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**Investigating the Molecular Basis of Volatile-
mediated Plant Indirect Defense against
Herbivorous Insects Using Functional and
Comparative Genomics**

A Dissertation

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Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Shuhua Yuan

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Dedication

The dissertation is dedicated to my wife, Susie Yuan Dai, who accompanied me through all the hardships and shared all of my joys, worries, frustrations, and dreams. The dissertation is also dedicated to my mom, Dengfen Gao, and my father, Guizhong Yuan, both of whom have brought me up with integrity, kindness, and aspiration to live a strenuous life with a higher purpose than one's own self-interest. 'Tolerance in Adversaries, and Rejoicing in Hope'.

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Brief Abstract

Volatile organic compounds (VOCs) play important roles in plant indirect defense against herbivorous insects by attracting the natural enemies. I first used a tritrophic model system involving rice, rice fall armyworm (*Spodoptera frugiperda*), and the parasitoid *Cotesia marginiventris* to discover and characterize the volatile terpenoids and *TPS* genes involved in the indirect defense of rice against the insect herbivory with integrated functional genomics analyses. Seven rice *TPS* genes were found to be significantly up-regulated by both microarray and real-time PCR analyses, with one characterized as a linalool synthase and two as sesquiterpene synthases. The products of all three characterized genes covered most of the volatile terpenoids emitted by the fall armyworm-damaged rice. The products of all three characterized genes covered most of the volatile terpenoids emitted by the fall armyworm-damaged rice. In addition to the insect treatment, responses induced by defense hormone jasmonic acid were also examined with volatile analysis, gene expression profiling and enzyme assays. Only one *TPS* gene in the microarray analysis was up-regulated during the early response to jasmonic acid, and the gene was characterized as *OsLMS* (rice limonene synthase). Moreover, a homolog gene with similar sequence was characterized with limonene synthase activity and named *OsLMS2*. Both genes exhibited a time-dependent expression up-regulation upon jasmonic acid treatment. The regulation of terpeneoid volatile emission was also examined from the perspective of diurnal cycle. Our results showed that the emission of volatile terpenoids was linked to the diurnal cycle;

however, the base level emissions were different among the products of three genes. The volatile terpenoid biosynthesis and emission were regulated at both *TPS* gene expression level and the substrate level. To further understand the molecular evolution of plant indirect defense against herbivorous insects, I also investigated the evolution of the TPS gene family using comparative genome analyses. These analyses revealed both a rapid evolution of the *TPS* gene family and a conserved group of monoterpene synthase with a deep evolutionary origin. Biochemical analysis of one of the poplar *TPS* genes in the conserved group showed linalool synthase activity.

Abstract

Volatile organic compounds (VOCs) play important roles in plant indirect defense against herbivorous insects by attracting the natural enemies. Volatile terpenoids are a major group of VOCs involved in the indirect defense based on tritrophic interaction among plants, herbivorous insects, and natural enemies of herbivorous insects. Molecular mechanisms of plant indirect defense were studied from the perspectives of insect induced responses, jasmonic acid regulated responses, diurnal cycle dependent regulation, and evolution of terpene synthase (*TPS*) gene family.

I first used a tritrophic model system involving rice, rice fall armyworm (*Spodoptera frugiperda*), and the parasitoid *Cotesia marginiventris* to discover and characterize the volatile terpenoids and *TPS* genes involved in the indirect defense of rice against the insect herbivory with integrated functional genomics analyses. The Y tube bioassay revealed the potential tritrophic interactions among rice, fall armyworm, and *Cotesia*, since the parasitoid *Cotesia* can be preferentially attracted by fall armyworm damaged rice. Volatile profiling demonstrated that terpenoid volatiles were a major group of VOCs induced by fall armyworm in *Nipponbare* rice. Global gene expression profiling indicated the coordinative changes of transcriptional regulation, signal transduction, and the secondary metabolism in the rice defense against fall armyworm. Seven rice *TPS* genes were found to be significantly up-regulated by both microarray and real-time PCR analyses, with one characterized as a linalool synthase and two as

sesquiterpene synthases. The products of all three characterized genes covered most of the volatile terpenoids emitted by the fall armyworm-damaged rice. One product, linalool, preferentially attracted parasitoids in the Y tube bioassay. Overall, our results showed that terpenoid volatile compounds and *TPS* genes are important components of the indirect defense of rice against herbivorous insects in the *Nipponbare* cultivar.

