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In Silico Analysis of *Arabidopsis thaliana* Gene Expression Changes Against Four Different Insect Attackers

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ABSTRACT

Understanding the gene regulatory network of plants in response to different attackers are important to elicit unique and common mechanisms of plant defense. Until recently, accumulations of the massive gene-expression data brought an opportunity to reach this aim. In silico-based approaches offer exclusion of regulatory mechanisms of every single gene and/or gene clusters in the genome by analysis. Collecting valuable biological information by this approach can give us an opportunity to make profound experimental sets and it can speed up the process on our understanding of gene regulatory mechanisms. In this study, we used in silico analysis of published *Arabidopsis thaliana* gene expression data against four insect species; *Pieris rapae*, *Frankliniella occidentalis*, *Myzus persicae*, *Brevicoryne brassicae*. We identified and compared plant genes that are differentially regulated upon infection by these insects. According to our results, although a small number of gene numbers were commonly upregulated by four of these insects, each insect species uniquely induced plant gene regulatory network. Functional analysis of these genes and the gene regulatory sequences (promoters, motifs etc.), as well as analysis of metabolic and physiological networks, can help on understanding the gene regulation mechanisms of plant defense networks against insects.

Keywords: *Arabidopsis thaliana*, Insects, Transcriptomics data, In silico analysis.

INTRODUCTION

Plants are sessile organisms and cannot escape from biotic or abiotic threats. Rather, they must cope with these stresses and evolve accordingly. Plants confront with a wide variety of biotic stress factors such as, viruses, bacteria, oomycetes, fungus, and insects. Plant must protect itself against all attackers with various mechanisms. Waxy cuticular layer and physical barriers and rigid cell walls are the first steps for plant protection (Nürnberger & Lipka, 2005). In addition to physical barriers, plants have different layers of immune system responses. The first layer of plant immunity is based on the specific recognition of pathogen-derived molecules (Chisholm, Coaker, Day, & Staskawicz, 2006; Nimchuk, Eulgem, Holt, & Dangl, 2003). Highly conserved microbe associated molecular patterns (MAMPs) can be recognized by plant receptors (Pattern recognition receptors (PRRs)) and activate Pattern-Triggered Immunity (PTI) (Jones & Dangl, 2006; Nishimura & Dangl, 2010; Zipfel, 2008). Plant-pathogen interactions have dynamic platforms. While plants evolve to be resistant against pathogens, pathogens also try to find ways to overcome these resistance mechanisms (Chisholm et al., 2006; Macho & Zipfel, 2014). Pathogens can secrete effector proteins into plant cell and can attenuate or block PTI (Abramovitch & Martin, 2004). These microbial pathogens are well-adapted to their hosts and can cause compatible plant/pathogen interactions, which is called as Effector Triggered Susceptibility (ETS) and called as basal defense (Chisholm et al., 2006; Glazebrook et al., 2003; Jones & Dangl, 2006). Basal defense can limit the spread of virulent pathogens in their hosts, however, it is not enough to prevent diseases. A second layer of plant immunity are held by disease resistance (R)-genes which recognize the presence or activity of effector proteins and induce effector-triggered immunity (ETI) (Elmore, Lin, & Coaker, 2011; Jones & Dangl, 2006). Disease resistance (R)-genes and ETI is a well-described phenomenon of gene-for-gene resistance or race-specific resistance (Flor, 1971; Jones & Dangl, 2006; Nimchuk et al., 2003). All these layers of immune system members utilize some set of signaling components to prevent diseases. In these systems; multiple regulatory

proteins, reactive oxygen intermediates (ROIs), phytohormones; salicylic acid (SA), ethylene (ET) and jasmonic acid and their levels on plant tissues effect plant's response to pathogen (Nimchuk et al., 2003; Spanu, 2012). Also, the level of these phytohormones change in accordance with the type of pathogen. While SA is mostly taking a role in response to biotrophic pathogens, JA and its underneath signalling network is important for necrotophic pathogens as well as herbivorous insects (Glazebrook, 2005; McDowell & Dangl, 2000). Studies using wide variety of pathogens are important to elicit whether these mechanisms are common on defense against various pathogens or does plants evolve differently against each pathogen.

