in more detail.

To further clear up the roles of mechanical wounding and chemical factors in plant defense explanation of the areas are organized and summarized in Table 1.

Table 1. Roles of mechanical wounding and chemical factors in gene regulation in *A. thaliana* leaf attacked by insect and MecWorm.

Mechanical wounding induced regulation	Chemical factors induced regulation	
c + d + (b)	a + d + (b)	Insect

Areas in Figure 9	Mechanical wounding	Chemical factors	Description	
a	No regulation	Inducing regulation	Regulation induced in insect treatment but not in MecWorm treatment, hence induced by chemical factors	
c	Inducing regulation	No regulation	Regulation induced by MecWorm and stays the same in insect treatment, hence induced by mechanical wounding and not influenced by chemical factors	MecWorm
d	Co-regulation	Co-regulation	Regulation induced by MecWorm and in insect treatment significantly changed, hence induced by both mechanical wounding and chemical factors	MecWorm
b	Inducing regulation	Inhibiting regulation	Regulation induced in MecWorm treatment but not in insect treatment, hence originally induced by mechanical wounding but inhibited by chemical factors in insect treatment	MecWorm

- Inducing regulation
- No regulation
- Co-regulation
- Inhibiting regulation

4.1.2 Overview of pathway regulations: Roles of mechanical wounding and chemical factors in both local and systemic samples

The insect saliva can only fully function with the combination of tissue damaging, thus the independent role of chemical factors is difficult to uncover. However, the role of chemical elicitors can be detected through comparing the defense results from mechanical wounding and insect treatment. In the insect wounded leaves, chemical regulation can:

Enhance the effect of mechanical wounding. This means that chemical factors induce a regulation of certain pathways in the same direction with mechanical wounding and the regulation of insect treatment is a combination of both. For example, as shown in Figure 11, photosynthesis or light reaction, jasmonic acid (JA) synthetic pathway, nucleotide synthesis, amino acid synthesis, and secondary metabolites synthesis. Especially in the lignin precursors synthetic pathway, only the co-operation of both mechanical wounding and chemical factors can induce the up-regulation of the whole pathway (large schemes can be found in Appendix Figure 4). These pathways are mostly involved in primary and secondary metabolism in plant herbivory.

Inhibit or suppress the effect of mechanical wounding. Area 'b' of Figure 10-I represents genes regulated by MecWorm but not by insect feeding. This indicates that these genes were regulated by mechanical wounding but regulation was suppressed by chemical factors in insect feeding treatments. Comparing these genes and the pathways in which they are involved, shows that many pathways induced by mechanical wounding are inhibited and 'fine-tuned' to the herbivory defense-specific direction by chemical elicitors. For example, signaling related genes, receptor kinases synthesis, and calcium regulation are all important pathways in plant insect defense signaling. A large number of these genes in biotic stress associated pathways were down-regulated in mere mechanical treatment (MecWorm) (Figure 12-1), but this down-regulation was suppressed and other genes in the same pathways were significantly up-regulated in insect treatments (Figure 12-2, 3 and 4). On the other hand a large number of other abiotic defense pathways genes such as heat shock proteins and abiotic stress were significantly up-regulated in pure mechanical treatment (Figure 12-1) but were suppressed in insect treatments (Figure 12- 2, 3, 4).

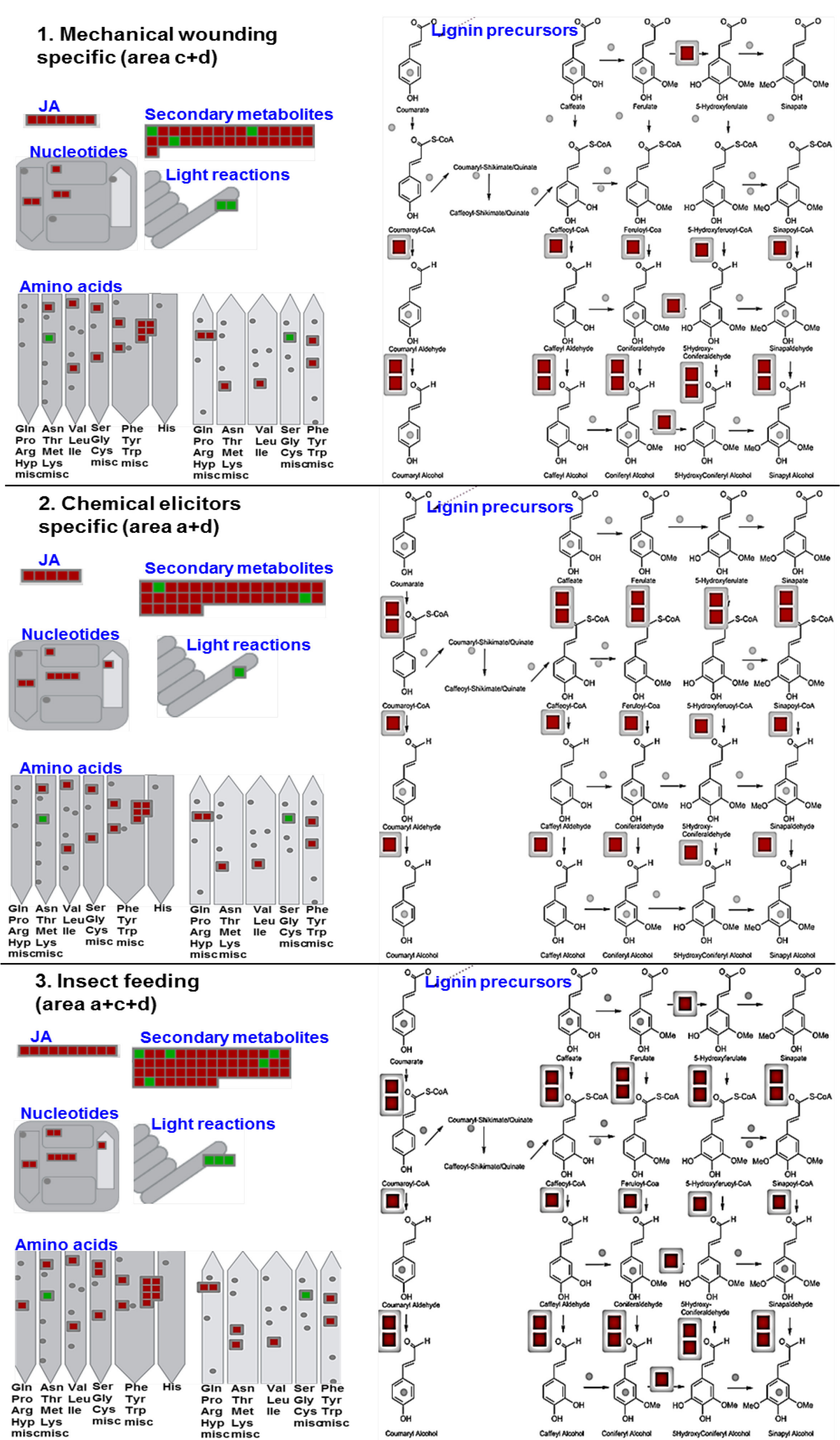


Figure 11. Pathways that indicate an enhancing effect of chemical elicitors to mechanical wounding. Areas a, b, c, and d refer to the corresponding areas on Figure 10-I. Red grids represent up-regulated genes and green down-regulated genes. Local 9 h treatment. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

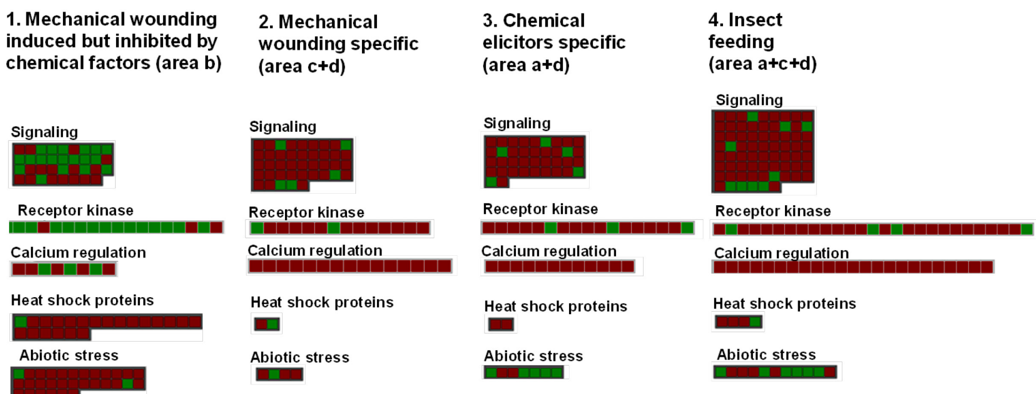


Figure 12. Pathways that show inhibiting and fine-tuning effect of chemical elicitors to mechanical wounding. The letters a, b, c and d are correspondent to the different areas on Figure 10-I. Red grids represent up-regulated genes and green down-regulated genes. Local 9 h treatment. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

No influence on the effect of mechanical wounding. For example, the whole area 'c' in Figure 10 represents genes that were regulated by mechanical wounding but not influenced by chemical factors. If we look at the pathway level, there are pathways which were regulated by MecWorm wounding and stay the same when treated by insect. For example, salicylic acid (SA) pathway, C-1 (one-carbon compounds) metabolism, and inositol phosphates synthetic pathway (Figure 13). SA signaling pathway has been proved playing an important role in plant disease or pathogen defense (Luu *et al.* 2015, Yang *et al.* 2016) and has an antagonizing role to the JA mediated defense (Vos *et al.* 2013). Metabolism of one-carbon (C1) compounds is shared by all living organisms and plays a central role in microbial metabolism (Feist *et al.* 2014), however, plant C1 biochemistry has remained relatively unexplored, partly because of the low abundance or the lability of many of its enzymes and intermediates (Hanson *et al.* 2000). Inositol phosphates are a group of mono- to polyphosphorylated inositols. It is believed (Irvine 1987, Hughes and Putney 1990) that IP3 (Inositol, 1,4,5-trisphosphate), has a role as a second messenger whose function is to mobilize Ca^{2+} from the endoplasmic reticulum,. The IPs were also found to be involved in SA signaling pathway (Lin *et al.* 2004). This up-regulation of IPs in insect treatment is consistent with signaling and calcium regulation, which indicates a wide-spread up-regulation of the defense signaling system.

In systemic leaves, mechanical wounding alone has less effect compared to chemical elicitors. There are less genes and pathways that are specifically and significantly induced. On the other hand, in the systemic leaves after insect treatment, the most regulated pathways are signaling, calcium, receptor kinases, PR-proteins (Figure 14), cell

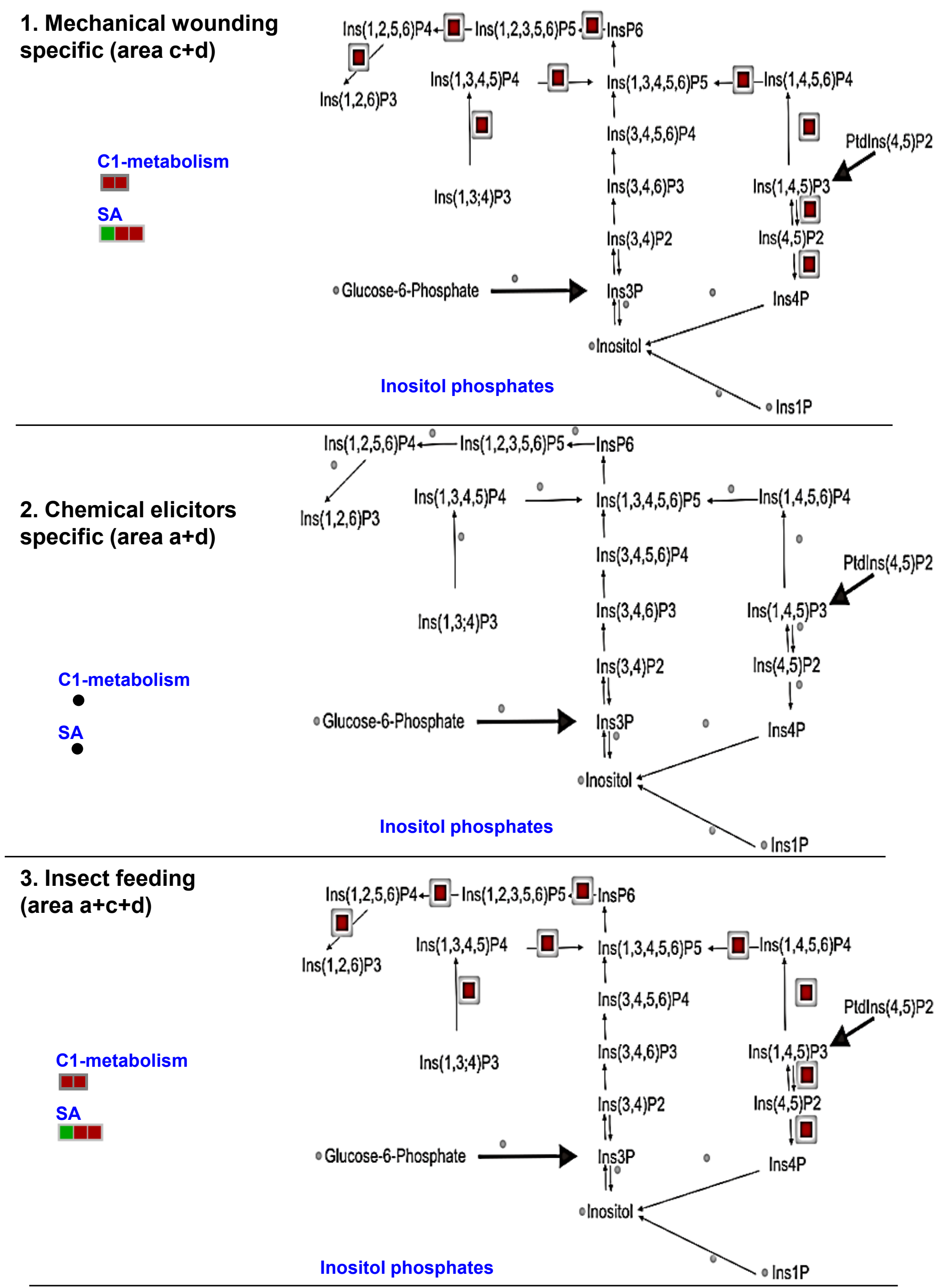


Figure 13. Pathways not affected by chemical elicitors. Areas a, b, c, and d refer to the corresponding areas on Figure 10-I. Red grids represent up-regulated genes and green down-regulated genes. Local 9 h treatment. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

construction (Figure 15), and primary metabolism (DNA, RNA and Protein; Figure 15). In lignin precursors pathway mechanical wounding doesn't have any regulation (Figure 14). In systemic leaves, there are much less secondary metabolism genes induced in comparison to wounded leaves. These results indicate that in systemic leaves, the defense reactions are mostly signaling and resource related while defense compounds (secondary metabolites) related genes are more regulated in damaged leaves.

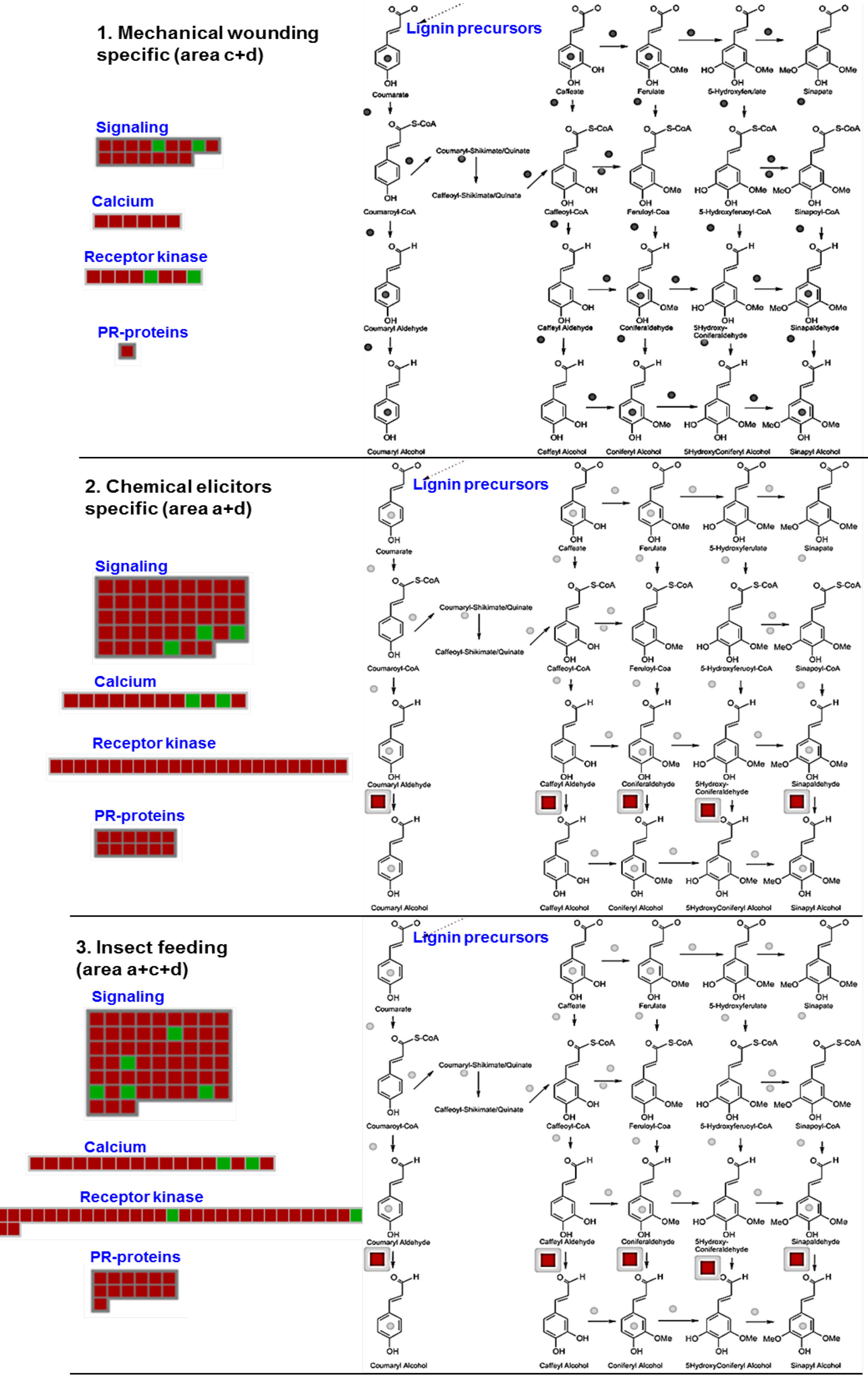


Figure 14. Pathways that are significantly regulated in systemic leaves. Areas a, b, c, and d refer to the corresponding areas on Figure 10-II. Red grids represent up-regulated genes and green down-regulated genes. Systemic 24 h S treatment. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

To summarize the analysis above, in insect damaged leaves, both mechanical wounding and chemical elicitors contribute to the plant defense. Mechanical wounding regulates more genes in number while chemical elicitors have a 'fine-tune' function by enhancing, inhibiting, or 'turning' the effect of mechanical wounding to maneuver the regulation to the more defense-specific direction. In systemic leaves, chemical elicitors are the major force in defense regulation, especially in signaling pathways. Figures with a complete gene expression overview of metabolic pathways, biotic stress pathways, regulation pathways, and lignin precursors synthetic pathways from both local (9 h) and systemic (24 hS) can be found in Appendix (Figures 1- 4); the overview of pathways that are regulated by MecWorm but inhibited by chemical elicitors in local 9 h treatments can be found in Appendix (Figure 5).

4.1.3 Significant pathways analysis

To study in more detail the roles of mechanical wounding and chemical factors in gene regulation of plant against insect feeding, the specific pathways are analyzed separately. Figure 15 shows all the significantly regulated primary metabolism and signaling pathways in all treatments. Red grids representing up-regulated and green grids down-regulated genes.

Photosynthesis: Photosynthesis was down-regulated in both MecWorm and *P. xylostella* damaged leaves but no significant regulation was found in systemic leaves. This clearly shows that down-regulation of the photosynthesis reaction pathway was caused by mechanical wounding, and enhanced by chemical signals.

Not only from gene expression level, it has been commonly observed from physiology level that plant photosynthesis rate is lowered after insect attack (Tang *et al.* 2006, Kerchev *et al.* 2012).

Results of this study showed no significant photosynthesis gene regulation in systemic samples. This phenomenon has also been observed with stem treated plants. When the stems of plants were undergoing herbivory, photosynthesis of the leaves was not influenced, although growth and fitness of plants were significantly reduced (Stephens and Westoby 2015).