In addition to the insect treatment, responses induced by defense hormone jasmonic acid were also examined with volatile analysis, gene expression profiling and enzyme assays. Volatile analysis revealed a time-dependent emission of different monoterpene volatiles in rice treated with jasmonic acid. To understand the molecular mechanisms of these differential volatile emission patterns, the early stages of jasmonic acid-induced responses were examined by global gene expression profiling. Microarray results indicated the up-regulation of relevant defense pathways, but only a limited increase in the expression of terpenoid biosynthesis genes. Only one *TPS* gene in the microarray analysis was up-regulated during the early response to jasmonic acid, and the gene was characterized as *OsLMS* (rice limonene synthase). Moreover, a homolog gene with similar sequence was characterized with limonene synthase activity and named *OsLMS2*. Both genes exhibited a time-dependent expression up-regulation upon jasmonic acid treatment. Comparative genomic analysis revealed that limonene synthase may have evolved across species through convergent evolution.

The regulation of terpenoid volatile emission was examined from the perspective of diurnal cycle. Our results showed that the emission of volatile terpenoids was linked to the diurnal cycle; however, the base level emissions were different among the products of three genes. *TPS* gene expression did not correlate well with the volatile terpenoid emission pattern, which indicated the importance of other molecular mechanisms in the regulation of volatile terpenoid biosynthesis and emission. Global gene expression profiling suggested differential gene regulation between night and daytime responses to the insect damage. Metabolic pathway analysis revealed on and off expression patterns of key enzymes in both mevalonate and non-mevalonate pathways during the diurnal cycle. Jasmonic-acid-induced volatile emission and gene expression were also studied. Overall, the volatile terpenoid biosynthesis and emission were regulated at both *TPS* gene expression level and the substrate level.

To further understand the molecular evolution of plant indirect defense against herbivorous insects, I investigated the evolution of the *TPS* gene family using comparative genome analyses. These analyses revealed both a rapid evolution of the *TPS* gene family and a conserved group of monoterpene synthase with a deep evolutionary origin. Biochemical analysis of one of the poplar *TPS* genes in the conserved group showed linalool synthase activity. The biochemical activity is discussed from the perspectives of gene function and evolution.

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**Chapter I. Literature Review: Plant Volatiles Involved
in Indirect Defense against Herbivorous insects:
Chemistry, Biosynthesis, Function, Evolution, and Gene
Discovery**

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Abstract

Volatile compounds play important roles in plant indirect defense against herbivorous insects by providing info-chemicals for the natural enemies to best locate their forage. A tremendous amount of research has been carried out to characterize both the tritrophic interactions and the volatiles involved in these interactions. In this chapter, we aim to provide a wide-ranging review on the biosynthesis, emission, function, and evolution of the volatiles involved in the plant indirect defense. A variety of volatile compounds are involved in plant indirect defense, and these compounds include terpenoid volatiles, green leaf volatiles, indole, volatile phenolic compounds and others. The biosynthesis of these compounds has been thoroughly studied and the regulation of biosynthesis and volatile emission are discussed in the review. Moreover, the detailed information of the active signals from volatile compounds was identified by testing pure synthetic compounds and genetic engineering. The evolution of volatile-mediated indirect defense was discussed with the perspectives of evolutionary driving force. Finally, the importance of gene function characterization and the role of functional genomics in gene discovery are also discussed.

Key words: Volatile, terpenoid, tritrophic interaction, indirect defense

I. Introduction

Insect damage accounts for about thirteen percent of pre-harvest crop lost, which surpasses pathogens and weeds to become the largest biotic threats for modern agriculture (Schoonhoven, 2005). In-depth study of plant interaction with herbivorous insects will have a profound impact on the sustainability and development of agriculture systems. Moreover, from the evolutionary perspective, the details of insect-plant interaction are still yet to be unveiled despite different theories attempting to explain the co-existence of a flourishing plant kingdom and a large insect kingdom including diverse and abundant herbivores. The study of plant defense mechanisms helps to answer the question imposed by many evolutionary ecologists: why the world is still green with all these herbivorous insects.