Barah, Winge, Kusnierczyk, Tran, & Bones (2013) studied with insect *Brevicoryne brassicae* and *Pseudomonas syringae* pv.tomato bacteria strain DC3000 to understand what are the common and specific defense mechanisms existing in plant systems for protection. Transcriptomic reprogramming and defense responses of *Arabidopsis thaliana* against two attackers were identified. Also, De Vos et al. (2005) designed an experiment to understand plant defense system against different insects and bacteria. Analysis of global gene expression profiles of *Arabidopsis thaliana* against these pathogens can give us an insight to understand interactions.

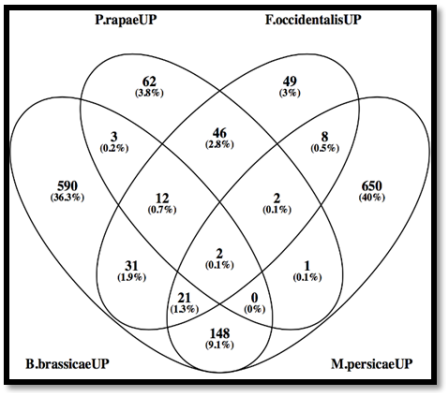
In our study, we aimed to analyse transcriptional responses of *Arabidopsis thaliana* plants against four different insect species. *Pieris rapae* is a tissue chewing caterpillar that is special to cruciferous plant species. *Frankliniella occidentalis* is a western flower thrips that cause extensive damage on many plant species. *Myzus persicae* is a green peach aphid that feed from plants phloem. *Brevicoryne brassicae* is commonly known as the cabbage aphid and cause significant losses in crop fields. Also, all these insects can attack Arabidopsis (Barah et al., 2013; De Vos et al., 2005). Upon perception of biotic stimuli from outside factors, plants can induce plant defense. This induction and the immune responses are tightly associated with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network (Tsuda, Sato, Glazebrook, Cohen, & Katagiri, 2008; Sato et al., 2010). Monitoring the unique regulatory networks to each insect as well as identifying common ones are important to elicit undefined key players of immune responses. In-silico analysis of global transcriptional patterns of *Arabidopsis thaliana* against insect species will help to understand the pattern through a set of computational approaches.

MATERIALS AND METHODS

Data collection and in silico analysis of differential gene expression

Global transcriptional patterns associated with four different insect species in Arabidopsis were obtained from related journal web sites and databases. To bioinformatically assess the functionality of identified genes, web tools that can be accessed online; "The Arabidopsis Information Resource (TAIR)", "The Bio-Analytic Resources for Plant Biology", "ShinyGO v0.61: Gene Ontology Enrichment Analysis + more", "ThaleMine," were used. The web tools validate the analysis of stress-responsive gene expressions. Gene Ontology (GO) enrichment was performed using The Arabidopsis Information Resource (TAIR) and The Bio-Analytic Resources for Plant Biology using the default settings. Other analysis retrieved from ThaleMine and ShinyGO v0.61 for cluster of genes or specific genes. The graphic and image results were downloaded from the Web sites.

RESULTS AND DISCUSSION



Functional analysis of global transcriptional patterns associated with four different insect species

We profiled global transcriptional patterns of *Arabidopsis thaliana* against four different insect species *Pieris rapae*, *Frankliniella occidentalis*, *Myzus persicae*, *Brevicoryne brassicae* (Barah et al., 2013; De Vos et al., 2005). We first

a **b**

identified common genes list with Venny (Fig. 1) (Oliveros, 2007), in order to understand transcriptional patterns unique to each insect species and shared by for all. According to results, 62 genes are exclusively upregulated by *Pieris rapae*, 590 genes are exclusively upregulated by *Brevicoryne brassicae*, 49 genes are exclusively upregulated by *Frankliniella occidentalis* and 650 genes are exclusively upregulated by *Myzus persicae*. For these genes, enriched gene ontology (GO) terms were calculated by the Botany Array Resource classification super viewer (Toufighi, Brady, Austin, Ly, & Provart, 2005). According to results, collective induction by individual insects are I) response to abiotic or biotic stimulus, II) response to stress, III) signal transduction and IV) other metabolic processes (Table 1).