Cell wall: In (9 h) insect damaged leaves, genes for cell wall precursors were up-regulated; degradation genes were down-regulated in damaged leaves but not significantly in systemic leaves. Cell wall is the structure that barriers harms and holds water

solution for all the basic reactions in the cell. When a cell is damaged, it is necessary for the neighbor cells to increase cell wall precursor production and reduce the cell wall degradation. However, the up-regulation of cell wall precursor genes in insect damaged leaves started at 9 h but not at earlier time points, while in MecWorm treatments there was an up-regulation at 1 h and both up- and down-regulation of these precursor genes at 9 h. This suggests that insect OS has an inhibiting effect on the cell wall strengthening of damaged leaves, which is beneficial for the insect to conduct feeding process. Same up-regulation of cell wall structure and modification genes at damaged leaves from whitefly feeding has also been published (Quintana-Camargo *et al.* 2015).

Cell: In cell organization and cell cycle pathways, MecWorm local treatments showed an up-regulation in the first hour but not later (3 h and 9 h). In insect local treatments there was almost no significant regulation while in systemic leaves, these processes were up-regulated broadly. Cell organization and cell cycle pathways are resources for later plant cell reproduction and plant growth.

DNA: DNA synthesis was significantly down-regulated in all treatments. It was co-regulated by both mechanical wounding and chemical factors. One cell needs only one set of genome DNA, DNA replication happens only during growth, repairing and reproduction. RNA and protein molecules are main conductors and consumers of energy in plant herbivory defense. It is economical for the plant to save the reproduction energy for defense consumption. This also explains the theory that costs can arise from the allocation of resources to defense and away from plant growth and development (Walters and Heil 2007).

RNA: RNA synthesis had a general up-regulation in all treatments, with a stronger regulation in systemic leaves than in wounded leaves, indicating that there is an increase on gene transcription in both damaged and systemic leaves and the undamaged systemic leaves are more effective in functioning.

Protein synthesis: In wounded leaves, protein synthesis was down-regulated at the first two time points (1 h and 3 h) and up-regulated at 9 h treatments. This can be explained by the fact that signaling and RNA transcription are up-stream events compared to protein synthesis. It is economic for plant to first utilize the energy and resource for the preparation i.e. signaling and RNA synthesis, and then for the defense

conduction i.e. protein synthesis. Protein degradation was generally down-regulated in all treatments, especially in wounded leaves. The induction of protein synthesis in insect treatments was stronger than the induction in MecWorm treatments. This indicates that OS has an enhancing effect in protein synthesis induction.

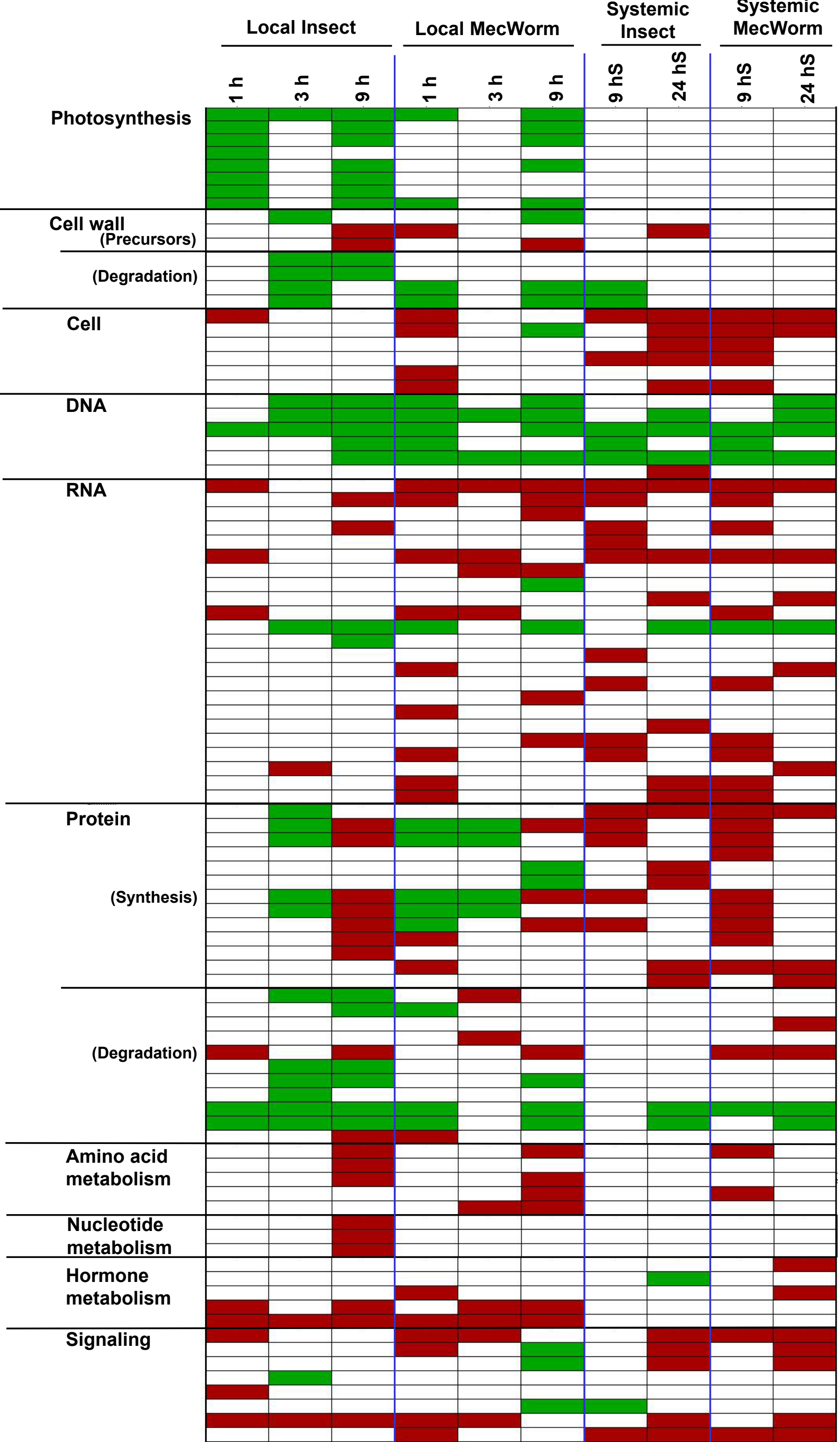


Figure 15. Major primary metabolism and signalling pathways. First three columns are *A. thaliana* treated with *P. xylostella* continuously for 1 h, 3 h, and 9 h, damaged leaves; second three columns are *A. thaliana* treated by MecWorm continuously for 1 h, 3 h, and 9 h, damaged leaves; the next 4 columns are *A. thaliana* systemic leaves from same plants treated with *P. xylostella* and MecWorm after 9 h and 24 h, respectively; Red grids represent up-regulated genes, green down-regulated genes. Wilcoxon rank sum test. Benjamini Hochberg correction. Software: MapMan, Pageman analysis. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

Hormone metabolism: Phytohormones, especially JA synthesis, were up-regulated in damaged leaves from the first hour in both insect and MecWorm treatments. This suggests that the up-regulation of hormone metabolism is driven by mechanical wounding and starts immediately after wounding in damaged leaves. There was no significant up- or down-regulation of phytohormones in undamaged leaves, again indicating that this regulation is induced by mechanical wounding. This quick induction of hormone synthesis and VOCs emission in damaged leaves has also been observed in down-stream work other than gene expression level (Arimura *et al.* 2008).

Signaling: In insect and MecWorm local treatments, signaling genes had stronger up-regulation in earlier hours (1 h and 3 h), indicating again that wounded leaves are the starting points for defense signaling which happens rapidly after insect wounding. However, compared to MecWorm local treatments, insect local treatments had weaker induction of signaling genes, which indicates that chemical factors in insect OS suppresses the fast signaling in plant to help the insect to avoid the fast produced toxic defense compounds during feeding. In both insect and MecWorm treated plant systemic leaves, there was a general up-regulation in all treatments. When combining with the non-significant hormone induction in systemic leaves above, a signaling from damaged leaves to systemic leaves is possible. This communication and signaling could be conducted through transportation systems of the plant or through priming by airborne signals (Perrigo and Bronson 1982, Kessler *et al.* 2006).

To sum up the primary metabolism pathway analysis, from gene expression level perspective, when plant is under insect attack, in damaged leaves, signaling and hormone metabolism are up-regulated immediately to conduct direct defense. The results from this study confirms that after insect feeding, the synthesis of amino acids and nucleotides are up-regulated, as well as the following RNA and protein synthesis. The induction of signaling pathway, protein and RNA production is more active in systemic leaves than in damaged leaves. A reason for this stronger induction in systemic leaves could be the distance of systemic leaves from the wounding site; another explanation would be that damaged leaves conduct fast defense such as strengthening of cell wall and releasing of secondary metabolites to prevent the plant from further damage of the insect, which consumes more energy and resources than more distant systemic leaves do. The strong signaling up-regulation in systemic leaves also sug-

gests a communication and signaling system between systemic leaves and damaged leaves.

It has also been well proven from physiology and metabolism studies that photosynthesis in damaged leaves is reduced, cell wall modification and structuring in damaged leaves is strengthened. This could be confirmed on the gene expression level in this study.

An interesting point of the analysis is that both cell cycle pathways and DNA synthesis are processes for cell reproduction. In insect damaged leaves, DNA synthesis is down-regulated and cell processes have only one gene up-regulated after 1 hour treatment. It is understandable that plant gives priority to defense over reproduction after insect feeding. But why there is an up-regulation of cell organization in systemic leaves? My hypothesis is that since RNA and DNA use the same resources for synthesis, RNA synthesis is for defense and it is up-regulated and more needed even in systemic leaves, thus DNA synthesis is down-regulated in both damaged and systemic leaves to give priority to RNA synthesis; resources for cell organization and cell cycle are up-regulated in undamaged systemic leaves as preparation for stronger growth of systemic leaves in case of the possibly upcoming herbivory.

4.1.4 Cell compartment level of regulation: chloroplast, cell wall and nucleus

Chloroplast: As shown above, in insect or MecWorm damaged leaves, photosynthesis associated genes were generally down-regulated after treatments. These genes are mostly light reaction or Calvin cycle related genes (Figure 16-A, B). Both light reaction and Calvin cycle processes are located in chloroplast. Other pathways such as starch degradation (Figure 20) and chlorophyll synthesis (Figure 16-C), which are also located in chloroplast, were additionally down-regulated.

The down-regulation of photosynthesis and other pathways in chloroplast (Figure 15) happened only in damaged leaves but not in systemic leaves and in both insect and MecWorm treatments. Mechanical wounding is the common feature shared by MecWorm and *P. xylostella* and there is no mechanical wounding in systemic leaves. Hence, this down-regulation is mechanical wounding induced.

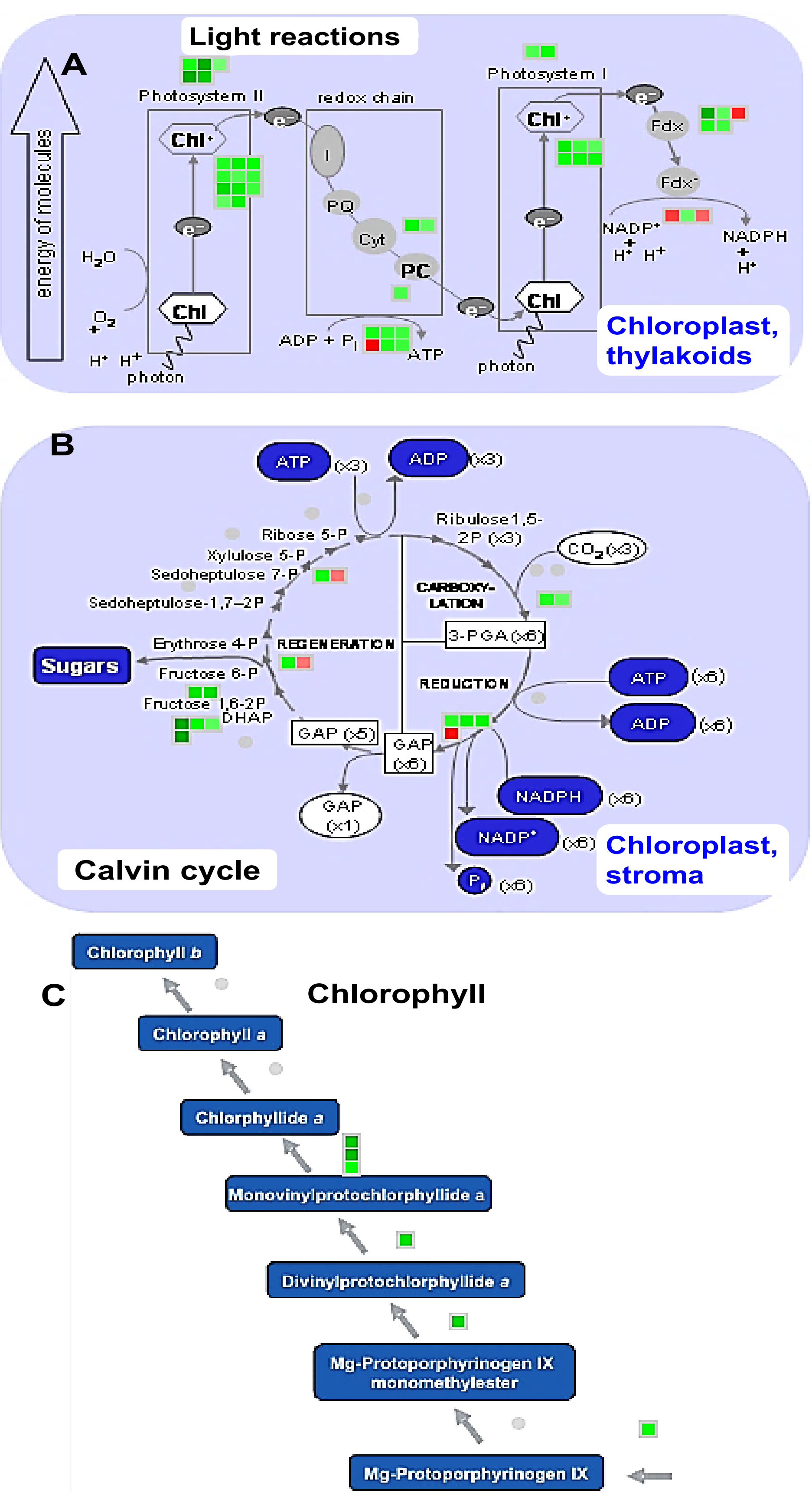


Figure 16. Chloroplast located pathways of *A. thaliana* damaged leaves after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. **A**: Light reaction; **B**: Calvin cycle; **C**: Chlorophyll synthesis. Threshold: 2.0; Software: MapMan.

Cell wall: There was an up-regulation of cell wall precursor synthesis genes and down-regulation of cell wall degradation genes, which indicate strengthening of cell wall, in both MecWorm and insect damaged cells. The up-regulation of cell wall precursor synthetic pathway genes is shown on Figure 17. This precursor synthesis of cell wall doesn't locate at cell wall but are cell wall structure genes.

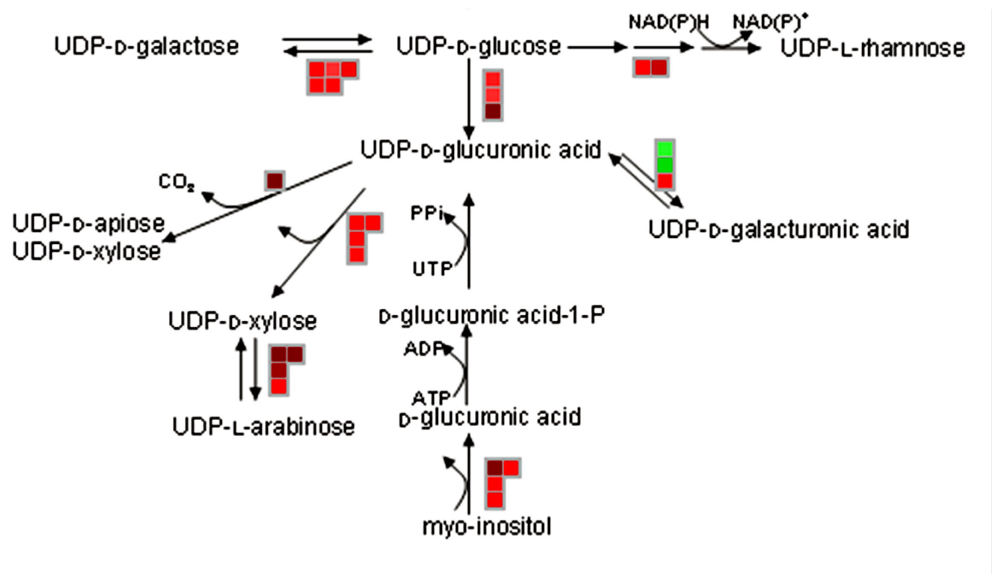


Figure 17. Cell wall precursors synthesis in damaged leaves of *A. thaliana* after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. Threshold: 2.0; Software: MapMan.

Nucleus: It can be seen from Figure 15 that DNA synthesis was down-regulated. If the distribution of regulated genes on the 5 major chromosomes of *A. thaliana* is examined, a pattern can be observed: around all the centromere areas there is a concentrated down-regulation (Figure 18). The centromere, which is one of the essential parts of a chromosome, controls kinetochore formation and chromosome segregation in mitosis and meiosis during reproduction (Feng *et al.* 2015). The down-regulation of this area, together with the down-regulation of DNA replication, can be explained by the theory that upon pathogen or insect attack, resources are allocated to defenses instead of to plant growth and reproduction (Vos *et al.* 2013).

From both Figure 15 and Figure 18, it can be seen that both the DNA synthesis pathway and chromosome areas, which are located in nucleus, were down regulated in both MecWorm and insect damaged leaves. This indicates that this down-regulation is induced by mechanical wounding. However, there was also down-regulation in systemic leaves for both treatments, which suggests a co-regulation of mechanical wounding and chemical factors, at least in systemic leaves.

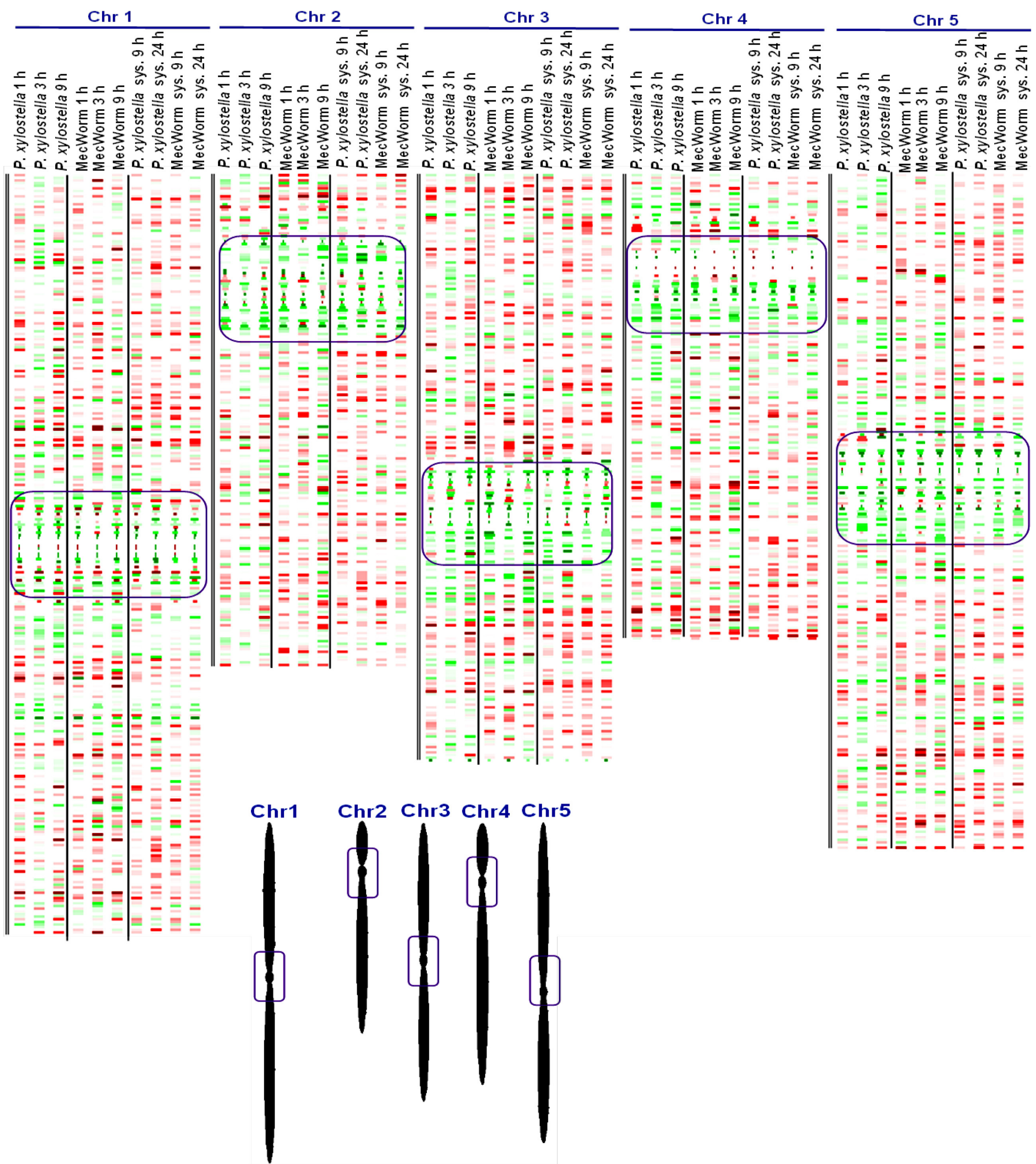


Figure 18. Chromosome overview. Every big lane represents one chromosome of *A. thaliana*. On every chromosome, first three columns are *A. thaliana* treated with *P. xylostella* continuously for 1 h, 3 h, and 9 h; second three columns are *A. thaliana* treated by MecWorm continuously for 1 h, 3 h, and 9 h; the next 4 columns are *A. thaliana* systemic leaves from same plants treated with MecWorm and *P. xylostella* after 9 h and 24 h, respectively; Red color bars represent up-regulated genes, green down-regulated genes. Purple squares highlight the areas with a concentrated down-regulation. Picture on the bottom shows the 5 chromosomes of *Arabidopsis* and purple squares show the position of centromere areas of each chromosome. Threshold: 2.0; Software: MapMan.