Plant defense against herbivore can be generally classified into two categories, the direct defense with repellents, toxins, deterrents and special structures, and the indirect defense involving increasing fitness of natural enemies of herbivorous insects. These fitness-increasing measures include providing host, alternative food source, and foraging signals for natural enemies of herbivorous insect (Takabayashi and Dicke, 1996). One example of indirect defense is the secretion of extrafloral nectar to provide alternative food for natural enemies. Another important components of indirect defense is the volatile-mediated indirect defense involving tritrophic interactions (Pare and Tumlinson, 1997). Tritrophic interactions have been subject to intensive studies for the last two

decades, ever since it was proposed by Price et al (Turlings et al., 1990). The so-called tritrophic interaction involves the interaction among plants, plant herbivore, and the natural enemy of plant herbivore. Volatile organic compounds (VOCs) are the cue linking the three players in the interaction. The classical tritrophic interaction model indicates that plants emit volatile compounds upon insect damage, which in turn attracts the natural enemies of the herbivorous insect (Turlings et al., 1990; Vet and Dicke, 1992). Tritrophic interaction is an important component of the plant defense system with impact on the ecological community.

A further understanding of the role of different volatile compounds in volatile-mediated indirect defense has important implications from both applicable and scientific perspectives. Identification of the key volatiles in tritrophic interaction will help to develop an agriculture system with sustainable and effective biological control (Aldrich et al., 2003; James, 2003). Only well characterized interactions between volatile compound(s) and parasitoid can lead to develop such systems (Degenhardt et al., 2003; James, 2003). When developing an agricultural system using transgenic plants, the influence on volatile-mediated indirect defense should also be considered (Turlings et al., 2005). Moreover, as key mediators between insects and plants, volatiles are believed to be actively involved in the evolutionary battle between the two kingdoms. Previous theories in evolution of plant defense largely ignored the role of volatile compounds and sometimes the higher trophic interactions (van Veen et al., 2006). From an

evolutionary ecologist's view, it is important to understand the origin and driving force for the volatile-mediated indirect defense.

Considering the importance of volatile compounds, we hereby discuss the role of volatiles in indirect defense from the perspectives of both historical research and recent advancements. We will first provide an attempting comprehensive survey of the volatiles involved in different volatile-mediated defense systems, and discuss the potential compounds serving as signals for parasitoid and predator forage behavior. Considering the hundreds of publications during the past two decades on the issue, an all-inclusive review on tritrophic system and volatiles involved is beyond the scope of this paper. However, a survey on the diversity of volatiles and tritrophic interactions thus confer important information regarding the qualitative and quantitative features of key volatiles in the tritrophic interactions. We will then discuss the information conferred by the qualitative and quantitative features of volatiles in volatile-mediated indirect defense. Recent research has indicated more sophisticated indirect defense roles of volatile compounds beyond the tritrophic interaction, and we will briefly discuss volatiles as signal compounds for neighboring plants, microbes, and animals in plant defense. The origin and driving force for the evolution of volatile-induced indirect defense will also be discussed. Regardless the more than twenty years of intensive research by scientists from several fields, there are still many questions remaining for the mechanisms of volatile induced

indirect defense. At the end of the review, we will briefly discuss these questions and future directions in the field.

II. Occurrence Of Indirect Defense Mediated By Plant Volatiles

Volatile-mediated plant indirect defense have been described for at least 17 plant species and 23 different systems. In Table 1.1, only the previous publication with characterized volatile profiling and well-defined tritrophic interactions were included. Most of these studies were carried out in laboratory environment, which allows both better identification of volatile profiling and precise characterization of tritrophic interactions with either Y-tube bioassays or wind tunnel systems. The field studies often fall short on either one of them. Even though an exhaustive list of the studies on tritrophic interaction would be difficult due to the tremendous amount of work in the area including both field and laboratory work, the well characterized interactions in the table suggest the prevalence of the phenomena. In fact, besides the laboratory studies, the field work in forestry, entomology, and ecology indicated more diverse indirect defense systems and the knowledge has been broadly applied in the biological control of pest insects (Boulter, 1993; James, 2003, 2005).

As shown in the table, the tritrophic interaction-based indirect defense is diverse in term of plant species, insect order, and types of natural enemies. For plant species, indirect defense was shown to exist broadly in both gymnosperm and angiosperm species. In angiosperm species, indirect defense was found in a wide

range of species including those in Leguminosae, Brassicaceae, Solanaceae, Rosaceae, and Poaceae. If the field research with less defined volatile profiling was counted, more species would be included. The broad distribution of indirect defense indicated its importance in plant defense against herbivorous insects and maintaining the balance of ecosystems.