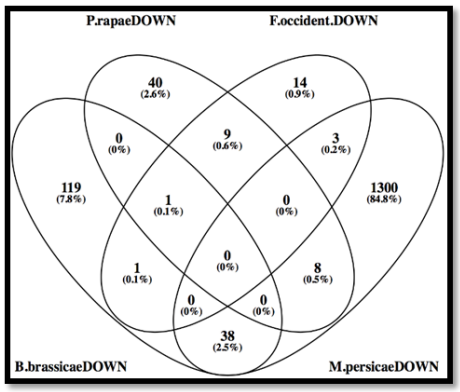
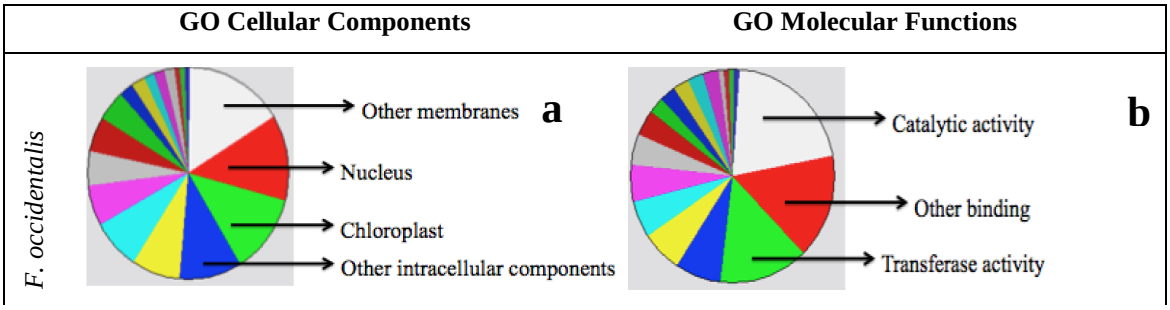


Fig. 1: Venn diagrams of number of genes that were (a) specifically up-regulated, or (b) down-regulated in response to *Pieris rapae*, *Frankliniella occidentalis*, *Myzus persicae* and*Brevicoryne brassicae*.

Table 1. Set of Arabidopsis genes uniquely expressed in response to *Pieris rapae*, *Frankliniella occidentalis*, *Myzus persicae* or *Brevicoryne brassicae*.

Gene Set	Number of genes in set	Enriched GO terms* (with p values)
Upregulated by *Pieris rapae*	*62*	**7.682e-04** response to abiotic or biotic stimulus
		6.279e-03 response to stress
		5.778e-05 other metabolic processes
Upregulated by *Brevicoryne brassicae*	590	2.368e-46 response to stress
		9.996e-28 response to abiotic or biotic stimulus
		5.961e-10 signal transduction
Upregulated by *Frankliniella occidentalis*	49	2.388e-09 response to stress
		2.466e-03 signal transduction
		3.587e-03 response to abiotic or biotic stimulus
Upregulated by *Myzus persicae*	650	1.980e-09 response to abiotic or biotic stimulus
		1.541e-05 signal transduction
		2.289e-07 response to stress

Further analysis for insect-specific genes were applied. Functional categorization for GO cellular component showed that *A. thaliana* up-regulated genes for these insects are mostly located on nucleus, other membrane, chloroplast and plasma membrane (Fig. 2a) with the molecular function on catalytic activity, transferase activity, protein binding and other binding activity (Fig. 2b). The induction of these molecular patterns suggested that plants try to defend themselves against attackers with extensive transcriptional- and metabolic–reprogramming controlled by a complex regulatory network.