From the above analysis of the three cell compartments (chloroplast, cell wall and nucleus), one can see that in these organelles, both compositing and functioning involved pathways within them showed common regulation patterns. What can be the explanation on the mechanism of the organelle level regulation? To answer this question, two common features of this organelle regulation can be found:

1. There is a gene regulation on organelle level in plants against herbivory.
2. This regulation is triggered by mechanical wounding, either alone or together with chemical factors.

These two points suggest that there is an organelle level gene regulation in plant attacked by insects, and this regulation is triggered by mechanical wounding. This rings a bell of the 'damaged-self recognition' theory (see page 4).

"*This damage results in the occurrence of plant molecules outside the compartments to which they are bound in the intact cell and releases fragments from such molecules because they become exposed to enzymes that, in the intact cell, are localized to different compartments. These chemical motifs are indicative of the 'damaged self' and can principally serve as elicitors of plant defense responses.*"--- damaged-self recognition (Heil 2009).

Taking organelles and compartments of the cells as the 'compartments' mentioned above, the 'fragments' as end products from their own synthetic pathway, then when the end product of the pathway is released from the damaged cells and contacts to the parallel compartment or organelle in the neighboring intact cells, could the released fragments function as an elicitor to give the 'feedback' information to the intact cell nucleus through the enzymes along the pathway? This information could be that the organelles at damaged leaves are facing insect feeding, it is more emergent to conduct defense activity than repairing the already damaged leaf area or recovering photosynthesis; or it could be that the vital system for self-protecting is damaged and more products are needed, e.g. cell wall strengthening.

For damaged-self recognition, it is the already damaged tissue, of which the biological function is dramatically (if not completely) lost, that generates the signal; and it is the surrounding undamaged area that receives the signal for conducting defense reaction. Thus the 'local treatments', with regulation of gene expression are actually the undamaged area directly around the damaged area. In these areas examined in this experiment, all the cells are actually intact functioning cells. The difference between local (damaged) leaves and systemic leaves is actually the distance for receiving and transmitting insect feeding signals. The down- or up- regulation of organelle pathways doesn't mean the damaging status of the examined cells; instead, it represents a strat-

egy of plant to receive and pass on signals of feeding and to allocate resources for defense and self-fitness economically.

Results in this study also showed that continuous mechanical wounding induces more genes in damaged leaves while insect chemical factors has a fine-tune effect. How does mechanical wounding conduct the signaling to elicit the plant defenses?

The damaged-self recognition is based on the general observation that applying plant-derived tissue onto a wounded plant can trigger plant defense reaction. This phenomenon underlies in feeding almost all the plant predators (pathogens, insects and animals) (Devaiah *et al.* 2009, Heil 2012). The damaged leaf compartments could contain elicitors that trigger the defense signaling. This theory is based on the observation that the wounded 'leaf juice' can trigger plant defenses (Green and Ryan 1972, Ryan 1974, Turlings *et al.* 1993, Mattiacci *et al.* 1995) and most of the studied elicitors can be totally (or partly) plant derived.

With the damaged-self recognition signaling, it is now also possible to explain why continuous mechanical wounding alone with MecWorm can induce volatile organic compounds (VOCs) emission while single cuts with razor blades or pattern wheel can not. It is the continuous wounding that generates enough plant self-derived elicitors to start the defense. But of course, the 'leave juice' does not serve the roles as chemical factors from the insect OS, which results in different gene regulation and plant defenses.

Although the damaged-self recognition remains to be a hypothesis that was based on observed phenomenon, it could to some extent explain the results from this study about the regulation on organelle level. The mechanisms of damaged-self recognition theory need to be further studied.

Compared to the more structurally complicated chloroplast, the cytosol is barely influenced, up-regulation of defense compounds biosynthesis were observed. For example, synthesis of glucosinolates, lignin and lignans, lignin precursors (Figure 19), UDP-cell wall precursors (Figure 17), nucleotides (Figure 15), and fermentation (red asterisk in Table 2.) pathways were upregulated.

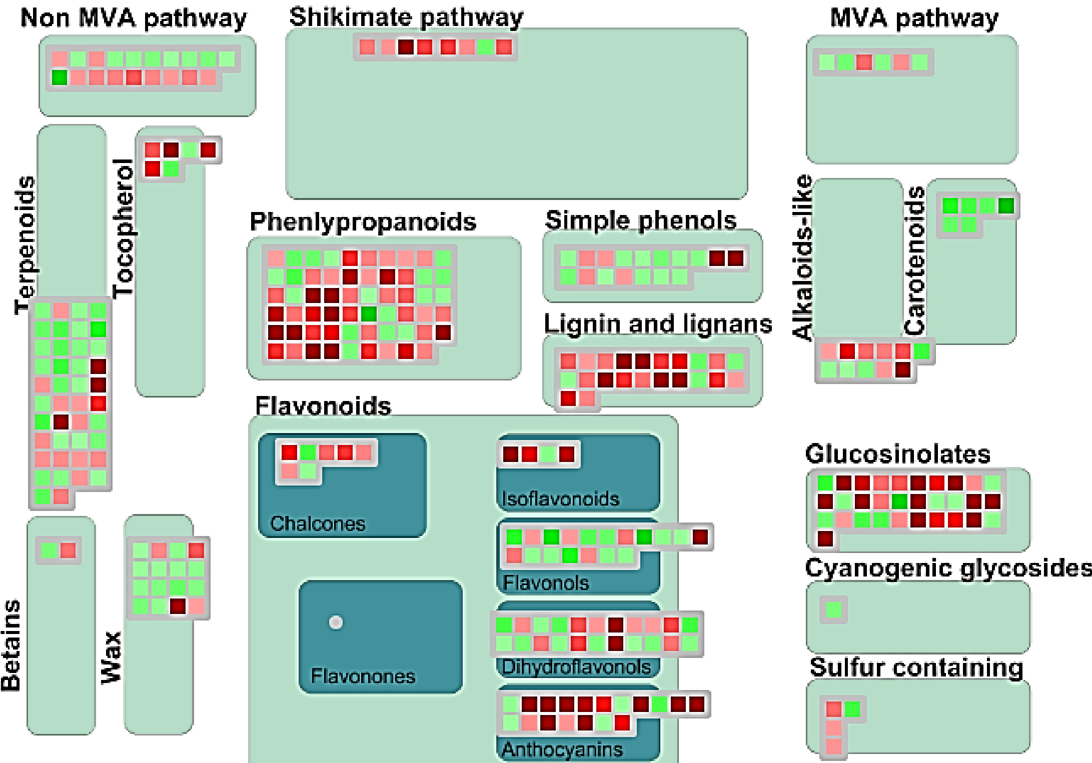


Figure 19. Secondary metabolism in damaged leaves of *A. thaliana* after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. Threshold: 2.0; Software: MapMan.

It is becoming apparent that plastid (except for chloroplast), mitochondria, and peroxisome functions influence a wide range of processes outside of the organelles themselves (Geigenberger and Fernie 2014). Probably due to the small sizes and amounts, these organelles were not damaged enough to generate enough signaling compounds to influence the structuring and functioning of the organelles. Pathways associated with them were not observed to be significantly regulated.

Table 2. Significantly regulated pathways of *A. thaliana* after 9 h feeding by *P. xylostella*, local Treatment. Wilcoxon rank sum test, *: $0.01 < p < 0.05$; **: $0.005 < p < 0.01$; ***: $p < 0.005$. Benjamini Hochberg correction. Software: MapMan. Threshold: 3.0 (only genes that are over 3 times regulated are shown).

Mapman bin codes	name	elements	p-value	
16	secondary metabolism	53	8.72E-07	***
1	Photosynthesis	7	1.46E-04	***
26.16	misc.myrosinases-lectin-jacalin	11	1.70E-04	***
13	amino acid metabolism	25	2.44E-04	***
26	misc	116	3.45E-04	***
13.1	amino acid metabolism.synthesis	18	4.03E-04	***
20.2	stress.abiotic	11	4.34E-04	***
17.2.3	hormone metabolism.auxin.induced-regulated-responsive-activated	32	6.96E-04	***
30.3	signalling.calcium	164	3.70E-04	***
34.4	transport.nitrate	10	3.80E-03	***
26.28	misc.GDSL-motif lipase	10	1.39E-03	***
20.2.99	stress.abiotic.unspecified	5	1.95E-03	***
16.5.1.3	secondary metabolism.sulfur-containing.glucosinolates.degradation	4	3.00E-03	***
16.8	secondary metabolism.flavonoids	15	3.00E-03	***
17.6.3	hormone metabolism.gibberelin.induced-regulated-responsive-activated	3	3.49E-03	***
29.3	protein.targeting	158	3.50E-03	***
17.2	hormone metabolism.auxin	36	3.87E-03	***
16.8.1	secondary metabolism.flavonoids.anthocyanins	9	4.09E-03	***
16.5	secondary metabolism.sulfur-containing	13	4.35E-03	***
16.5.1	secondary metabolism.sulfur-containing.glucosinolates	13	4.35E-03	***
23	nucleotide metabolism	10	4.38E-03	***
21	redox	18	4.69E-03	***
17.5.2	hormone metabolism.ethylene.signal transduction	6	6.24E-03	**
26.2	misc.UDP glucosyl and glucoronyl transferases	17	6.43E-03	**
20.1	stress.biotic	44	6.48E-03	**
13.1.6	amino acid metabolism.synthesis.aromatic aa	9	7.38E-03	**
17.7.1	hormone metabolism.jasmonate.synthesis-degradation	9	7.69E-03	**
11.2	lipid metabolism.FA desaturation	3	9.94E-03	**
16.1.5	secondary metabolism.isoprenoids.terpenoids	3	1.03E-02	*
1.1	PS.lightreaction	3	1.04E-02	*
17.6	hormone metabolism.gibberelin	4	1.12E-02	*
23.1	nucleotide metabolism.synthesis	3	1.20E-02	*
23.4.99	nucleotide metabolism.phosphotransfer and pyrophosphatases.misc	3	1.36E-02	*
34.6	transport.sulphate	7	1.36E-02	*
29.5.1	protein.degradation.subtilases	3	1.39E-02	*
17.7	hormone metabolism.jasmonate	10	1.63E-02	*
28.2	DNA.repair	3	1.70E-02	*
16.5.1.3.1	secondary metabolism.sulfur-containing.glucosinolates.degradation.myrosinase	2	1.88E-02	*
8	TCA / org. transformation	4	1.99E-02	*
17.4	hormone metabolism.cytokinin	3	2.08E-02	*
16.2.1	secondary metabolism.phenylpropanoids.lignin biosynthesis	6	2.23E-02	*
1.3	PS.calvin cyle	2	2.50E-02	*
1.3.6	PS.calvin cyle.aldolase	2	2.50E-02	*
10	cell wall	413	2.52E-02	*
5	fermentation	10	2.54E-02	*
11.2.1	lipid metabolism.FA desaturation.desaturase	2	2.62E-02	*
34.12	transport.metal	78	2.67E-02	*
8.3	TCA / org. transformation.carbonic anhydrases	3	2.74E-02	*
27.3.24	RNA.regulation of transcription.MADS box transcription factor family	3	2.85E-02	*
1.1.1	PS.lightreaction.photosystem II	2	2.98E-02	*
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	2	2.98E-02	*
35.1.26	not assigned.no ontology.DC1 domain containing protein	4	3.12E-02	*
23.1.2	nucleotide metabolism.synthesis.purine	2	3.32E-02	*
23.1.2.8	nucleotide metabolism.synthesis.purine.SAICAR lyase	2	3.32E-02	*
26.13	misc.acid and other phosphatases	2	3.54E-02	*
13.1.6.5	amino acid metabolism.synthesis.aromatic aa.tryptophan	7	3.68E-02	*
26.9	misc.glutathione S transferases	13	4.45E-02	*
27.3.22	RNA.regulation of transcription.HB,Homeobox transcription factor family	4	4.72E-02	*

4.1.5 Compensatory energy system of chloroplast

Herbivory causes loss of leaf area and reduces photosynthesis (Table 2, MapMan bin code 1, green asterisk) (Giri *et al.* 2006, Velikova *et al.* 2010, Nabity *et al.* 2013). However, the fermentation process in this study was up-regulated (Table 2, MapMan bin code 5, red asterisk), indicating a compensation of down-regulated chloroplast function.

This analysis also shows that after 9 h insect feeding, the degradation of starch to glucose is down-regulated while to maltose is up-regulated (Figure 20).

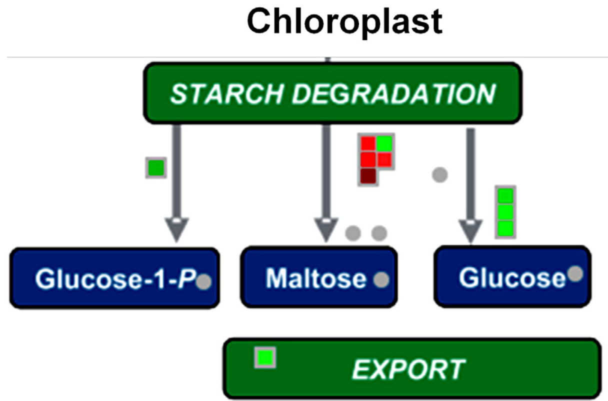


Figure 20. Starch degradation in damaged *A. thaliana* leaves after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. Threshold: 2.0; Software: MapMan.

Most higher plants accumulate starch in their leaves during the day, while in subsequent dark periods it is degraded (Scheidig *et al.* 2002). Transitory starch is stored during the day inside chloroplasts and broken down at night for export. Maltose is the primary form of carbon export from chloroplasts at night (Servaites and Geiger 2002, Ritte and Raschke 2003, Weise *et al.* 2004, Lu *et al.* 2005, Lu *et al.* 2006).

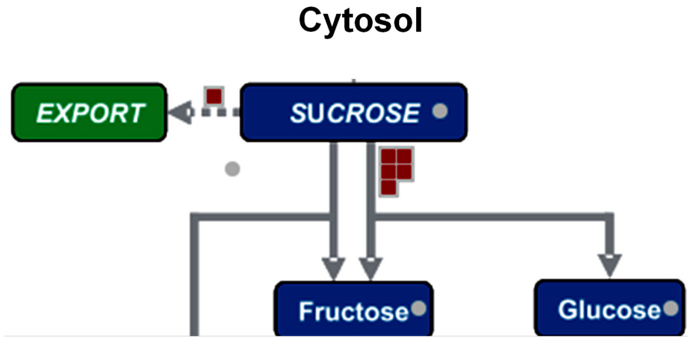


Figure 21. Sucrose degradation in damaged *A. thaliana* leaves after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. Threshold: 3.0 (only genes that are over 3 times regulated are shown); Software: MapMan.

Another compensation for glucose is the degradation of sucrose, which happens in the cytosol. Data of the experiments show that after insect feeding, generation of glucose from starch in chloroplast is down- (Figure 20) but degradation of sucrose in cytosol is up-regulated (Figure 21). More evidence needs to be obtained in future work.

4.1.6 Growth-defense tradeoff, signaling and resource communication between local and systemic leaves

Plant defense against herbivores relies on synthesis of phytochemicals that adversely affect the growth and development of the attacking pest (Howe and Ryan 1999). According to the timing of the deployment, defenses can be categorized as constitutive (or 'static') and induced (or 'active') defenses (Gatehouse 2002). Both are costly to plants and can compromise plants' growth and reproduction, ultimately reducing their fitness (Baldwin & Preston 1999; Tian *et al*. 2003; Zavala *et al*. 2004). Therefore, plants use sophisticated regulatory networks to maintain a balance between growth and defense response when attacked by herbivores.

It has been shown above that after insect feeding, in the damaged leaves, plant up-regulates defense genes such as cell wall precursors, RNA and protein synthesis, secondary metabolism, and signaling, while down-regulate reproduction and growth genes such as DNA synthesis. This suggests that after herbivory plants save energy in damaged leaves and invest more into defense.

In non-damaged systemic leaves, signaling genes are up-regulated at all the time points (Figure 15, systemic MecWorm and systemic insect treatments). This is an accumulation of signaling resources. If put together the accumulation of signaling resources, cell building and organization material, RNA, and protein resources in systemic leaves, it can be concluded that after insect attack, there is an allocation of energy and resources in the plant and there is an accumulation of signaling and cell repairing resources in the systemic leaves, which suggests the existence of a signaling, communication and transportation of energy and resources from systemic leaves to damaged leaves after the early defense responses.

As also mentioned above, the down- or up-regulation of organelle pathways doesn't mean the damaging status of the examined cells; instead, it represents a strategy of plant to receive and pass on signals of feeding and to allocate resources for defense and self-fitness economically.

These results indicate a growth and defense trade-off, meaning that processes for reproduction are down-regulated and more resources are used in defense production, especially in damaged leaves. Results of this work also show that plant herbivory growth and defense trade-off currency nitrogen (Ullmann-Zeunert *et al.* 2013) has up-regulated transportation (Table 2, purple asterisk).

Since forty years it is well known that there is a signal transmitted to other parts of the plant as result of herbivory (Green and Ryan 1972). Although the exact mechanisms are not yet clear, recent publications show that the vascular system and jasmonic acid pathway are essential in the systemic signaling (Green and Ryan 1972, Maffei *et al.* 2004, Maffei *et al.* 2006, Zimmermann *et al.* 2009). This study confirmed an active and fast up-regulation of the JA signaling pathway (Figure 22 and Figure 15-hormone metabolism).

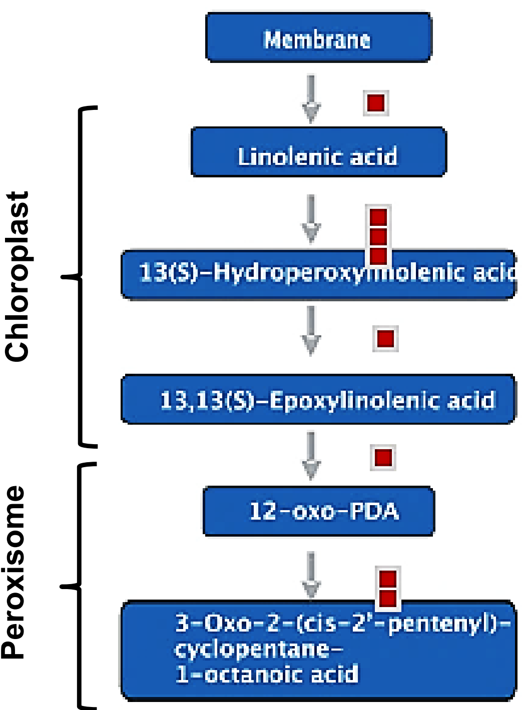


Figure 22. JA synthetic pathway in damaged *A. thaliana* leaves after 9 h *P. xylostella* treatment. Red grids represent up-regulated genes and green down-regulated genes. Threshold: 3.0; Software: MapMan.