The tritrophic interaction was also found to be against a wide range of insects including both specialist and generalist insects, as well as both sap-sucking insects and chewing insects. Different types of insects tend to induce different profiles of volatile compounds in plants, and these different volatile profiles allow natural enemies to best locate their forage. In order to further understand the tritrophic interaction and its evolution, more studies need to be carried out for characterizing plant insect interactions at the molecular level.

Plant indirect defense is also diverse in terms of natural enemies. Both carnivore species and parasitoid species can serve as natural enemies. In fact, recent studies indicate a broader spectrum of trophics in the indirect defense, where microorganisms, animals and non-host plants can all have effects on the interaction between herbivorous insects and plants (Dudareva et al., 2006; Rostas et al., 2006; Snoeren et al., 2007). The concept of multitrophic interactions has been introduced to describe these complicated interactions in ecosystems. The diversity in indirect defense indicates that more studies need to

be carried out to define the occurrence of indirect defense mediated by plant induced volatiles in different ecosystems, especially agro-ecosystems.

III. Volatiles Potentially Involved In Indirect Defense: A Comprehensive Survey

Many volatile compounds have been described to be inducible by herbivorous insects from a broad spectrum of plant species (Dudareva et al., 2006). Table 1.1 and Table 1.2 summarize research about the volatile compounds potentially involved in plant indirect defense. Table 1.1 focus on the well-described tritrophic interaction and volatile organic compounds induced during the defense. Tritrophic interaction has been described for more than thirty species combinations, however, most of the research focuses on the entomological and ecological perspectives, where no volatile compounds were profiled. On the other side, there are many reports about insect or elicitor treatment induced volatile production, yet no well-defined study on the plant-insect-natural enemies interaction. In Table 1.1, we only included the studies that had both components, a well defined ecological study of tritrophic interaction and a volatile profiling identifying candidate compounds potentially involved in the tritrophic interaction. It should be noted that the table reflects a history of the field, where early work tends to identify fewer inducible compounds as compared to recent research. Table 1.2 provides a list of volatiles induced by herbivorous insect damage, elicitors, or plant defense hormones such as jasmonic acid. Enormous amount of work has been expended in volatile

profiling of plants, and we only included research with a clear indirect defense perspective since we attempt to identify key elements or common constituents for indirect defense across the species. The studies included in the table normally derive from the system that the plant species, herbivorous insects, or the donor of elicitor have been well characterized for their involvement in tritrophic interaction. For example, the volatiles induced by volicitin treated maize were included. The maize plant has been shown to be able to emit volatiles attracting *Cotesia* genus parastoids when damaged by armyworm, and volicitin is isolated from armyworm regurgent as an elicitor mimicking the insect damage induced effects (Alborn et al., 1997).

From Table 1.1 and Table 1.2, we can find a highly diverse spectrum of volatile organic compounds involved in indirect defense. The amount and composition of the compounds differs in different systems. The factors involved in the diversity of compounds and the meaning of different compound complex will be discussed later in the article. Despite the diversity, volatile compounds induced by herbivore can be classified into several groups according to their molecular structure and biosynthesis. The groups include green leaf volatiles, terpenoids, phenolic compounds, indolic compounds etc.

Green Leaf Volatiles and their Derivatives

Green leaf volatiles are low molecular weight molecules normally with six to ten carbons that are induced by wounding or insect damage. The green leaf volatiles

include many C₅, C₆ and even C₁₀ compounds and acetate, aldehyde, and other derivatives from these base compounds (Wilson et al., 1996; Ruther and Furstenu, 2005). Green leaf volatiles are synthesized from lipid pathway, where lipoxygenase (LOX), fatty acid hydroperoxide lyase (HPL), and alcoholdehydrogenase (ADL) play important roles for their biosynthesis. For example, for the biosynthesis of the most common six carbon green leaf volatiles, the 18 carbon unsaturated fatty acids linolenic acid and linoleic acid can be converted to hexanal or cis-3-hexenal by LOX. Hexanal and cis-3-hexenal can be further oxidized by ADH to produce hexanol-1 and hexen-1-ol, respectively (Feussner and Wasternack, 2002).