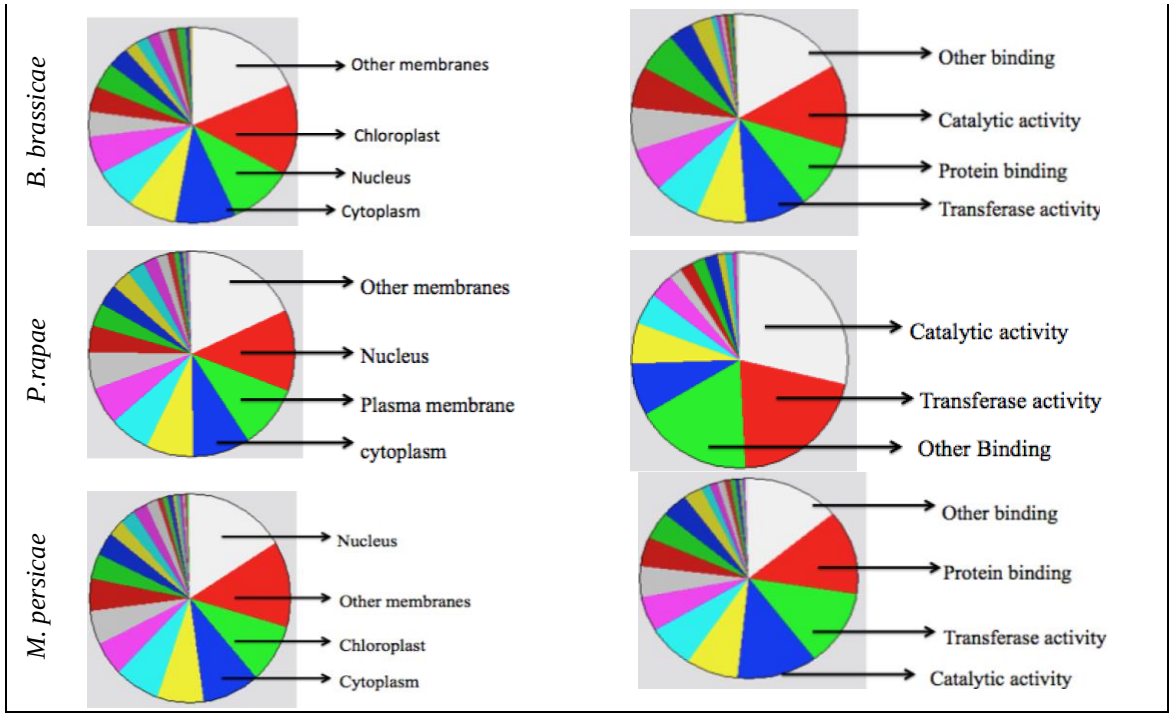


Fig. 2: Functional categorization of insect specific up-regulated genes for GO cellular components (a) and GO molecular functions (b).

Most of the up-regulated genes are specific for each insect species, while some are common for only two or three insect species. These results suggest that plants show extensive unique transcriptional reprogramming in response to insect attacks (Fig. 1). Although these genes are not common, their functional analysis are mostly in the same groups such as response to abiotic or biotic stimulus and/or response to stress. When we analysed down-regulated genes, the unique expression profile was more dramatic. Most of the down-regulated genes are unique to each insect. Very limited numbers of genes are common for two or more insect species. According to this, we can claim that, the plant response is mostly species specific.

According to Fig.1, *At5g13220* (*JAZ10*) and *At4g31800* (*WRKY18*) genes that are commonly induced among all insects were identified and cis-regulatory elements were analysed (Table 2). According to Table 2; *JAZ10* and *WRKY18* shares some common binding sites which are mostly related to plant defense, such as *WRKY*, Homeobox or bZIP transcription factors. *JAZ10* is a member of the JASMONATE ZIM-domain (JAZ) family. JAZ family proteins take crucial role on the regulation of jasmonate (JA)-dependent signalling pathways. JA is a lipid-derived hormone, which regulates plant responses against insect herbivores, necrotrophic pathogens, and various abiotic stresses. Moreover, it has roles on some plant growth and development processes, such as cell division, cell fate determination, photomorphogenesis, and sexual reproduction. Since JA has wide impact on both plant growth and plant defense, controlling the level of this hormone is crucial for plant (Browse, 2009). According to JA signaling model; when the JA is absent, JAZ family of proteins represses *MYC2*, which function as a transcriptional activator. This JAZ family of proteins interact with *MYB2* physically and prevents activation of downstream JA-regulated gene expressions. On the contrary, when the level of JA hormone increased, *MYB2* expression is released by turnover of JAZ. JAZ proteins bind to *CORONATINE INSENSITIVE1* (*COI1*) in the component of the ubiquitin E3 ligase SCF^{COI1} and it results with ubiquitination and degradation of JAZs by the 26S proteasome (Moreno, 2013). *JAZ10* is one of the members of this family and establishes a negative feedback loop to attenuate JA responses. In this study, JAZ10 was one of the two commonly upregulated genes and it is interesting that *JAZ10* gene expression is increase. The induction of *JAZ10* might stop JA-regulated defense signalling, although JA signalling network provides protection against herbivorous insects. There are some possibilities that I) insects can induce *JAZ10* to block JA signalling defense