Another very active process connected to transportation is protein targeting (Table 2, black asterisk and Figure 23). The up and down-regulated transporters and targeting genes indicate very active communication within and between cell compartments, both in damaged and systemic leaves.

It is important to study the energy and resource allocation during and after herbivory since herbivore defense is a whole plant mission. A lower photosynthesis rate results directly in a smaller carbon source in damaged leaves. Producing and releasing defense products such as secondary metabolites has a high cost. Catabolism of carbohydrates is another backup for defense production. Although significant positive relationships have been observed between herbivory and carbon allocated to roots, root exudates, and root and soil respiration (Holland *et al.* 1996), which may increase plants' ability of herbivory tolerance; there are other studies showing that attacked plants import more resources into the leaves to support plant defenses (Arnold and Schultz 2002, Arnold *et al.* 2004, Appel *et al.* 2012, Ferrieri *et al.* 2013). Therefore there is a resource defense-tolerance trade-off. Leaf derived jasmonates were identified as major regulators of this root-mediated resource-based trade-off. Jasmonate- and auxin-dependent mechanisms may lead to divergent defensive plant strategies against herbivores in nature (Machado *et al.* 2013).

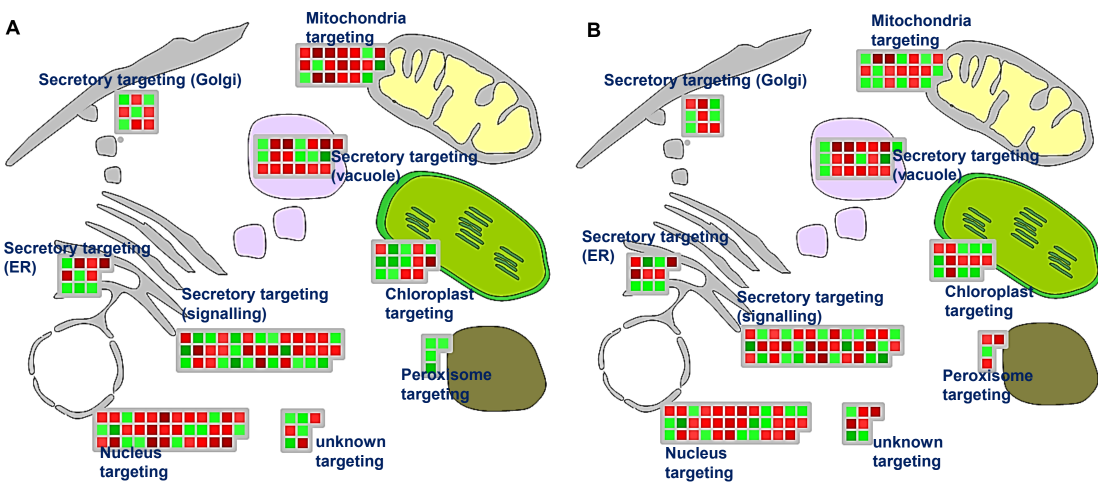


Figure 23. Protein targeting in *A. thaliana* leaves after *P. xylostella* treatment. 9 h damaged leaf (A) and 24 hS systemic leaf (B). Red grids represent up-regulated genes and green grids represent down-regulated targeting genes. Threshold: 2.0; Software: MapMan.

There are two aspects involved in intra- and inter-cellular communication in plant: energy (ATP, NAD(P)H) and resources (H_2O, carbohydrates, proteins, fatty acids, enzymes, metabolites etc.).

Reduction-oxidation status is the magic operator that transfers and transports energy through the intra- and inter-cellular environment of living organisms. In this analysis, redox associated genes were also significant regulated (yellow asterisk Table 2). In the chloroplast, reductants such as ferredoxin (Fdx) and NADPH are produced by the

photosynthetic electron transport chain, and along with ATP, used to generate sugar phosphates, amino acids, and many other metabolites that are supplied to the rest of the cell. Peroxisomes are single membrane-bounded subcellular organelles with an essentially oxidative type of metabolism and are probably the major sites of intracellular H_2O_2 production. These organelles also generate superoxide radicals and besides catalase they have a complex battery of antioxidative enzymes (del Rio 2011).

In addition to this, NAD(P)H metabolism is involved in central processes such as glycolysis, fermentation, and oxidative pentose phosphate pathway (OPP) in the cytosol, tricarboxylic acid (TCA) cycle, respiratory electron transport, and biosynthetic processes in mitochondria, and photorespiration in plastids, mitochondria, and peroxisomes. Therefore, redox status is a major integrator of subcellular and extracellular metabolism and is simultaneously itself regulated by metabolic processes. While knowledge of the network of metabolic pathways and their intra-organellar redox status regulation has increased in the last years, little is known about the inter-organellar redox signals coordinating these networks (Geigenberger and Fernie 2014).

A deeper looking into the energy and resource allocation, growth and defense trade-off, and cell communication during and after herbivory are all interesting topics in future work.

4.1.7 Quantitative RT-PCR

To verify the microarray data, four candidate genes were taken based on their expression patterns with a 10 times difference between the highest and lowest expression levels to avoid bias. The four expression patterns (Figure 24-A) are: not induced by MecWorm but up-regulated by *P. xylostella*; not induced by MecWorm but down-regulated by *P. xylostella*; down-regulated by MecWorm but up-regulated by *P. xylostella*; and up-regulated by MecWorm but down-regulated by *P. xylostella.* The four genes are: Benzoate-CoA ligase (*BZO1*, At1g65880.1), Clathrin adaptor complex small chain family protein (*CAP*, At1g60970.1), Pectinesterase family protein (*PE,* At2g26440.1) and acid phosphatase 5 (*ATACP5*, At3g17790.1). Figure 24 (B-E) shows the RT-qPCR result of all four candidate genes.

BZO1 is acting in the phenylpropanoid pathway shown above (see Figure 11, first row of enzymes, adding CoA); it is one of the genes that are regulated by chemical factors but not influenced by mechanical wounding.

CAP is involved in cell reproduction. In damaged leaves, genes for reproduction are down-regulated to give energy and resource priority to defense. Thus this gene is down-regulated in insect damaged 9 h treatments and not influenced by mechanical wounding alone.

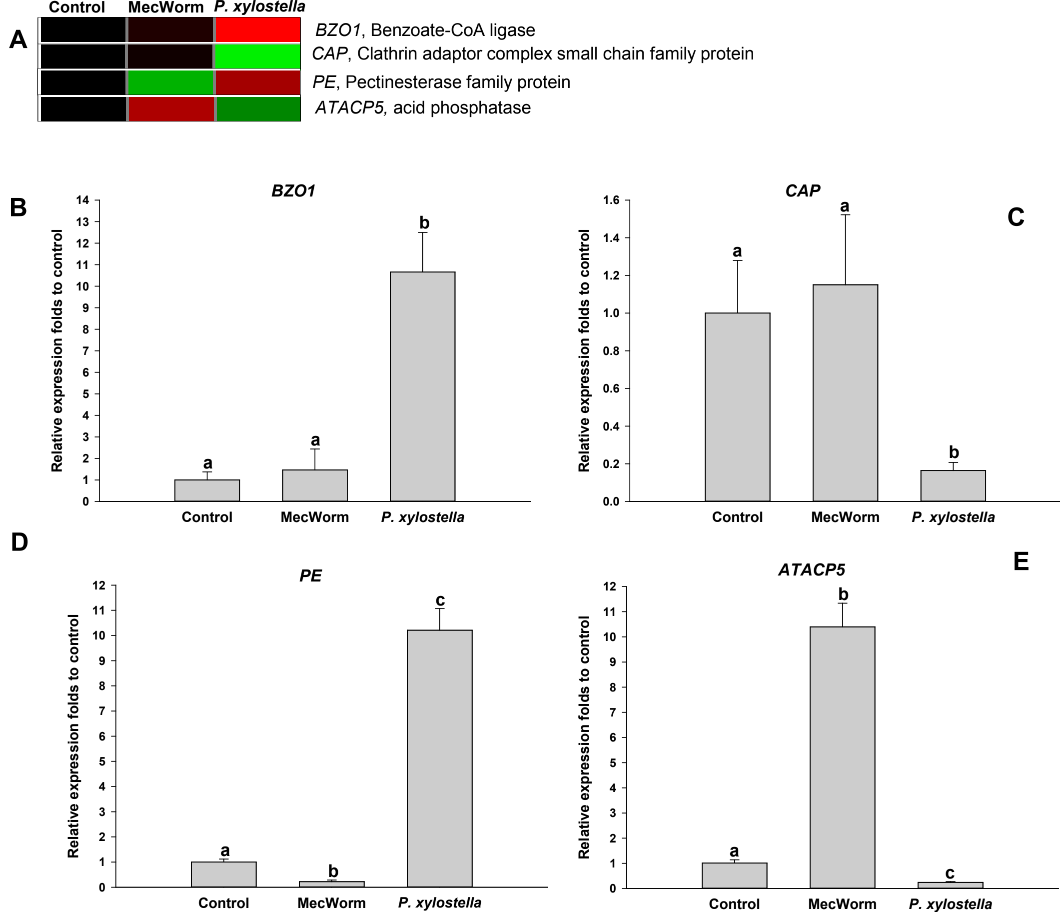


Figure 24. Verification of microarray results by quantitative real-time RT-PCR, *A. thaliana* plants 9 h treatments by MecWorm and *P. xylostella*. **A:** Expression patterns of the selected genes from microarray analysis results, for all the patterns the difference between the lowest and highest expressions are 10 times; Black squares are control genes or genes with same expression levels as controls; red squares are genes that are up-regulated, green down-regulated. **(B-E)** Quantitative real-time RT-PCR analysis of the selected genes. *BZO1*, Benzoate-CoA ligase **(B)**; *CAP*, Clathrin adaptor complex small chain family protein **(C)**; *PE*, Pectinesterase family protein **(D)** and *ATACP5,* acid phosphatase 5 **(E)**. Mean ±SD, n=9, one-way ANOVA, Post hoc test: Tukey.

PEs are involved in cell wall modification like improved cell elongation and cell adhesion. As shown in above microarray analysis, cell wall precursors are significantly up-regulated in damaged leaves to help plant to keep water and to strengthen battle against insect feeding. The up-regulation of this gene in insect 9 h local treatment in RT-qPCR is again consistent with result from above analysis, showing down-regulation by sole mechanical wounding which is turned to a strong up-regulation by chemical factors in insect feeding.

ATACP5, in *A. thaliana* transgenic plants (ATACP5) showed responses to phosphate starvation and to ABA and salt stress. So this gene is involved in abiotic stress reaction. It was up-regulated by MecWorm but suppressed in insect treatment. The chemical elicitors have a fine-tuning effect to mechanical wounding from abiotic stress to biotic stress direction. This is one of the ‘b’ area (Figure 10-I, area b) genes. This result is also consistent with the microarray analysis above.

All four candidate genes showed in RT-qPCR the same regulation pattern as in microarray analysis. In addition the results of published down-stream works are consistent with the microarray results, e.g. the down-regulation of photosynthesis, damaged-self recognition signaling, up-regulation of hormone signaling pathways and the fine-tuning function of chemical elicitors to mechanical wounding.

4.2 Turning MecWorm into SpitWorm: robotic mechanical wounding with simultaneous application of salivary components

To build up an insect mimicking tool combining both mechanical wounding and chemical elicitors, 'SpitWorm' was developed, which extends the original MecWorm (Mithöfer *et al.* 2005) with the ability of simultaneous delivery of oral secretions or other bioactive compounds.

The novel SpitWorm was expected to mimic the action of a feeding insect as close as possible, and provides the possibility to study the effect of individual bioactive molecules in combination with the effects of continuous mechanical damage. It would be ideal if the MecWorm could exactly mimic the biting pattern and deliver the same amount of insect OS onto the same wounding size in the same time period as insect feeding. However, different insects have different biting patterns, thus the microstructure of wounding surface and OS deliver patterns into the leaf veins are different. In addition, due to the conical shape of the aglet 'teeth' of the MecWorm, about 200 µm behind the cutting boarding of the wounding area is more frayed and flawed than insect cutting (Mithöfer *et al.* 2005). This small flaw may influence the delivery pattern and amount of the OS delivered. By using fluorescent labeled OS in both insect and SpitWorm, the fluorescent quantities delivered into the wounding area from insect and SpitWorm were compared with a serial of OS dilutions, to determine the right dilution times of OS so that same amount of effective OS is delivered to the same wounding area size at the same biting time period, despite the different biting patterns of insect and SpitWorm.

In this study lima bean (*P. lunatus*) was used because of its specialty to emit strongly a variety of induced VOCs (volatile organic compounds) after insect attack. This specialty has been used as an effective standard to study plant defense activities against insects. This plant was also used for the testing of MecWorm when it was developed and proved that continuous mechanical wounding alone can induce similar bouquet of VOCs compared to insect feeding. Since SpitWorm is developed based on MecWorm, it is reasonable to use the same plant-herbivore system for comparing and testing the functionality. For the same reason *S. littoralis* larvae were chosen as feeding insect, despite the fact that *S. littoralis* is a generalist instead of specialist like *P. xylostella* which doesn't feed on lima bean.

As mentioned in the introduction (page 15), the quantities of OS applied to mechanical wounded plants by using tools such as razor blade or pattern wheel were mostly more than thousand times higher than the real amount left behind at the wounding site by an insect. To precisely mimic insect feeding, it is necessary to determine the real amount left at the wounding zone by insect feeding.

To calculate OS delivery amount by chewing insect per bite, a workflow chart is shown in Figure 25: the insect is injected with fluorescence solution into the foregut, and then allowed to feed on plant leaves. The fluorescence amount left at the wounding zone can be quantified and used to calculate the amount of OS left by the insect per bite.

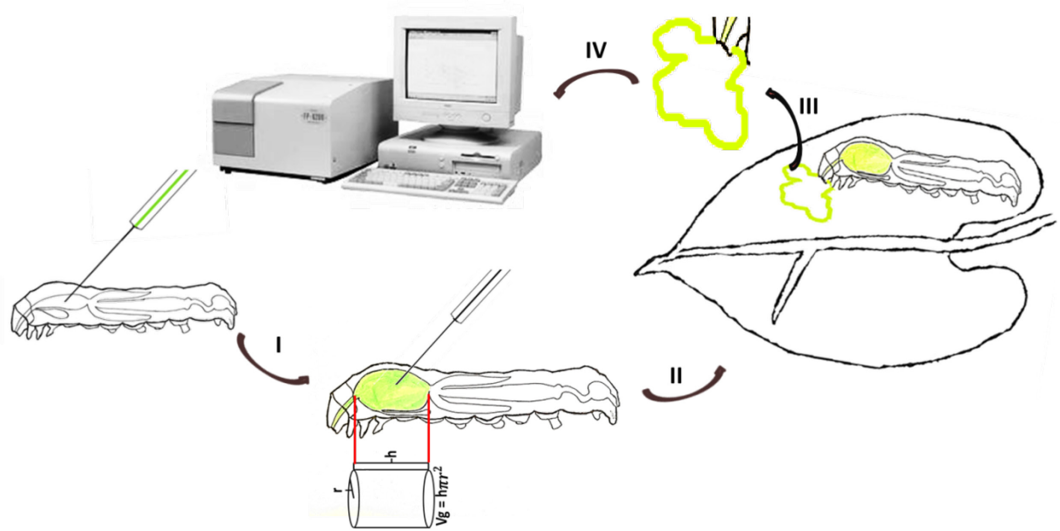


Figure 25. Workflow to measure insect saliva amount left on the leaf wounding area. I. *S. littoralis* larva injected with fluorescent dye into the foregut; II. *S. littoralis* larva foregut dissected and measured as a cylinder; III. fluorescence treated larva fed on *P. lunatus* leaf; IV. fluorescence dye signal at the wounding area of the leaf being quantified.

With the optimized system, typical herbivory-induced plant responses such as VOC induction and gene expression with special emphasis on JA responsive genes were investigated: lipoxygenase (LOX) in the octadecanoid pathway; phenylalanine ammonia-lyase (PAL) in the phenylpropanoid pathway; and pathogen-related (PR) proteins PR2 (b-1, 3-glucanase) and PR3 (chitinase) (Arimura, 2000). Using the SpitWorm, it is possible to study insect mechanical wounding and chemical factors both individually and collectively.

4.2.1 Wounding sizes of leaves fed by *Spodoptera. littoralis*

To make sure that SpitWorm and MecWorm have the same wounding size as *S. littoralis* feeding after certain time period of treatment, wounding sizes of insect feeding

after different time points were measured. With four replicates for each treatment, the mean wounding sizes after insect feeding were after 5 min (0.30 cm^2), 1 h (0.93 cm^2), 3 h (1.81 cm^2), 9 h (5.49 cm^2), and 17 h (7.25 cm^2), see Figure 26.

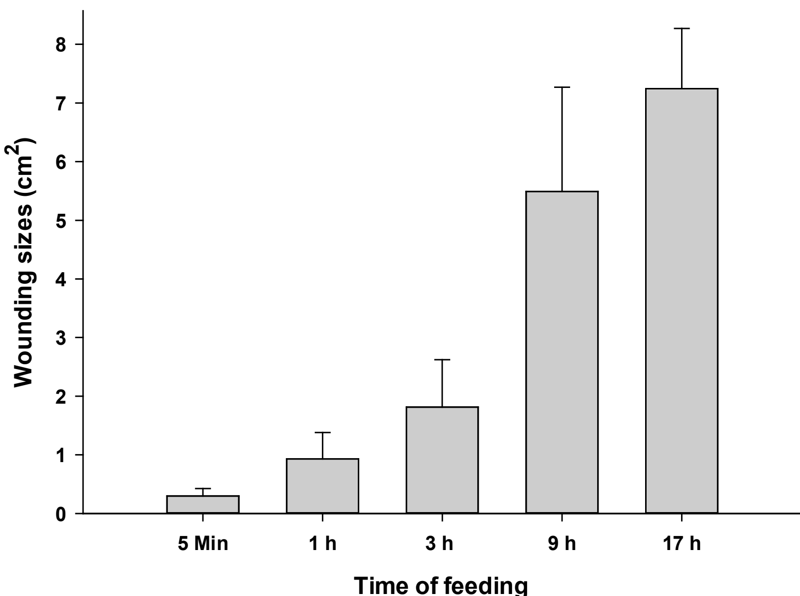


Figure 26. Wounding sizes of *S. littoralis* feeding on lima bean leaves after 5 min, 1 h, 3 h, 9 h and 17 h. Mean ± SD, n = 4.

To determine the optimal concentration of fluorescent dye to inject into insect foregut, insect activity after injection of different concentrations of dye was examined by measuring the wounding sizes 5 min after feeding.

Mean wounding sizes of *P. lunatus* leaves after 5 min feeding of *S. littoralis* (Figure 27) injected with 1 µL, 5 µL, 10 µL and 15 µL of a saturated solution of Lucifer yellow were: 0.29 cm^2, 0.35 cm^2, 0.14 cm^2, and 0.09 cm^2 respectively; mean control wounding size with larvae that were not injected was 0.30 cm^2. Four replicates for each treatment.

It can be seen from Figure 27 that insects with 10 µ*L* and 15 µ*L* injections showed significantly smaller wounding sizes comparing with the 5 min wounding size of control. This indicated that 10 µ*L* and 15 µ*L* injections influenced the feeding ability of the larvae. Larvae injected with 1 µL and 5 µL have the same wounding sizes as non-injected larvae, however, 5 µL solution was chosen as the treating dose for later experiment for a better detection and quantification of fluorescence (Figure 28).

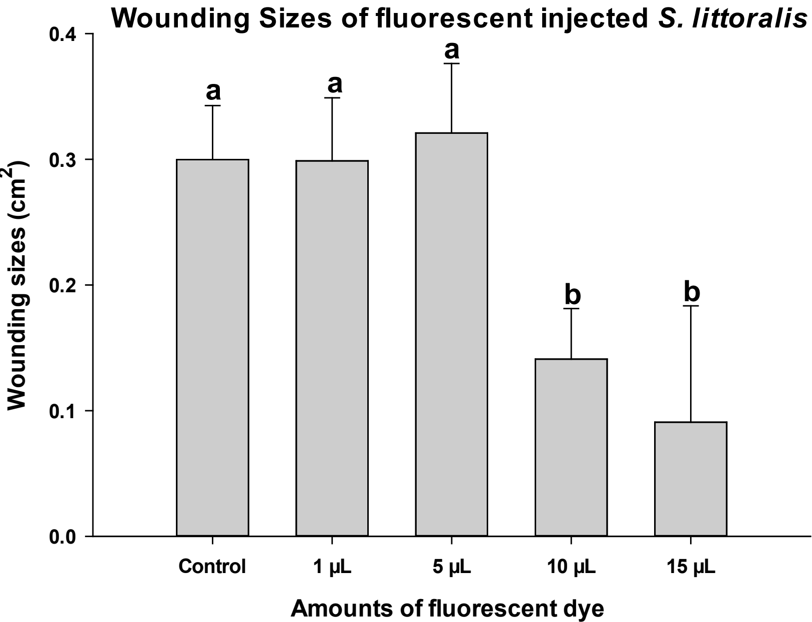


Figure 27. Wounding sizes of lima bean leaves after 5 min feeding of *S. littorals* injected with different amounts of fluorescence dye solution. Control means no injection to the insect. Bars with same letters are treatments showing no significant difference. Mean ±SD, n = 4 , One way ANOVA, Post hoc test: Tukey.