Green leaf volatiles are part of the spectrum of volatile organic compounds emitted during the herbivorous insect damage. However, the emission of green leaf volatiles is not specific to herbivorous insect damage. Mechanical wounding damage can also induce green leaf volatiles (Mithofer et al., 2005). As shown in Table 1.1, most of the volatile mixtures involved in plant indirect defense have some types of green leaf volatiles. Green leaf volatiles along with terpenoids constitute two major group of constitutive volatile organic compounds potentially involved in indirect defense. Several common green leaf volatiles induced by insect damage across species are E-2-hexenal, Z-3-hexen-1-ol, and Z-3-hexen-1-yl acetate. Most of the green leaf volatiles emit quickly after insect damage, but some volatiles like Z-3-hexen-1-yl acetate begin to emit three to four hours after insect damage (Hatanaka, 1993; Ruther and Furstenu, 2005).

The immediate release of green leaf volatile is believed to result from the quick conversion of linolenic acid and linoleic acid into volatile compounds such as hexenal. De novo synthesis of green leaf volatiles are also increased in response to the insect damage (Pare and Tumlinson, 1997), and such increase is attributed to the increase of expression of genes involved in the pathway.

The role of green leaf volatiles in indirect defense is still controversial. In some ecosystems, green leaf volatiles seem to provide no attraction for parasitoids (Scutareanu et al., 1997), whilst the results are the opposite in other ecosystems (Du et al., 1998). Scutareanu et al. (1997) tested different volatiles produced by *Psylla*-infested pear trees for their capacity to attract natural enemy of *Psylla*, anthocorid predators. The *Psylla*-induced green leaf volatile compounds do not provide a cue for anthocorid predators to locate the forage, whilst other compounds such as (E)- β -farnesene serves as a signal for *Psylla* anthocorid predators (Scutareanu et al., 1997). However, the results seem to be contradictory to those in other ecosystems. Both (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate are induced when beans (*Vicia faba*) are treated with aphids, and both compounds can attract *Aphidius ervi*, the natural enemy of aphids (Du et al., 1996). Considering that both (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate are the most common compounds induced during the insect damage among different plant and herbivorous insect species, the tritrophic interaction exploiting green leaf volatile will not be specific. It is likely that a synergetic effect of green leaf volatiles and other compounds that render herbivore natural

enemy a fitness increase by locating the forage. Besides the role in indirect defense, green leaf volatiles are indicated for plant-plant communication (Ruther and Furstenau, 2005).

Another compound relevant to green leaf volatile in term of biosynthesis is methyl jasmonate, which is commonly induced in many plant indirect defense systems. Methyl jasmonate biosynthesis is the downstream of lipoxygenase pathway, where allene oxide synthase (AOS) catalyze the formation of 12,13-octadecatrienoic acid, which can be further processed by several steps to form jasmonic acid. Jasmonic acid can in turn be synthesized into methyl jasmonate by a SABATH gene family member, JMT. Methyl jasmonate serves as an important component of plant indirect defense. Applying methyl jasmonate alone to the wild tobacco plant showed significantly decreased herbivore damage (Kessler and Baldwin, 2001).

Terpenoids

Terpenoids constitute the most diverse group of volatile organic compounds induced by insect damage. Terpenoids are a group of secondary metabolites with common five-carbon isoprene base structure. Based on the number of the five carbon based structure, terpenoids can be classified into hemiterpene (five carbon), monoterpene (ten carbon), sesquiterpene (fifteen carbon), and diterpene (twenty carbon). Most of the volatile compounds involved in indirect defense are monoterpenes and sesquiterpenes. Besides the common terpenoid

compounds, two homoterpenes derived from sesquiterpene and diterpene were also shown to be major volatile organic compounds in several species (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000).