mechanism, II) JA-regulated defense response takes role on those insects, or III) various isoform of *JAZ10* are insensitive to *MYB2*. Previous studies demonstrated that alternative splicing is a mechanism in *JAZ10* protein to increase the repressive activity of *JAZ10* proteins. Alternative splicing of *JAZ10* pre-mRNA generates various protein variants that differentially interact with *COI1*. For example, *JAZ10*.1 isoform strongly binds to *COI1*, whereas *JAZ10*.3 and *JAZ10*.4 isoforms interact weakly with *COI1* that effect activation of gene expression on JA signalling pathway. In this sense, although the mRNA expression of *JAZ10* was commonly high in this study, it is not certain that protein variant of all transcriptomes are similar. It is important to design an experiment to check all *JAZ10* protein isoforms against all of these insect species (Moreno, 2013).

Another commonly up-regulated gene is *WRKY18*, which is the member of the WRKY transcription factor gene family. WRKY factors take roles in diverse signaling pathways such as biotic and abiotic responses and some developmental processes. All WRKY factors contain a highly conserved domain of 60 amino acid residues that have *WRKYGQK* sequence motif (Eulgem, 2006; Eulgem, Rushton, Robatzek, & Somssich, 2000). Member of this family has interconnected transcriptional networks as both positive and negative regulators as well as auto-regulatory and cross-regulatory properties of these transcription factors (TFs) (Eulgem, Rushton, Schmelzer, Hahlbrock, & Somssich, 1999). *WRKY18* has functionally redundant roles in plant basal defense with *WRKY40* and *WRKY60*. Previous studies with *wrky18wrky40wrky60* triple mutants, *wrky18wrky40* and *wrky18wrky60* double mutants showed susceptible response to *Botrytis cinerea* but displayed increased resistance against *Pst*. Also positively modulates the expression of some key JA-signaling genes by partly suppressing the expression of JAZ repressors (Xu, Chen, Fan, & Chen, 2006; Pandey, Roccaro, Schön, Logemann, & Somssich, 2010). This demonstrates that *WRKY18* is taking a role in response to these insect species. Further experimental design with knockout mutants may reveal actual role of *WRKY18* in defense mechanism.

Table 2. Detailed cis-regulatory element annotation for JAZ10 and WRKY18

Binding Site Name	Binding Site sequence	Binding Site Family/TF
RAV1-A binding site motif	caaca	ABI3VP1
AtMYC2 BS in RD22	cacatg	BHLH
ATB2/AtbZIP53/AtbZIP44/GBF5 BS in ProDH	actcat	bZIP
Bellringer/replumless/pennywise BS1 IN AG	aaattaaa	Homeobox
LFY consensus binding site motif	ccattg	LFY
MYB binding site promoter	aaccaaac	MYB
W-box promoter motif	ttgact	WRKY
W-box promoter motif	ttgacc	WRKY

CONCLUSION

Biotic stresses are a major threat for plants and agricultural production around the world. Understanding the genetic and molecular mechanisms of plant defense that are activated under biotic stresses is important for plant scientists. The availability of the next generation sequencing technologies and accumulation of data collections can be advantage to reach some predictive information for plant defense pathway. The aim of this study was to identify common and unique stress-responsive genes and to characterize the function of these genes. According to results, although minimal common genes are available for these herbivorous insects, most of the up regulated genes were insect specific. These results are more dramatic for down-regulated genes, which are almost entirely unique to each pathogen. The functional analyses information can be a start point to establish experimental design to find actual/specific role of the genes in plant defense.

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