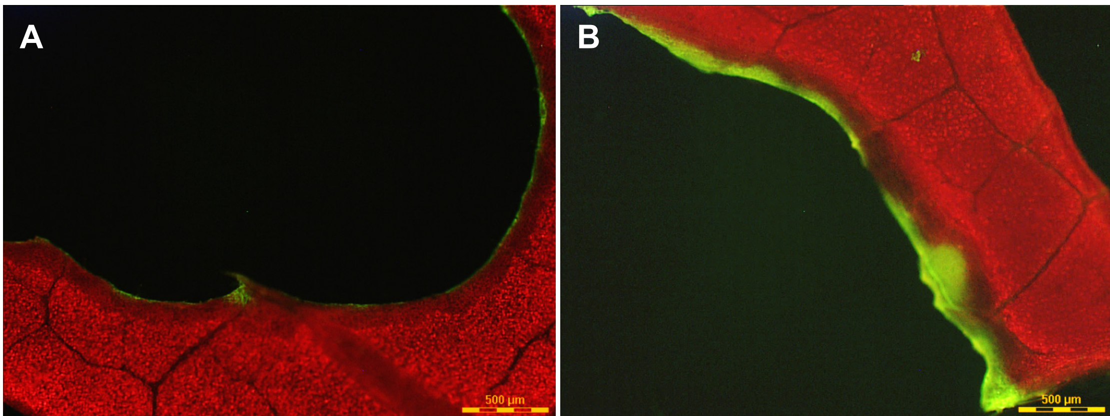


Figure 28. Comparison of fluorescent signals left in plant wounded sites by insects injected with fluorescent dye solution. **A**: wounded by insect with 1 µ*L* injection; **B**: wounded by insect with 5 µ*L* injection of a saturated solution of Lucifer yellow in water.

4.2.2 Determination of insect foregut volume

Based on the relative simple structure of the foregut of *S. littoralis* larva, it's shape was taken as cylinder. The dissection was conducted right after feeding to make sure that the foregut is in a fully expanded condition to be close to the shape of the cylinder.

Dissection of insect foregut after feeding with 5 replicates resulted in an average foregut length h = 4 mm; average width d = 3.75 mm; thus average foregut volume *Vg* was calculated as

$$Vg = h\pi\left(\frac{d}{2}\right)^2 = 4\ \text{mm} \times \pi\left(\frac{3.75\ \text{mm}}{2}\right)^2 \approx 44\ \mu\text{L}$$

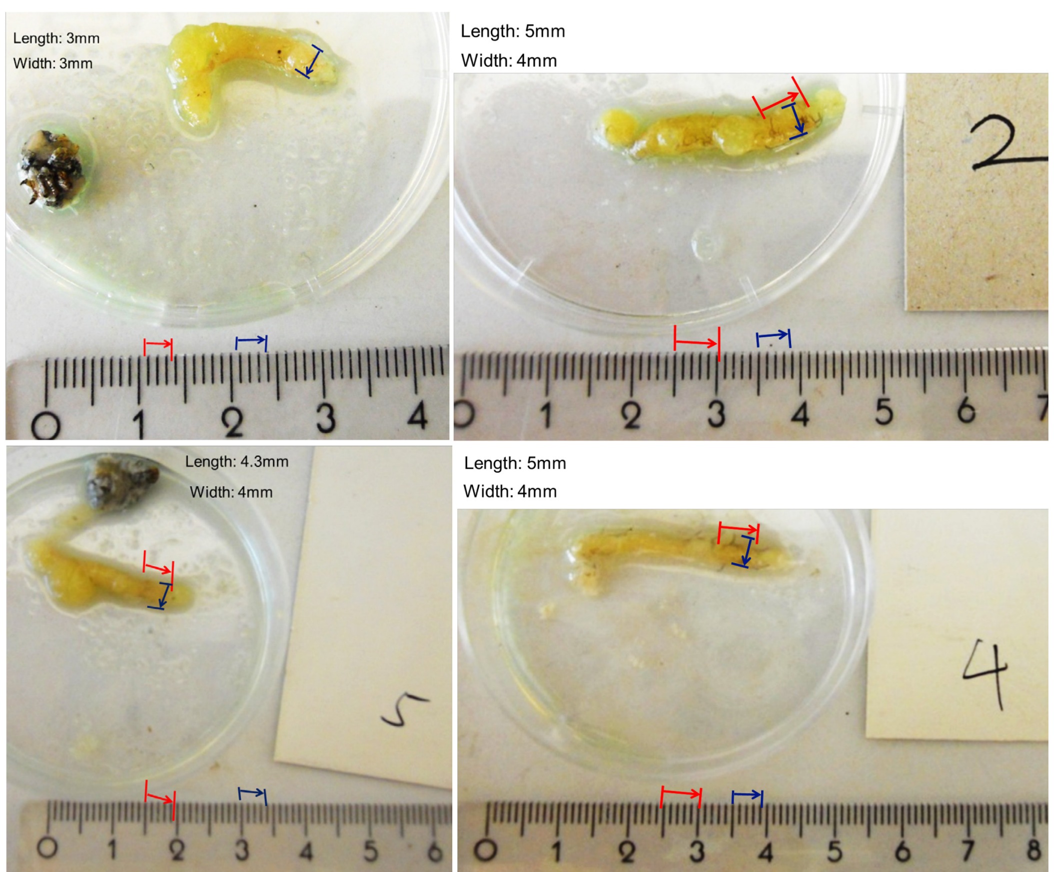


Figure 29. *S. littoralis* foregut volume measurement. 5 replicates.

4.2.3 Calculation of spit amount left by insect onto plant wounds per bite

The concentration of Lucifer yellow dye left by 5 µL injected insect after 5 min feeding in leaf wounded site was determined by fluorometer to be *Cd* (dye concentration) = 0.01 µL/mL in a sample volume *Vs* of 1 mL (Figure 33), percentage of dye in the injected insect foregut was calculated by foregut volume (*Vg* = 44 µL) divided by the injection volume (*Vi* = 5 µL). Treating time *t* was 5 min (300 *s*). Biting rate (*BR*) of insect larva was counted through close-up slow motion video to be 3-5 bites per second (bite/s). In the following calculations a biting rate of 4 bite/s was used.

Based on biting rate of insect larva and fluorescence amount left by insect within 5 min, OS volume left on plant leaf wounding area by insect per bite (*Vb*) was calculated as

$$Vb = \frac{Cd \times Vs \times \frac{Vg}{Vi}}{t \times BR} = \frac{0.01\frac{\mu\text{L}}{\text{mL}} \times 1\ \text{mL} \times \frac{44\ \mu\text{L}}{5\ \ \mu\text{L}}}{300\ \text{s} \times 4\frac{\text{bite}}{\text{s}}} \approx 73\ \text{pL/bite}$$

4.2.4 Oral secretion delivery rate of SpitWorm

As long as the delivery of liquid was high enough to form a droplet (Figure 30, C), a continuous trail of ink was obtained on a piece of white paper (instead of a leaf, Figure 30, D). By using ink with SpitWorm on white paper, the smallest rate to keep a continuous delivery or to leave continuous ink trail on the paper was 10 nL/sec.

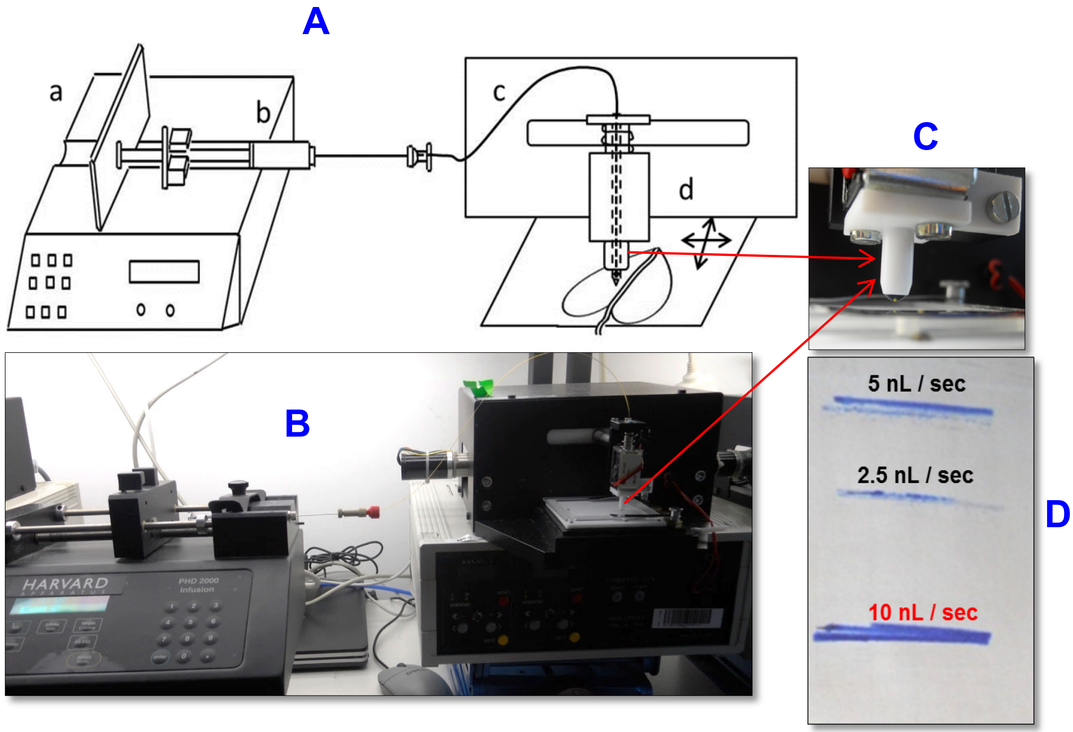


Figure 30. **A and B**: SpitWorm. a). syringe pump to control the delivery rate; b). 50 µL syringe to deliver the spit; c). capillary to connect the syringe to MecWorm through the hollow needle which has a little hole at the bottom to allow the OS fluid to go through to the leaf; d). MecWorm, a robotic system to mimic continuous wounding of insect. **C**: an enlarged picture of the 'tooth' of SpitWorm, with an ink droplet on the tip. **D**: ink marks left by SpitWorm with different delivery speed of ink.

4.2.5 Fluorescence imaging

After Lucifer yellow solution injection, larva was put under UV light immediately; the foregut area can be viewed glowing (Figure 31) from both front and back sides. In addition to the regurgitation of OS to plant during feeding, there is also a food and OS fluid going to the direction of midgut. In the fluorescence imaging, it could be observed that it takes 45 min to 1 hour for the fluorescence dye to start going through the whole body of insect till the anus. Therefore all experiments with fluorescent dye and insect dissection were conducted within 30 min after injection or feeding, respectively. The injection amount of fluorescence dye should not be over 10 µL to not influence the vitality and should not be less than 5 µL to have a detectable trace on plant later during feeding.

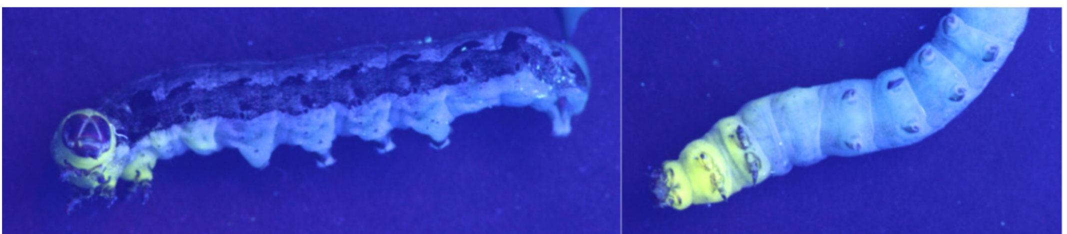


Figure 31. Larva after Lucifer yellow (5 µL, saturated solution) injection into the *S. littoralis* foregut area under UV light wavelength at λ_{Ex} = 428 nm. Fluorescent foregut area can be seen from both back and bottom.

After the insect was injected with fluorescent dye and then allowed to feed on plant leaves, fluorescent dye was detected around the wounding area. Figure 32-A shows that insect OS was left to plant during herbivory and it traveled within the vascular bundles in the leaf. By comparison with the OS trail left by SpitWorm Figure 32-B, the insect OS went deeper. Treatment with MecWorm and razor blade cut without using fluorescent dye did not show fluorescent trails (Figure 32-C, D), this clarifies that mechanical wounding itself has not caused any fluorescence effect.

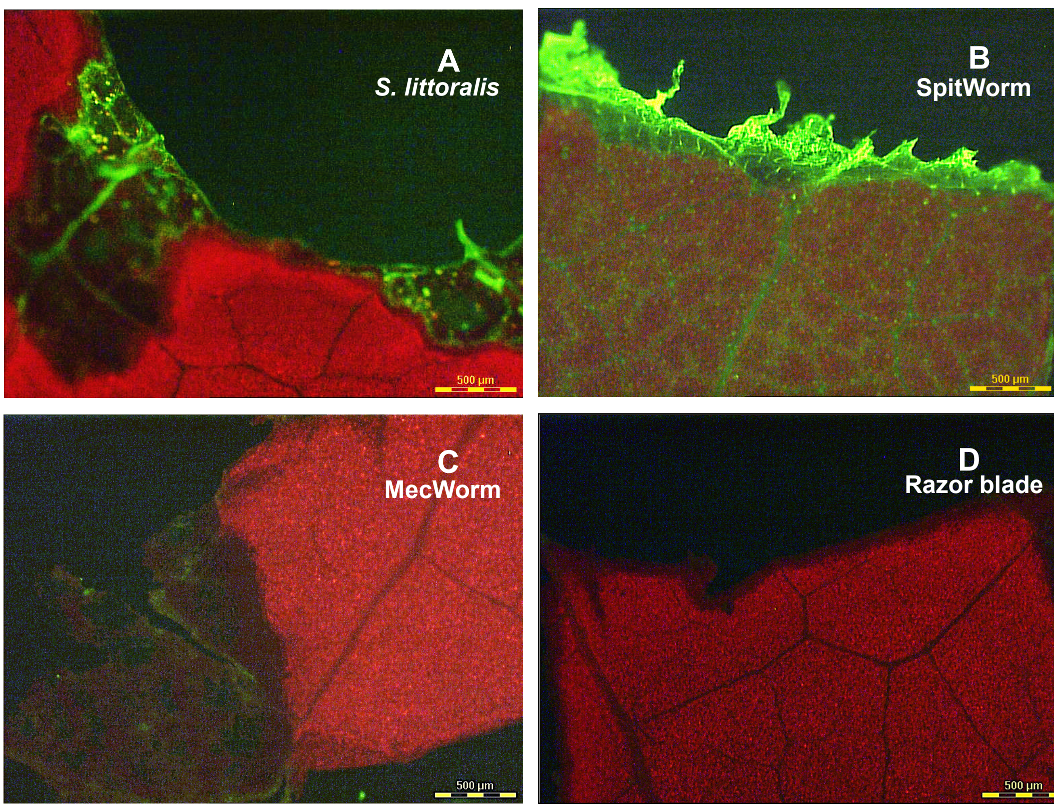


Figure 32. **A, B, C, D**: Fluorescent (λ_{Em} = 535 nm) microscopic pictures of lima bean leaves treated with *S. littoralis* larvae (**A**), SpitWorm (**B**), MecWorm (**C**) and razor blade (**D**). Larval and SpitWorm OS was labeled with Lucifer yellow (5 µL saturated solution into 44 µL OS). MecWorm and razor blade was not labeled.

Compared to insect, SpitWorm leaves a slight different pattern of OS trail. The OS of insect feeding goes deeper and faster into the plant tissue following the veins than that of the SpitWorm (Figure 32-A, B). The possible cause could be the difference between biting patterns of larvae, which forms a straighter border and MecWorm, which forms to some extend a small, frayed zone at the borderline, at the site of wounding as observed under the electron microscope (Mithöfer *et al.* 2005).

4.2.6 Quantification of fluorescence on the leaf wounded site left by insect and SpitWorm

From the calculation of OS amount left by insect larva per bite, it can be calculated that the OS delivered by insect to plant per second is 73 pL × 4 bite/sec = 292 pL ≈ 0.3 nL/sec. The lowest continuous delivery ability of SpitWorm (10 nL/sec) is about 30 times of the amount. The viscosity of the OS can also slow down the delivery speed. Therefore the OS delivered by SpitWorm needs to be filtered and diluted. To determine the optimal dilution times, which can enable SpitWorm to leave same amount on the wounding site, SpitWorm treatments with different concentrations of insect OS were compared.

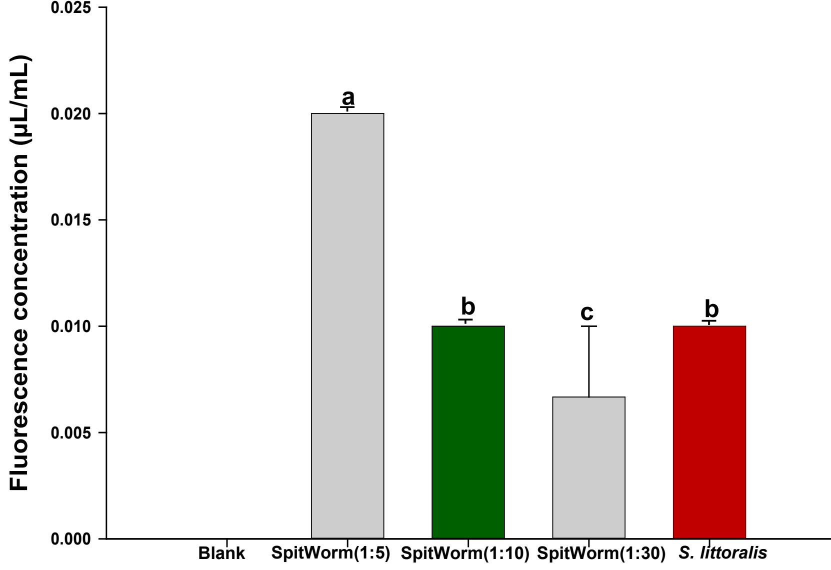


Figure 33. Comparison of fluorescent dye concentrations on leaves after treatment with larvae (injected with 5 µL saturated Lucifer yellow solution) and SpitWorm (44 µL OS and 5 µL saturated Lucifer yellow solution) with water dilution range of 1:5, 1:10 and 1:30. Mean ± SD, n = 3, one way ANOVA, Post hoc test: Tukey.

The concentration of Lucifer yellow was measured from the leaf samples treated by fluorescent labeled larvae (5 µL, saturated solution, injection) and SpitWorm (5 µL, saturated dye solution in every 44 µL OS) with different dilution serials (1:5, 1:10 and

1:30). Results showed that a SpitWorm treatment with a dilution of 1:10 of OS plus dye had the same dye concentration as larvae treatment (Figure 33), which accounted for the same concentration of OS left in the leaf.

With the delivery speed of 10 nL per second and 10 times diluted OS SpitWorm delivers 10 nL/10 = 1 nL effective OS per second, which is still about 3 times the amount of OS left by insect (0.3 nL per second). Here it needs to be taken into account that the feeding track of insect is not linear, it is normally in circles. The OS that was left in last circle is eaten by the insect in the next round and taken back to the insect, while SpitWorm needs to continue delivering without taking back OS because the damaged tissue is left outside. What is made sure in this experiment is the overall effect: the amount of OS that was left at the final wounding site, which is functioning as an elicitor for plant herbivory defense, from insect and SpitWorm, are the same.

To reduce the viscosity caused by large polysaccharides, fat and food residues in the insect regurgitate, freshly harvested spit was filtered through a 200 µm filter. Delivery of OS to SpitWorm was conducted under room temperature. Although the insects are raised and healthy in the same temperature range, it is not ensured that all the active compounds in the OS can keep the same activity, especially with long time delivery. This problem may be compensated by over delivery of OS. More experiments need to be done in the future to test the activities of chemical factors by using SpitWorm.