As compared to the green leaf volatiles, terpenoids involved in plant indirect defense exhibits high diversity, which might have made terpenoids the volatile components conferring the specific information for plant indirect defense. It is common to have different mixtures of terpenoids from different plant species when treated with the same insects. Indeed, different terpenoids mixtures have been found in different cultivars of same species when treated with the same type of insects in maize, cabbage and rice (Degen et al., 2004; Lou et al., 2006). Even though there is no single terpene serving as a universal signal for plant indirect defense, some terpenoids do appear at a higher frequency across the species induced by herbivorous insects. Several of such terpenoids include monoterpenes E- β -ocimene and linalool, as well as sesquiterpenes β -carophyllene and (E)- β -farnesene. Besides the monoterpenes and sesquiterpenes, the two homoterpenes DMNT and (3E,7E)-4,8,12-dimethyl-1,3,7,11-tridecatetraene also seem to be prevalent signal induced by insect damage. Several of these compounds including linalool, E- β -ocimene, (E)- β -farnesene and DMNT has been shown to be able to attract natural enemies in different ecosystems as listed in Table 1.3. For example, linalool has also shown to be able to reduce the *Manduca sexta* larvae infestation on wild tobacco by more than 90% in field experiments when natural enemies for the herbivorous insect

exist (Kessler and Baldwin, 2001). Combining the prevalence, the results from field experiments, and the Y tube assay showing attraction of natural enemies, linalool and other terpenoid compounds might play a key role in the indirect defense signaling.

Besides the above experiments, recent metabolic engineering work also confirmed the importance of terpenoid in indirect defense (Degenhardt et al., 2003; D'Alessandro and Turlings, 2005; Turlings and Ton, 2006).

Overexpressing maize sesquiterpene synthase *TPS10* can directly lead to increase of the attraction of generalist parasitoids from the *Cotesia* genus, which indicates the role of *TPS10* products in indirect defense (Schnee et al., 2002; Kappers et al., 2005; Schnee et al., 2006).

Indole and Indolic Compounds

It was speculated that indole is a key component in attracting natural enemies of herbivorous insects since indole can attract parasitoids in the Y tube assay and IGL gene has been shown to be up-regulated in response to insect damage.

However, recent experiments blocking the indole biosynthesis during insect damage has shown no significant impact on the attraction of parasitoid by host plants (D'Alessandro et al., 2006). The results indicate that indole might not be a necessary component for indirect defense, or at least in the system studied by D'Alessandro et al.

Phenolic Compounds

Another class of volatile compounds induced by insect damage are the phenylpropanoids and benzenoids derived from phenylalanine. This group of compounds includes benzaldehyde, benzyl alcohol, and methyl salicylate. Phenylpropanoids and benzenoids include several major compounds actively involved in plant pollination signaling. The existence of benzaldehyde and phenylpropanoids in insect induced defense volatiles seem not to be as prevalent as terpenoids and green leaf volatiles across the species. However, considering the important role of benzaldehyde compound in reproduction signaling and the fact that insect responds to phenolic compounds effectively according to electrophysiology studies, the importance of phenolic compounds in indirect defense should not be ignored.

Other Compounds

Besides the above compounds, other compounds derived from amino acid or fatty acid pathways can also be found in the volatile mixture emitted by plants after insect damage. These compounds include dimethyl disulfide and methyl(iso)thiocyanate. The existence of these compounds in plant herbivore induced volatiles is not predominant across species. They are usually species specific and the amounts are normally low. These compounds have not been studied much with regards to indirect defense.

Overall, insect induced volatiles display a very diverse pattern among different plant species or even cultivars. Among different classes of volatiles, terpenoids seem to be the most diverse group of volatile compounds that may present more specific information for natural enemies in indirect defense. It is also believed that the combined signature of volatile compounds determines the affectivity of the system.

IV. Control And Regulation Of Production And Emission Of Insect-Induced Volatiles

Specificity, sensitivity, complexity and diversity are all important consideration of plant indirect defense based on tritrophic interactions. In term of specificity, plants are able to emit different sets of volatile compounds when damaged by different types of insects, or same insects at different developmental stages.

Parasitoid or predators of herbivorous insects are proven to be able to distinguish these differences to maximize the efficiency of forage (De Moraes et al., 1998). Since many of the volatiles produced by plants upon herbivore damage are at trace amount, the detection sensitivity for predators and parasitoids is an important consideration. Limited information has been obtained regarding the sensitivity of the tritrophic interaction since the volatile compounds involved in tritrophic interactions are always a complex mixture.