To prove that SpitWorm can closely mimic insect feeding, with all the parameters evaluated so far, experiments such as VOCs induction and RT-qPCR, comparing the performance of MecWorm, SpitWorm and insect were conducted.

4.2.7 Volatile organic compounds induction

Comparison of volatile induction in *S. littoralis*, MecWorm and SpitWorm treatments (Figure 34) showed that all three treatments resulted in induction of a blend of VOCs with similar qualitative composition.

2-Hexen-4-olide, (or 5-Ethyl-2(5H)-furanone, $C_6H_8O_2$) was induced by MecWorm and SpitWorm but not by *S. littoralis* feeding, this result is consonant with a former report where 2-Hexen-4-olide was only detected in MecWorm treatment but not in insect feeding (Bricchi *et al.* 2010). This indicates that 2-Hexen-4-olide is an artifact which could be induced by mechanical punching of the machine, by leaving behind smashed plant material which can degrade over the time course of the experiment.

This doesn't happen in the insect treatments because the insect takes away the tissues from the damaged plant. A contamination from MecWorm itself can be excluded because without leaf wounding this compound could not be detected after 24 h volatile collection (pers. commun. M. Kunert).

When compared with insect feeding, the MecWorm treatment did not induce three compounds, TMTT, indole and nerolidol. The absence of the latter two was consonant with the result of Mithöfer *et al.* who found that they were only induced by *S. littoralis* feeding but not MecWorm (Mithöfer *et al.* 2005). Although TMTT was detected in MecWorm treatment by both Mithöfer *et al.* and Bricchi *et al.* work with a relative low amount compared to insect feeding, it was not detectable in MecWorm treatments of this study.

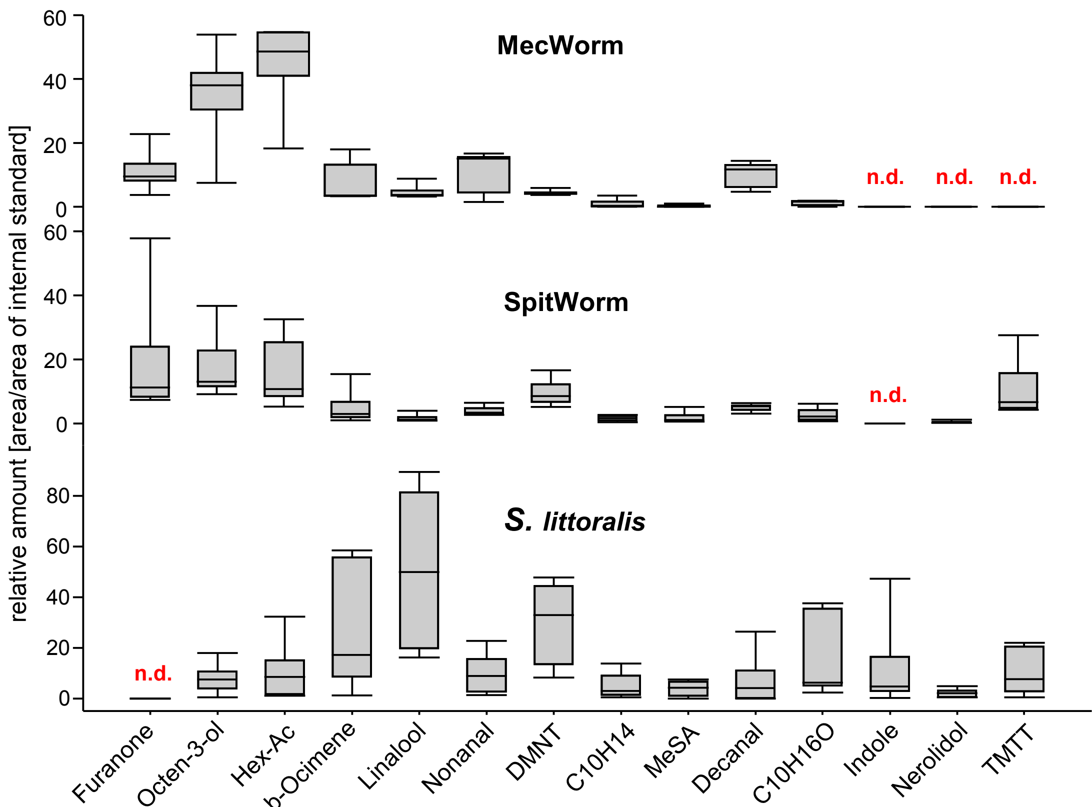


Figure 34. VOCs collected for 24 h after 17 h treatment. 14 compounds were detected and relative amounts were calculated by dividing sample peak area size by internal standard peak area size. The 14 compounds are: 2-hexen-4-olide ($C_4H_4O_2$); oct-1-en-3-ol ($C_8H_{16}O$); Hex-Ac (3-hexenyl acetate, $C_8H_{14}O_2$); (E)-β-ocimene ($C_{10}H_{16}$); 1-octanol ($C_8H_{18}O$); linalool ($C_{10}H_{18}O$); nonanal ($C_9H_{18}O$); DMNT (4,8-dimethyl-1,3,7-nonatriene, $C_{11}H_{18}$); $C_{10}H_{14}$ (degradation of ocimene on the charcoal filter); MeSA (methyl salicylate $C_8H_8O_3$); $C_{10}H_{16}O$ (degradation of ocimene on the charcoal filter); indole (C_8H_7N); nerolidol ($C_{15}H_{26}O$); TMTT ((3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, C_6H_{26}). 'n.d.': compounds not detected. n=5 for each treatment.

When compared with insect feeding, SpitWorm treatment had one compound, indole, not detected. However, in Mithöfer *et al.* work it was also very slightly induced in insect feeding.

Although SpitWorm has one artifact compound due to mechanical effect (2-Hexen-4-olide), it is effective in mimicking the pattern of insect feeding both mechanically and chemically. These results confirm the conclusion that mechanical wounding is the major cause of VOCs emission (Mithöfer *et al*., 2005); on the other hand, insect OS plays a relative minor role in this part of defense.

4.2.8 Comparative RT-qPCR

To test SpitWorm treatment of *P. lunatus* leaves by RT-PCR, four JA responsive genes were chosen. They were also used in earlier studies (Arimura *et al.* 2000). The four genes are: lipoxygenase (LOX3), phenylalanine ammonia-lyase (PAL), pathogenesis-related (PR) proteins (PR2 (b-1, 3-glucanase)) and (PR3 (chitinase)).

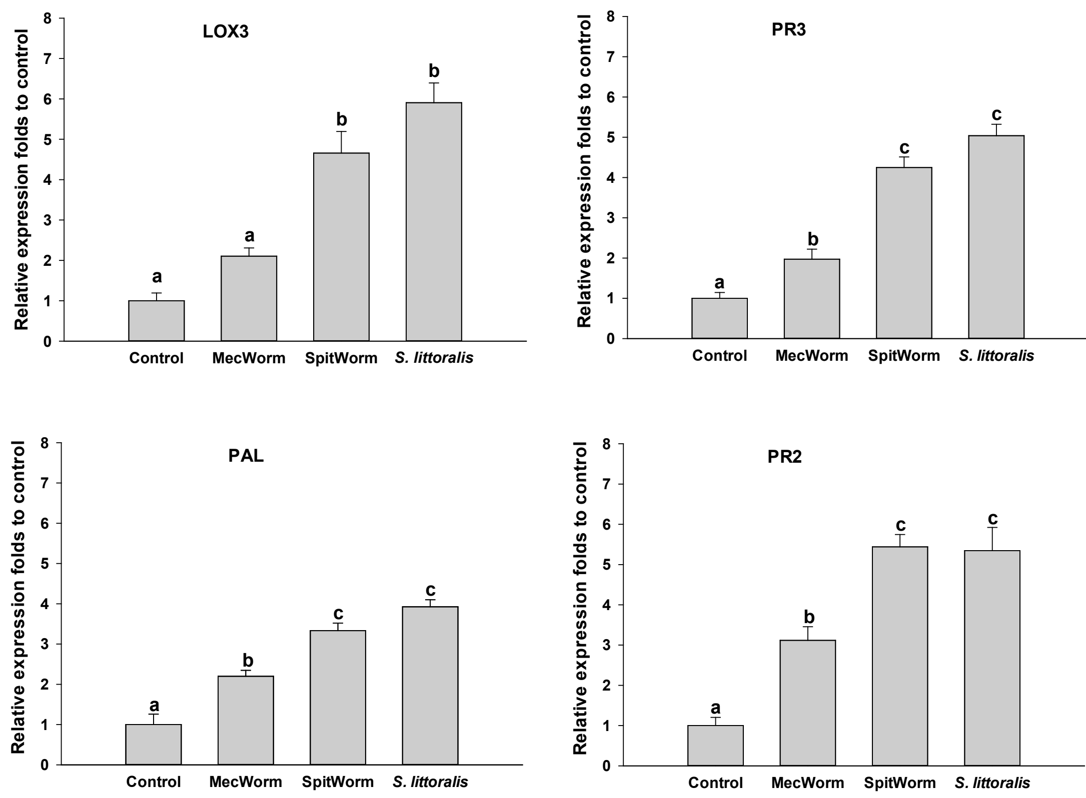


Figure 35. Gene expression of 4 JA responsive genes (*LOX3, PAL, PR2, and PR3*) from lima bean at 1 h. SpitWorm with 10 times diluted OS and delivery speed of 10 nL/s. Bars with same letters are expression levels with no significant difference. Mean ± SD, n = 6, one-way ANOVA, post hoc test: Tukey.

For all the four JA responsive genes (Figure 35), all the three treatments showed an induction. SpitWorm and larvae treatments showed stronger induction and there was

no significant difference between the inductions of SpitWorm and larvae feeding. The result confirmed that 10 times diluted OS is the optimal dilution factor to add OS to SpitWorm to mimic *S. littoralis* feeding.

Expression of all the 4 JA responsive genes was all up-regulated after MecWorm and *S. littoralis* treatments. This result is also consistent with the microarray analysis using *A. thaliana* treated by MecWorm and *P. xylostella* (Figure 15, hormone signaling).

As for defense reaction, SpitWorm treatments showed stronger induction of JA responsive genes, compared to only continuous mechanical wounding from MecWorm (Figure 35.). This indicates that mechanical wounding induces the JA responsive genes pathway and chemical factors enhance this induction. The results emphasize that both mechanical wounding and chemical factors play prominent roles in the gene regulation and defense reaction, which further proves that SpitWorm can be used as an effective tool in mimicking insect feeding. This result also confirmed the result in above microarray analysis (see page 36) that in local wounded leaves, mechanical wounding can trigger most of the defense reactions while chemical factors in insect OS have a ‘fine-tune’ function by enhancing or reducing the induction of gene regulation from mechanical wounding only.

4.3 Mechanical wounding and chemical factors - MecWorm and SpitWorm

Plant insect defense is a complex event which is triggered by a collaboration of both mechanical and chemical wounding. However, there are specific defense activities which response to either mechanical wounding or chemical factors. For example, mechanical wounding alone can cause the releasing of the major volatile organic compounds, the reduction of photosynthesis and the destruction of cell wall structures while chemical elicitors FACs (Fatty acid amino acid conjugates) and phytohormones are able to induce PR genes and whole plant responses (Gatz 1997, Klarzynski and Fritig 2001).

However, the more complicated regulation of gene expression is the result of both, namely mechanical wounding and chemical factors. According to results of this study, in damaged leaves, mechanical wounding is main trigger while chemical factors are fine-tuner. In systemic leaves, chemical factors were observed to be the major force of gene regulation induction in this study. In addition, there are already down-stream studies other than gene expression level showing this fine-tuning system of chemical

factors. For example, a significant increase in cytosolic Ca^{2+} concentrations was observed in *S. littoralis* treated lima beans, whereas single cut wounding and MecWorm wounding did not affect this second messenger (Bricchi *et al.* 2010). Musser *et al* (Musser *et al.* 2006) have compared insect mechanical wounding and insect feeding by either surgically removing the labial salivary gland or cauterizing the insect's spinneret. Both methods showed influence of insect saliva in plant anti-herbivore defense compounds production. "*These two methods are useful techniques for determining the role that saliva plays in manipulating plant anti-herbivore defenses.*"

Chemical factors can only fully function with the combination of mechanical wounding. Studies investigating the plant defense responses have been done by applying insect OS onto non-damaged leaves (Halitschke and Baldwin 2003). However, in such case it is difficult to get the full performance of chemical factors in gene regulation due to the physical barriers of the plant. By studying mechanical wounding and insect wounding separately, it has been proven that insect OS to wounds enhances insect performance (Consales *et al.* 2012). By comparing the gene regulation at different time points in both damaged and systemic leaves, this study showed that mechanical wounding itself can already trigger most of the defense pathways, while insect OS has an enhancing or reducing effect in regulating defense responses when combined with mechanical wounding. This enhancing or reducing effect directs plant defense to a more economical and defense-priory direction.

MecWorm offered a platform to study mechanical wounding of chewing insects to plant, and offered a platform to develop SpitWorm, which is a combination of mechanical and chemical mimicking of insect feeding. This enlarges the possibility of elicitor research in future work. It is possible to study the mechanical wounding and insect spit compounds one after another with different environment conditions and different combination of compounds. With the help of other comparative genomic, transcriptomic or proteomic methods, it is possible to go further and deeper in understanding of gene regulation of plant defense against insect feeding.

5 SUMMARY

Plant defense against herbivory is a broad field of scientific interest. I tried to understand and study the topic from the most basic area: gene expression. The interactions between insects and plants are among the basic and oldest ecological forms of interaction on our planet. Beyond ecology, morphology, physiology, and chemistry, of both insect and plant, this work focuses first on the molecular biology processes on the plant side in response to insect feeding and second on the development of a tool to mimic as similar as possible insect feeding both mechanically and chemically.

With these aims, first gene regulation analysis using microarray data of *Arabidopsis thaliana* leaves was conducted. Besides untreated control leaves, treatments were *Plutella xylostella* (diamondback moth) larvae feeding and continuous mechanical wounding by a computer controlled artificial system (MecWorm) mimicking the feeding damage and biting pattern of *P. xylostella*. By comparison of microarray data of both treatments it was possible to clearly separate the influence of mechanical wounding from chemical effects of larval saliva on plant gene expression patterns.

This refined analysis of the different wounding scenarios for damaged leaves revealed that insect mechanical wounding can trigger gene regulation in most of the metabolic pathways while chemical factors from insect oral secretion fine-tune the gene expression by enhancing, inhibiting, or additionally inducing the regulation.

In damaged leaves, metabolic pathways in chloroplast, cell wall and nucleus are down-regulated while most pathways in cytosol are up-regulated. For example, when photosynthesis of the damaged leaves is reduced, the genes for proteins involved in other chloroplast metabolic pathways are also down regulated. These observations support the “Damaged-Self Recognition Theory”, which supposes that substances from damaged cell compartments represent signals for healthy cells in order to direct their energy and resources to functional parts for a more efficient defense and self-recovery.

In contrast to damaged leaves where the regulation of defense compounds such as secondary metabolites is enhanced, in systemic leaves the regulation of signaling and resources for recovery is stronger. This up-regulation of signaling, resource accumulation, and active transporter genes in systemic leaves indicates a transportation and communication system between damaged leaves and intact leaves for later recovery

of plant. Interestingly, different to wounded leaves, in systemic leaves the insect derived chemical factors are the major driving force for regulation.

As a consequence the following conclusions can be drawn: After insect feeding, damaged leaves are the direct battle field against insect attack. Plants react by reducing photosynthesis and cell reproduction and shift their major activities in the damaged leaves to defense. The damaged compartments themselves signal neighboring undamaged cells for defense and resource allocation. Systemic leaves conduct resources production for plant recovery and defense with powerful signaling and communication. In plant-insect interaction gene regulation, mechanical wounding is the major trigger, while chemical factors are fine tuners for a more efficient and biotic stress focused defense machinery.

Inspired by the results obtained from microarray analysis, it was of interest to build up an effective tool to mimic insect feeding more precisely. To further study the roles of mechanical wounding and chemical elicitors both under controlled conditions, the well-established MecWorm system for mechanical leaf wounding was developed further to a so called 'SpitWorm' which can mimic additionally the delivery of insect saliva during the feeding process. This new robotic device was designed according to the plant-herbivore system *Phaseolus lunatus* (lima bean) / *Spodoptera littoralis* (African cotton leafworm), because *P. lunatus* emits on insect feeding a well-defined bouquet of volatiles which are not inducible by simple scratch wounding and only partly inducible by MecWorm. After studying larval features and behavior in detail, it was possible to make sure that the robot could deliver the same effective amount of saliva onto the wounding site with the same sizes at the same time points as the feeding of *S. littoralis* larvae. Comparing profiles of emitted volatiles from *P. lunatus* fed by *S. littoralis* or treated by SpitWorm by GC-MS revealed that SpitWorm is able to induce a volatile bouquet almost identical to herbivory induction, qualitatively and quantitatively. On gene expression level RT-qPCR of four jasmonic acid responsive genes from *P. lunatus* showed that different to sole mechanical wounding by MecWorm, SpitWorm induced a gene regulation pattern identical to *S. littoralis* feeding.

With this new developed SpitWorm, it will be possible to conduct future studies on mechanism of insect feeding by combination of mechanical wounding and chemical

elicitors in different combinations and concentrations and to decipher the influence of individual saliva constituents on plant defense reactions.

6 ZUSAMMENFASSUNG

Die Abwehr der Pflanzen gegenüber Fraßschädlingen ist ein weites Feld von wissenschaftlichem Interesse. In dieser Arbeit versuche ich dieses Thema, ausgehend von dem Grundlegendsten, der Genexpression, zu studieren und zu verstehen. Die Interaktionen zwischen Pflanzen und Insekten ist eine der ältesten und grundsätzlichsten Formen der Ökologie unseres Planeten. Jenseits von Ökologie, Morphologie, Physiologie und Chemie der Insekten und Pflanzen, konzentriert sich diese Arbeit erstens auf die molekularbiologischen Reaktionen der Pflanze gegenüber Insektenfraß und zweitens auf die Entwicklung eines Systems, welches diesen Insektenfraß sowohl mechanisch als auch chemisch künstlich nachahmen kann.

Vor diesem Hintergrund, wurden Mikroarray-Analysen der Genregulation in *Arabidopsis thaliana* Blättern durchgeführt. Neben unverwundeten Blättern wurden Blätter untersucht an denen Larven von *Plutella xylostella* (Kohlmotte) gefressen hatten. Darüber hinaus wurden Blätter, welche durch eine computergesteuerte „mechanische Raupe“ (MecWorm), die die mechanischen Fraßschäden und Bissmuster von *P. xylostella* imitieren kann, verletzt wurden, untersucht. Durch den Vergleich der Mikroarray-Daten aus beiden Verletzungsszenarien konnten der Einfluss der mechanischen Verwundung und die chemischen Effekte der Inhaltsstoffe des Raupenspeichels auf das Genexpressionsmuster der Pflanzen klar voneinander unterschieden werden.

Diese tiefergehende Analyse der verschiedenen Verletzungsszenarien zeigte für die verletzten Blätter, dass grundsätzlich die reine mechanische Verwundung die Genregulation für die meisten die Stoffwechselwege auslösen kann. Die chemischen Faktoren, aus dem oralen Sekret der Laven bewirken hingegen eine Feinabstimmung der Genexpression durch Verstärkung, Inhibierung oder zusätzlicher Induktion.

In den verletzten Blättern werden die Stoffwechselwege im Chloroplasten, sowie im Zellkern und der Zellwand heruntergeregelt. Hingegen werden die meisten Stoffwechselwege im Cytosol heraufgeregelt. Wird zum Beispiel die Photosyntheseaktivität in den Chloroplasten verletzter Blätter reduziert, so werden gleichzeitig auch die Gene für Proteine, die an anderen Stoffwechselwegen im

Chloroplasten beteiligt sind, heruntergeregelt. Diese Beobachtungen unterstützen die "Damaged-Self Recognition Theory" (Theorie über die Erkennung des verletzten Selbst), welche davon ausgeht, dass Substanzen aus zerstörten Zellkompartimenten Signalwirkung auf unverletzte Zellen haben, um diese zu veranlassen ihre Energien und Ressourcen zur effizienteren Verteidigung bzw. Regeneration der Pflanze einzusetzen.

Im Gegensatz zu den verletzten Blättern, in denen die Produktion von Abwehrstoffen, wie Sekundärmetabolite, verstärkt wird, zeigen systemische Blätter eine stärkere Regulierung der Gene, die mit Signal- und Zellreparaturprozessen verknüpft sind. Diese Hochregulierung von Genen, die für Informationsübertragung, Akkumulation von Ressourcen oder aktive Transportprozesse verantwortlich sind, weist auf ein ausgeprägtes Transport- und Kommunikationssystem zwischen beschädigten und intakten Blättern der Pflanze hin. Diese könnten späteren Wiederherstellungsprozessen der Pflanze dienen. Interessanterweise zeigte sich im Gegensatz zu verwundeten Blättern, dass hier überwiegend die chemischen Faktoren die treibende Kraft der Genregulation darstellen.