Previous research showed that a single volatile is sufficient to attract parasitoids and the content of the mixture can also confer information for the natural enemies. Basically, both the amount of key volatiles and the composition of the

complex mixture are critical to deliver information to natural enemies regarding the status of herbivore interaction with plants, but the details of the mechanisms for information delivered by volatile compounds still need to be addressed. Diversity is another important consideration of the tritrophic interactions, which has been described in more than thirty systems and proven to be a predominant phenomena in nature. These different systems sometimes involve generalist herbivorous insects such as armyworm and generalist parasitoids such as those in *Cotesia* genus and sometimes involve specialist insects and parasitoids that were proven to be able to tell the differences between host and non-host herbivorous insects (Du et al., 1996; Rose et al., 1997). Much difference in the content and composition of volatile compounds have been found in these different tritrophic interaction systems. Considering the prevalence of the tritrophic interaction in ecosystems and involvement of generalist insects and predators in many systems, it would be constructive to know if there is any common group of effective signal compounds across different systems or not, which will help to answer the diversity of volatile compounds relevant to the diversity of the tritrophic interaction phenomena across the ecosystems.

Volatile Biosynthesis

When different species of plants are damaged by the same type of insects, the volatile mixture are different in content and composition. In fact, even different cultivars from same species can emit quite different volatile compounds when damaged by the same type of insects (Geervliet et al., 1997; Hoballah et al.,

2002; Degen et al., 2004; Lou et al., 2006). Since host plant chemistry might have an effect on the fitness of parasitoids, being able to identify the proper host plants is important for parasitoids. Experiments have shown that generalist parasitoid *Cotesia marginiventris* can discriminate the volatile signal from different species as well as different cultivars of maize within the same species (Hoballah et al., 2002).

Despite of their diverse structures, most of the terpenes are synthesized from terpene synthase with GPP, FPP, and GGPP as the substrates. For example, monoterpene synthase use GPP as substrate to synthesize monoterpenes, whilst sesquiterpene synthase converts FPP into sesquiterpenes. The up-stream biosynthesis of terpenoids involves both mevalonate pathway in cytosol and non-mevalonate pathway in plastids. Since substrate availability is an important consideration for terpenoid biosynthesis, the coordinative up-regulation of genes in terpene biosynthesis pathway is important for the insect induced terpenoid release. Considering the multiple steps involved, the de novo biosynthesis of terpenoid has made terpene release always at later stage of infestation, normally a few hours after the initial damage (Pare and Tumlinson, 1997; Miller et al., 2005).

Volatile indolic compounds and indole comprise another small class of volatiles observed often in indirect defense. Indole appears in a more species-specific pattern, where in some species like tobacco, no indole was found during the

insect damage. Indole was synthesized from shikimic acid pathway derivatives, where anthranilate was synthesized into indole-3-glycerol phosphate, which can be converted to free indole by indole glycerol lysase (IGL) (Frey et al., 2000). The release of indole is often in late stage too, which reflected the relative complex biosynthesis of indole.

The biosynthesis of volatile phenolic compounds intervenes with the lignin pathway, where phenylalanine ammonia-lyase (PAL) first converts phenylalanine into trans-cinnamic acid, the common precursor of monolignol and some phenolic compounds. The subsequent hydroxylation and methylation lead to form different types of phenolic compounds. The biosynthesis of volatile phenolic compounds is still the subject of intensive studies (D'Auria et al., 2002).

Variation of Volatile Biosynthesis

As shown in Table 1.1 It is well known that the same type of insect will induce different volatile compounds in different plant species as shown in Table 1.1. Moreover, the volatile profilings induced by the same type of insects are also quite different among different cultivars in the same species (Degen et al., 2004). Such variation highlights the diversity of volatile-mediated indirect defense. Plants seem to be able to evolve different volatile profiles in the local ecosystem to adapt to changes in the environment. The molecular mechanisms of such diversity were studied. Enzyme specificity changes have been proposed to be an important mechanism of volatile biosynthesis diversity among or within species

(Kollner et al., 2004). Moreover, gene expression level may also contribute to the diversity in herbivore induced volatile contents and compositions.

Genetic Regulation by Signaling Molecules

1. The level of damage as indicated by quantity of volatiles

Different levels of damage are also able to induce different volatile mixtures. Experiments using a mechanical caterpillar have revealed that green leaf volatiles and monoterpenes are produced in proportion to the level of damage, whilst other compounds are not (Mithofer et al., 2005). However, the influence of such information on the foraging behavior of natural enemies is still unknown.