Diese Ergebnisse lassen folgende Schlussfolgerungen zu: Bei Insektenfraß bilden die verwundeten Blätter die vorderste Frontlinie gegen den Insektenangriff. Photosyntheseaktivität und Zellreproduktion werden reduziert und die Hauptaktivitäten des Stoffwechsels in Richtung Verteidigung verschoben. Substanzen aus den Kompartimenten der zerstörten Zellen regen die unzerstörten Zellen an, ihre Verteidigung heraufzufahren. Systemische Blätter hingegen verwenden den Großteil ihres Ressourcenverbrauchs zum Wiederaufbau und zur Kommunikation. Es zeigte sich, dass bei den durch die Pflanze-Insekt-Interaktion bewirkten Genregulationsprozessen die mechanische Verwundung der Hauptauslöser ist. Chemische Faktoren dienen eher der Feinregulierung einer effektiveren und auf den biotischen Stress fokussierten Verteidigungsmaschinerie.

Angeregt durch die Ergebnisse der Mikroarray-Analysen war es von großem Interesse ein Gerät zu entwickeln, welches den Insektenfraß noch besser und effektiver nachahmt. Um die Rolle von mechanischer Verletzung sowie den Einfluss chemische Elizitoren unter kontrollierten Bedingungen besser studieren zu können, wurde das bereits etablierte MecWorm-System zur mechanischen Blattverletzung zum sogenannten SpitWorm weiterentwickelt. Dieser kann zusätzlich die Zufuhr des Spei-

chels der Raupe während des Blattfraßes imitieren. Dieses neue Gerät wurde in Anlehnung an das Pflanze-Herbivor-System *Phaseolus lunatus* (Limabohne) / *Spodoptera littoralis* (Afrikanischer Baumwollwurm) entwickelt, weil *P. lunatus* bei Insektenfraß ein definiertes Bouquet von Duftstoffen emittiert, welche durch einfache Riss- oder Stichverletzungen der Blätter nicht und durch eine kontinuierliche Verletzung (MecWorm) nur unvollständig induziert werden. Nach intensivem Studium der Eigenschaften und des Fraßverhaltens der Raupen war es mittels SpitWorm möglich, die gleiche effektive Speichelmenge bei einem vergleichbaren Zeitverlauf, wie bei *S. littoralis*-Fraß, an die Verwundungsstellen des Blattes zu applizieren. Gleichzeitig wurden die Größen der Flächen des pro Zeiteinheit durch SpitWorm zerstörten Blattmaterials dem Fraßmuster von *S. littoralis* angepasst.

Der Vergleich der GC-MS-Profile der von *P. lunatus* emittierten Duftstoffe nach Raupenfraß bzw. nach artifizieller Verletzung durch SpitWorm, welcher verdünntes, von *S. littoralis* gesammeltes Regurgitat applizierte, zeigte, dass SpitWorm in der Lage ist, ein zum Raupenfraß qualitativ und quantitativ nahezu identisches Duftstoffbouquet zu induzieren. Auf der Ebene der Genexpression zeigte die Auswertung der qualitativen real time PCR von vier Jasmonsäure-responsiven Genen von *P. lunatus* ein Regulationsmuster identisch zu *S. littoralis* Fraß. Dies ist bei rein mechanischer Verletzung durch MecWorm in dieser Form nicht zu beobachten.

Mit dieser neu entwickelten „mechanisch-chemischen“ Raupe wird es möglich sein, weitergehende Untersuchungen der Mechanismen des Insektenfraßes durch die Verknüpfung von mechanischer Verwundung mit chemischen Elizitoren in unterschiedlichen Kombinationen und Konzentrationen durchzuführen, um die Einflüsse der verschiedenen, im Raupenspeichel enthaltenen Substanzen auf die Verteidigungsreaktionen der Pflanze zu entschlüsseln.

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8 APPENDIX

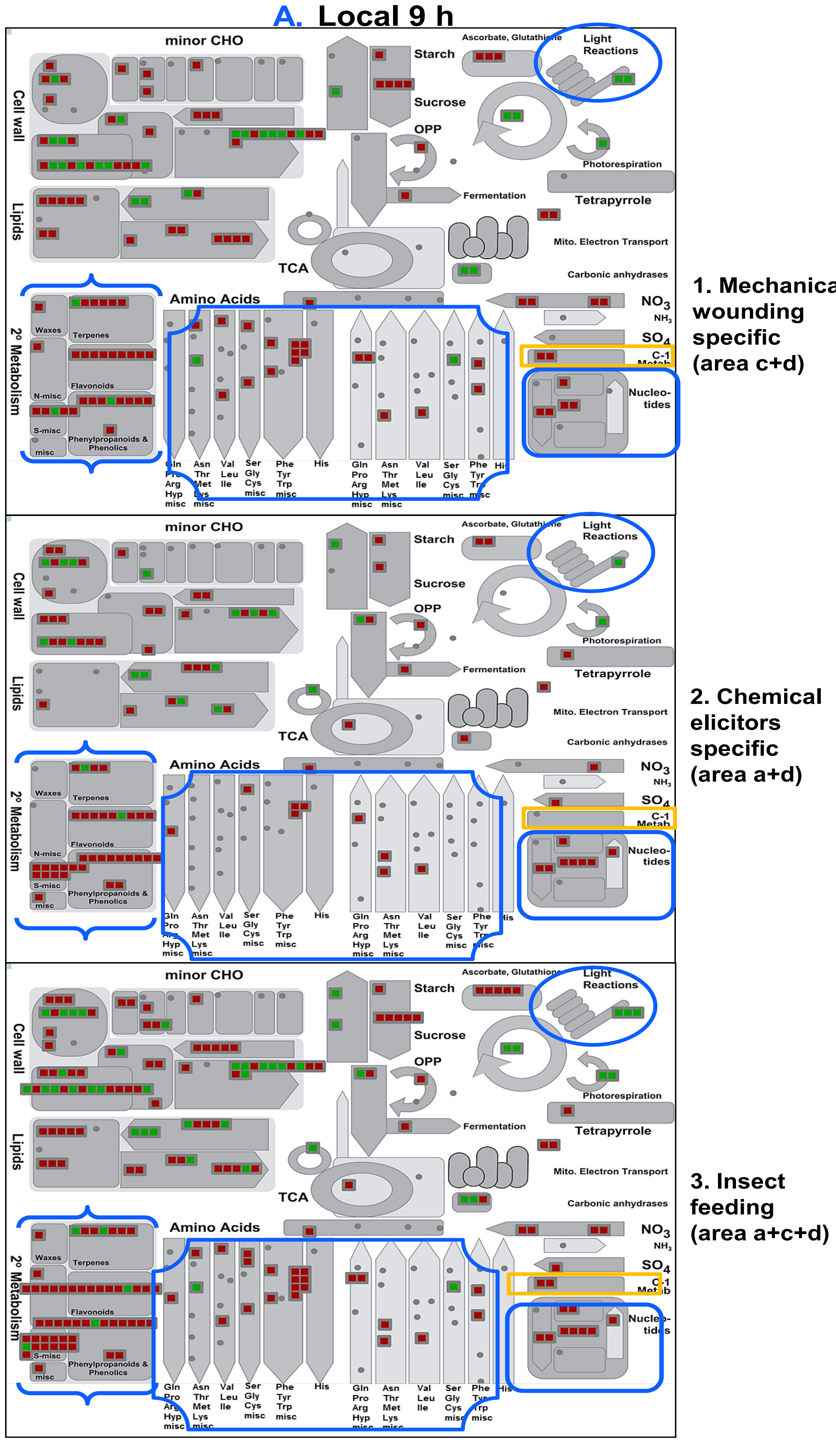
In the section “Overview of pathway regulations: Roles of mechanical wounding and chemical factors in both local and systemic samples”, the pathways that highlight the roles of mechanical wounding and chemical factors were shown in figures 11, 12, 13 and 14. The complete maps of gene expression overview of all metabolic pathways, biotic stress pathways, regulation pathways, and lignin precursors synthetic pathways from both local 9 h and systemic 24 h are listed as below as Appendix figure 1-5, for more general and detailed view.

The highlighted pathways with different shapes and colors are according to the 3 roles of chemical factors in fine-tuning the roles of mechanical wounding:

Enhance or induce the effect of mechanical wounding: photosynthesis or light reaction (Appendix figure 1-A, blue circles), jasmonic acid (JA) synthetic pathway (Appendix figure 2-A and Appendix figure 3-A, blue asterisks), nucleotide synthesis (Appendix figure 1-A, blue squares), amino acid synthesis (Appendix figure 1-A, blue plaques), and secondary metabolites synthesis (Appendix figure 1-A and Appendix figure 2-A, blue double braces).

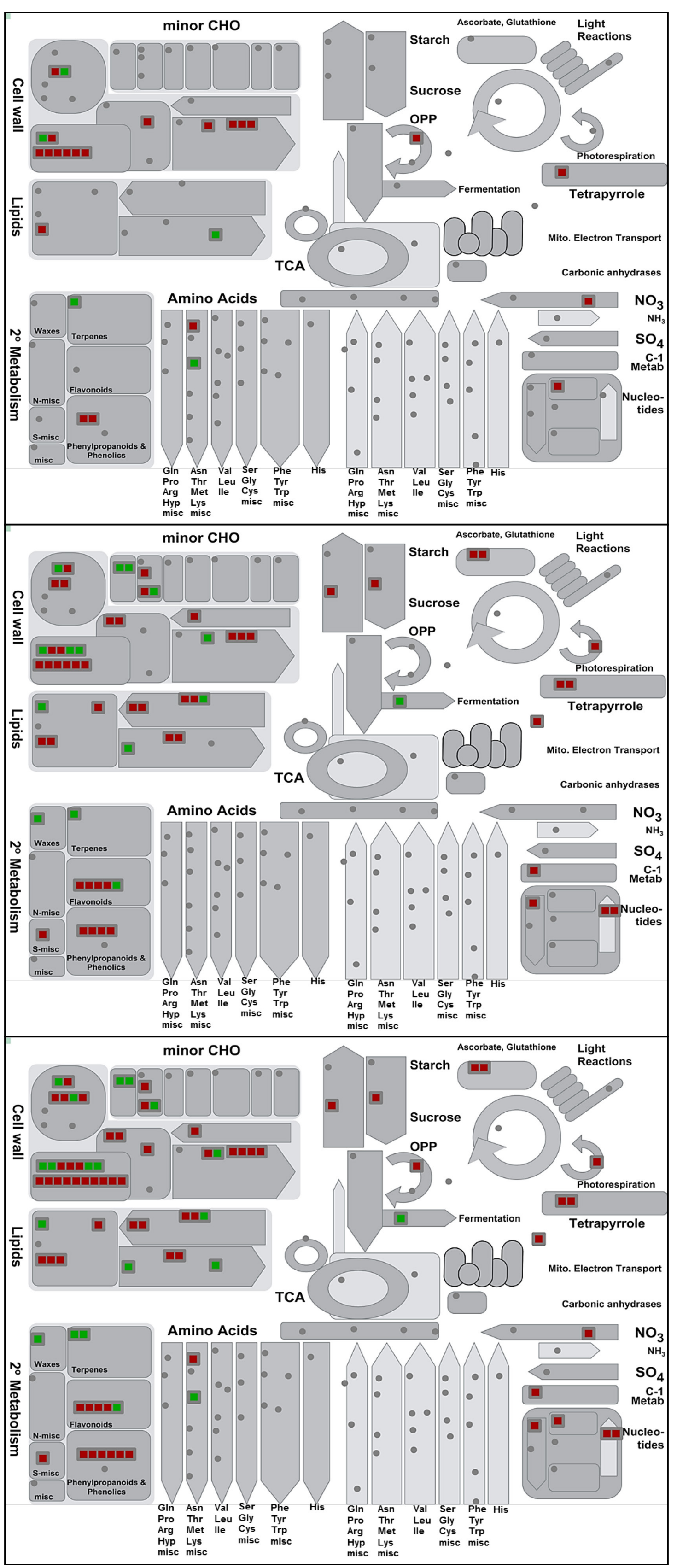
Inhibit the effect of mechanical wounding: In the whole ‘b’ area of Figure 10-(II) are the genes regulated by MecWorm but regulation totally inhibited by insect OS. Signaling related genes (Appendix figure 2-A, Appendix figure 5, purple hexagons), calcium regulation (Appendix figure 3-A, Appendix figure 5, purple triangles), receptor kinases synthesis (Appendix figure 3-A, Appendix figure 5, purple diamonds), heat shock proteins (Appendix figure 3-A, Appendix figure 5, purple rounds) and abiotic stress (Appendix figure 3-A, Appendix figure 5, purple asterisks) in Appendix figure 2-A.

No influence on the effect of mechanical wounding: salicylic acid (SA) pathway (brown circle in Appendix figure 3-A) and C-1 metabolism.



A. Local 9 h
minor CHO
Cell wall
Lipids
2° Metabolism
Waxes
Terpenes
N-misc
Flavonoids
S-misc
Phenylpropanoids & Phenolics
misc
Starch
Sucrose
OPP
Ascorbate, Glutathione
Light Reactions
Photorespiration
Fermentation
Tetrapyrrole
Mito. Electron Transport
Carbonic anhydrases
TCA
Amino Acids
NO3
NH3
SO4
C-1 Metab
Nucleo-tides
Gln Pro Arg Hyp misc
Asn Thr Met Lys misc
Val Leu Ile
Ser Gly Cys misc
Phe Tyr Trp misc
His
1. Mechanical wounding specific (area c+d)
2. Chemical elicitors specific (area a+d)
3. Insect feeding (area a+c+d)

B. Systemic 24 h



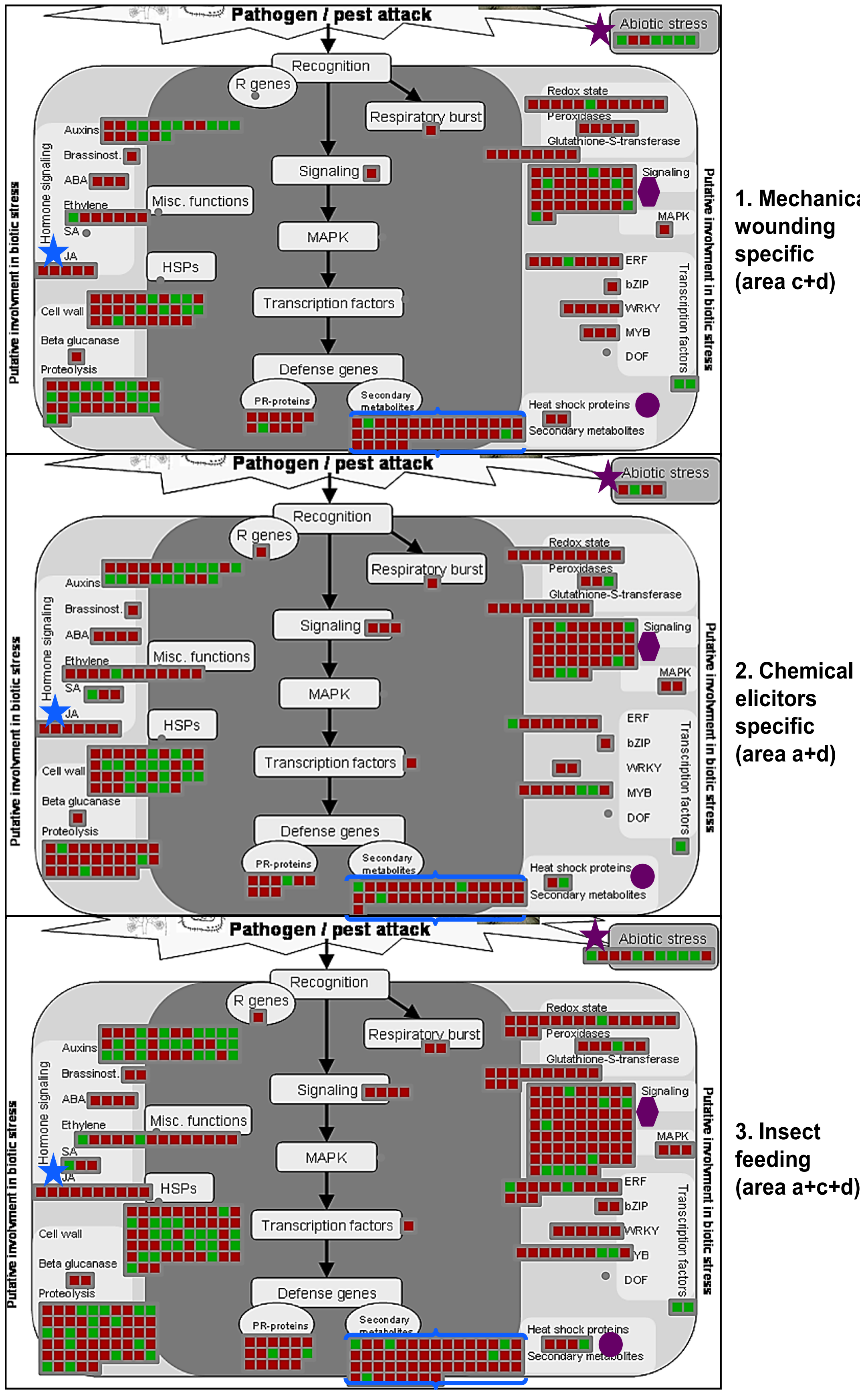
1. Mechanical wounding specific (area c+d)

2. Chemical elicitors specific (area a+d)

3. Insect feeding (area a+c+d)

Appendix figure 1. Gene expression overview of *A. thaliana* metabolic pathways after *P. xylostella* feeding. A: 9 h damaged leaves (red circle in Figure 9-II); B: 24 h systemic leaves (red circle in Figure 9-III). Pathways showing an enhancing effect of chemical factors to mechanical wounding are indicated with blue color shapes; pathways showing an inhibiting effect of chemical factors to mechanical wounding are indicated with purple color shapes; pathways showing no effect of chemical factors to mechanical wounding are indicated in yellow color shapes. Letters a, b, c, d are correspondent with the areas on Figure 10. Software: MapMan. Threshold: 3.0 (Only genes that are over three times regulated are presented).

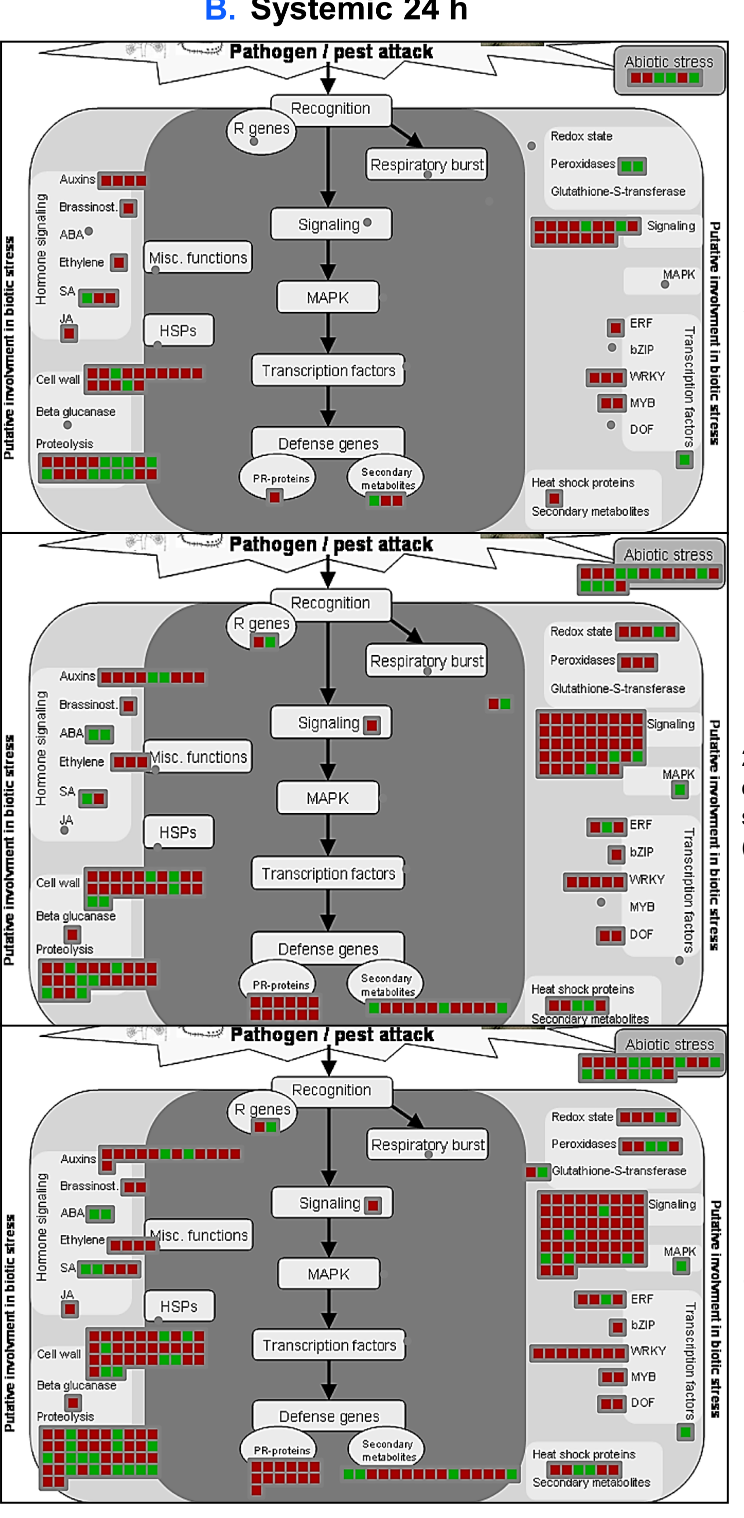
A. Local 9 h



1. Mechanical wounding specific (area c+d)

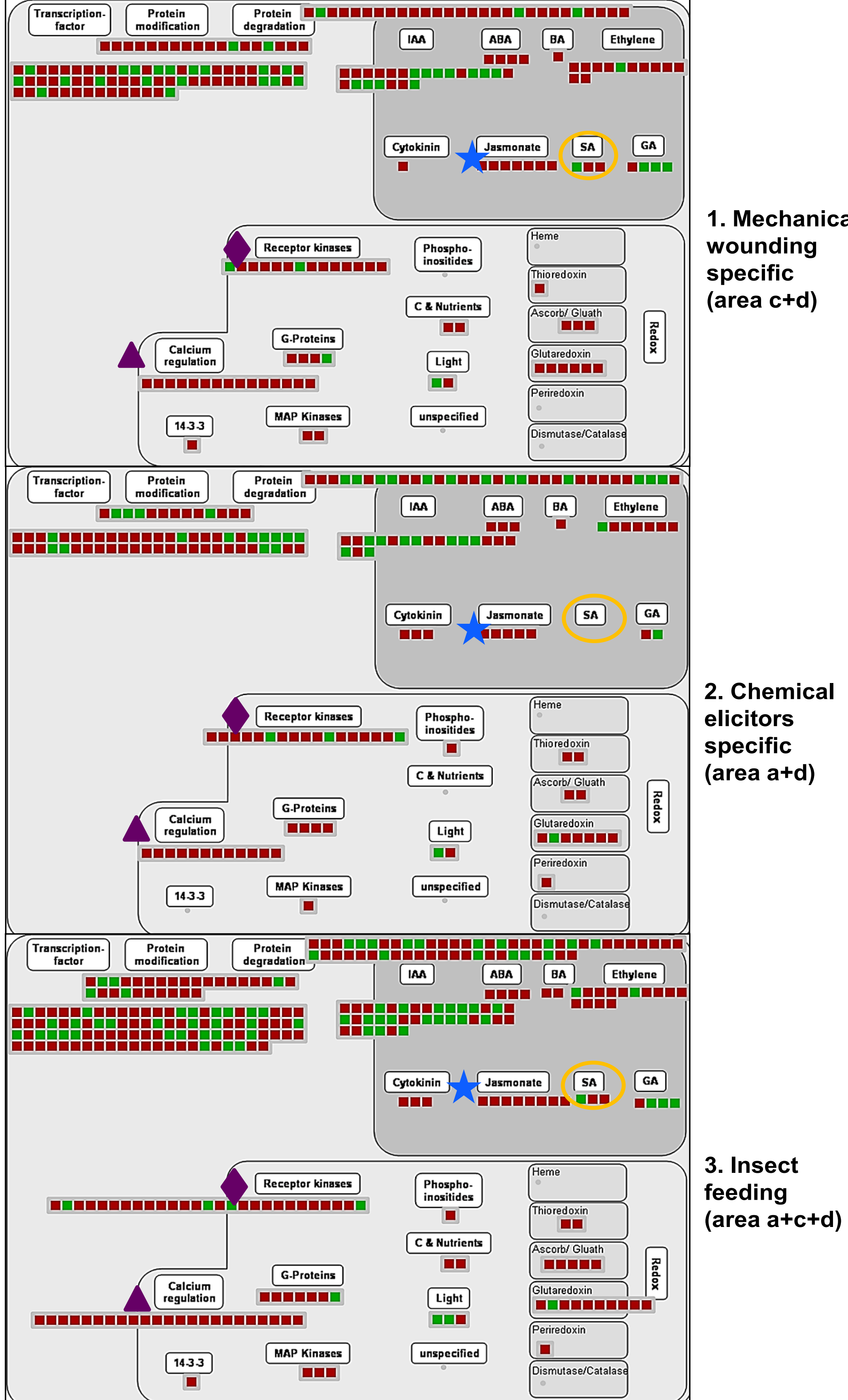
2. Chemical elicitors specific (area a+d)

3. Insect feeding (area a+c+d)



Appendix figure 2. Gene expression overview of *A. thaliana* biotic stress pathways after *P. xylostella* feeding A: 9 h damaged leaves (red circle in Figure 9-II); B: 24 h systemic leaves (red circle in Figure 9-III). Pathways showing an enhancing effect of chemical factors to mechanical wounding are indicated with blue color shapes; pathways showing an inhibiting effect of chemical factors to mechanical wounding are indicated with purple color shapes; pathways showing no effect of chemical factors to mechanical wounding are indicated in yellow color shapes. Letters a, b, c, d are correspondent with the areas on Figure 10. Software: MapMan. Threshold: 3.0 (Only genes that are over three times regulated are presented).

A. Local 9 h



B. Systemic 24 h

Transcription-factor
Protein modification
Protein degradation
IAA
ABA
BA
Ethylene
Cytokinin
Jasmonate
SA
GA
Receptor kinases
Phospho-inositides
C & Nutrients
G-Proteins
Light
Calcium regulation
MAP Kinases
unspecified
14-3-3
Heme
Thioredoxin
Ascorb/ Gluath
Glutaredoxin
Periredoxin
Dismutase/Catalase
Redox

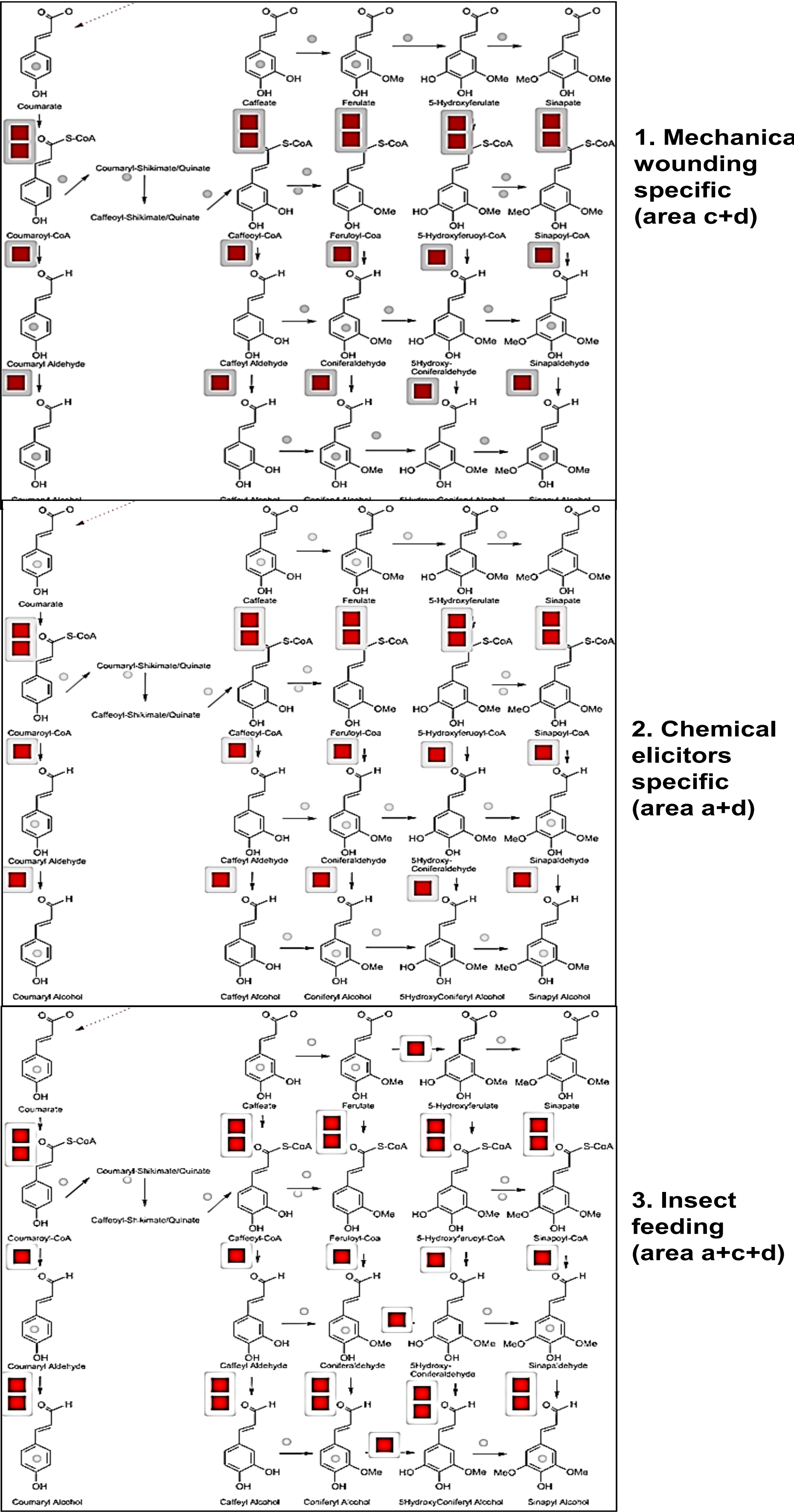
1. Mechanical wounding specific (area c+d)

2. Chemical elicitors specific (area a+d)

3. Insect feeding (area a+c+d)

Appendix figure 3. Gene expression overview of *A. thaliana* regulation pathways after *P. xylostella* feeding A: 9 h damaged leaves (red circle in Figure 9-II); B: 24 h systemic leaves (red circle in Figure 9-III). Pathways showing an enhancing effect of chemical factors to mechanical wounding are indicated with blue color shapes; pathways showing an inhibiting effect of chemical factors to mechanical wounding are indicated with purple color shapes; pathways showing no effect of chemical factors to mechanical wounding are indicated in yellow color shapes. Letters a, b, c, d are correspondent with the areas on Figure 10. Software: MapMan. Threshold: 3.0 (Only genes that are over three times regulated are presented).

A. Local 9 h

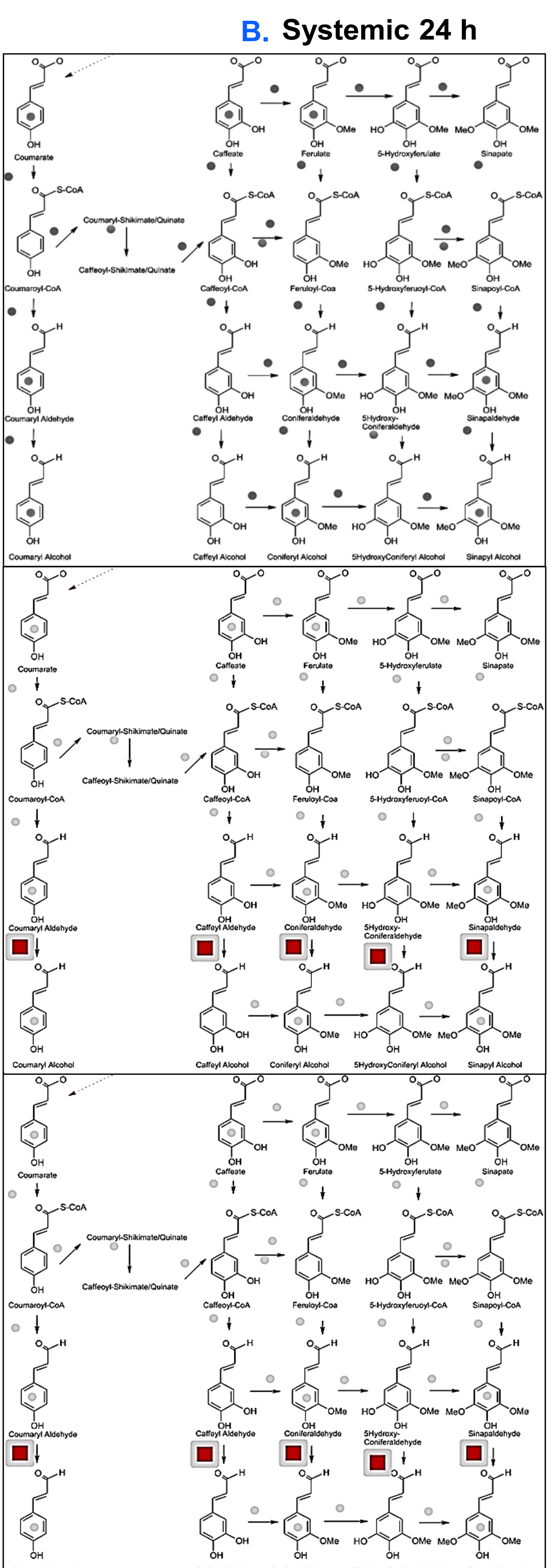


1. Mechanical wounding specific (area c+d)

2. Chemical elicitors specific (area a+d)

3. Insect feeding (area a+c+d)

B. Systemic 24 h

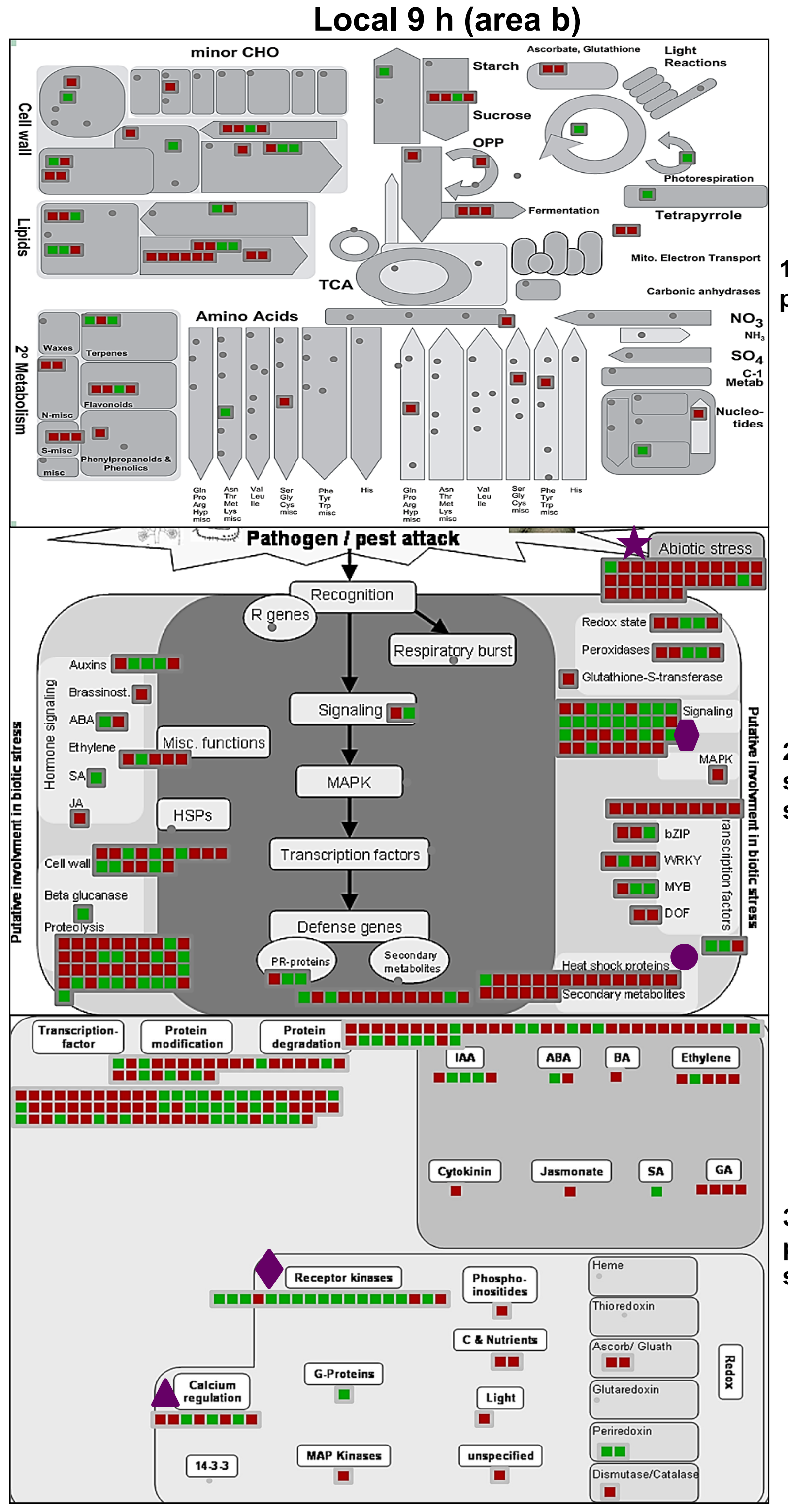


1. Mechanical wounding specific (area c+d)

2. Chemical elicitors specific (area a+d)

3. Insect feeding (area a+c+d)

Appendix figure 4. Gene expression overview of *A. thaliana* phenylpropanoids synthetic pathways after *P. xylostella* feeding. A: 9 h damaged leaves (red circle in Figure 9-II); B: 24 h systemic leaves (red circle in Figure 9-III). Pathways showing an enhancing effect of chemical factors to mechanical wounding are indicated with blue color shapes; pathways showing an inhibiting and fine-tuning effect of chemical factors to mechanical wounding are indicated with purple color shapes; pathways showing no effect of chemical factors to mechanical wounding are indicated in yellow color shapes. Letters a, b, c, d are correspondent with the areas on Figure 10. Software: MapMan. Threshold: 3.0 (Only genes that are over three times regulated are presented).



Appendix figure 5. Pathway overview of genes that are regulated by mechanical wounding (MecWorm) but inhibited by oral secretion (area 'b' in Figure 10-I), 9 h damaged leaf. Pathways showing an inhibiting and fine-tuning effect of chemical factors to mechanical wounding are indicated with purple color shapes Software: MapMan. Threshold: 3.0 (Only genes that are over three times regulated are presented).

9 CURRICULUM VITAE

Education

- **B. Sc., Biotechnology** (2003), Department of biotechnology, Shandong Agricultural University
- **M. Sc., Biochemistry & Molecular Biology** (2006), Maize institute, Sichuan Agricultural University
- **PhD, Biochemistry & Molecular Biology** (2009-), Department of organic chemistry, Max Planck institute for Chemical Ecology

Work Experience

2007 - 2009, Scientist, Competitive Prospecting Team, Monsanto Research Center, Bangalore, India.

- Technology Assessment for Ag. Biotech.
- Translation of technical documents (Chinese-English, English-Chinese).
- Business Analysis for technology evaluation.
- Preparing Company / Institution Profiles.

Thesis for Bachelor

Tissue culturing of 4 rare peony species.

Theses for Master

Two-Dimensional Electrophoresis and Peptide Mass Fingerprinting Analysis of Leaf Proteins under Drought Stress in Maize.

Theses for Doctor

Gene Regulation in Plant Herbivory Defense: Effect of Insect Mechanical Wounding and Chemical Oral Secretion Factors.

10 DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the school of Biology and Pharmacy of the Friedrich Schiller University, Jena that the submitted thesis was written only with the assistance of the literature cited in the text.

I was not assisted by a consultant for doctorate theses.

The theses has not been previously submitted whether to the Friedrich Schiller University, Jena or to any other university.

Guanjun Li

Jena, September 2016

SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena bekannt ist. Die vorliegende Arbeit wurde gemäß § 5 Abs. 4 PromO selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt.

Die vorgelegte Arbeit wurde zu keinem früheren Zeitpunkt, weder im Inland noch im Ausland in gleicher oder ähnlicher Form an einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens eingereicht.

Guanjun Li

Jena, September 2016

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