2. The developmental stage of herbivorous insects as indicated by volatile signal

Herbivores at different developmental stages are known to be able to induce volatile compounds with different qualitative and quantitative features (Takabayashi et al., 1995). Subtle differences have been found when maize are treated with different instar of noctuid moths, and parasitoids. *littoralis* does not seem to prefer an early instar damaged leave when two choice experiments were carried out (Gouinguene et al., 2003). However, in a different system where maize was treated with armyworm, the parasitoid *Cotesia* can readily distinguish between early and late instar insect-induced damage. Since *Cotesia* normally can only attack early instar armyworm, such capacity in recognizing the developmental stage of host insects by plant volatile is important for parasitoids to most efficiently identify the forage (Takabayashi et al., 1995). Like many

other features, the ability for parasitoids or predators to tell the different developmental stage depends on the system.

3. The timing of the damage as shown by volatile content

The releasing of volatile compounds was different during the time-course of treatment. Normally, green leaf volatiles are first released and then the terpenoids and methyl salicylate. After the damage, the green leaf volatiles ceased to produce much sooner than other compounds like terpenoids. It seems that parasitoid can exploit these information to know the timing of the damage. Experiments have shown that naïve *Cotesia glomerata* prefers newly damaged leaf over the old damaged leaves when maize plants are damaged by lepidopteron (Hoballah and Turlings, 2005).

4. Diurnal cycle as shown by volatile qualitative and quantitative features

Diurnal cycle can be indicated by the differences in volatile mixture. During a long-term treatment, terpenoid volatiles emit in a diurnal pattern, where maximal emissions occurred in the afternoon. For the jasmonic acid induced terpenoid volatile emission, the emission follows a diurnal cycle dependent pattern (Martin et al., 2003). The green leaf volatile seems to emit without diurnal cycle and ceased emission soon after the removal of herbivore (Loughrin et al., 1994). The daytime emission of terpenoids might be beneficial for both plants and natural enemies of herbivorous insects since most of the natural

enemies forage during the daytime (McCall et al., 1994; Turlings et al., 1995; Turlings et al., 1998).

5. Systemic Emissions

Besides the emission of volatile from the damaged site, plants are known to be able to elicit the systemic response by releasing the volatiles to attract parasitoids or predators (Turlings and Tumlinson, 1992; Rose et al., 1996; Rose et al., 1998; Neveu et al., 2002; Arimura et al., 2004; Rose and Tumlinson, 2005). Basically, systemic emission of plant volatiles referred to the emission of plant volatiles in whole plant including parts of the plants that are not damaged by herbivorous insects (Turlings and Tumlinson, 1992; De Moraes et al., 1998). The regulation and level of systemic response still needs to be further studied (Wu et al., 2007). The systemic signal would increase the fitness of plants by providing stronger signal attracting the natural enemies of herbivorous insects and prepare the plant organs from incoming damage.

Overall, the qualitative and quantitative differences of volatile compounds induced by herbivores confers complicated information to the parasitoids and predators regarding the situation of the infestation to allow the natural enemies of herbivorous insects to best locate the forage and thus increase the fitness of these natural enemies.

Environmental Regulation

Environmental factors are known to be able to influence the volatile production (Gouinguene and Turlings, 2002). Terpenoid volatiles are known to be up-regulated in high temperature, where they may serve as protective compounds (Zvereva and Kozlov, 2006; Hance et al., 2007; Veteli et al., 2007). Carbon dioxide and ozone as green-house gases often up-regulate terpenoid volatile production and sometimes result in the changes in tritrophic interactions (Vuorinen et al., 2004; Vuorinen et al., 2004; Vuorinen et al., 2004; Vuorinen et al., 2005; Noe et al., 2006). The volatile production can also be influenced by the nutrient availability, where nitrogen containing compounds are limited by the nitrogen availability. Terpenoids do not have nitrogen and thus are not limited by the nitrogen availability, however, the production of terpenoids are believed to be regulated by light, at least by the diurnal cycle, where significantly less terpenoids are produced during the night (Loughrin et al., 1994).

Emission from Below-ground

Even though parasitoids and predators are the most prevalent natural enemies involved in volatile-mediated indirect defense, recent research has enlisted fungi as an active component against green mites (Hountondji et al., 2005). Moreover, the underground volatile production induced by herbivore anthropol *M. virgifera* has been proved to be able to attract the parasitoid nematode (Rasman

et al., 2005), which expand the tritrophic interaction to the underground ecosystem. Moreover, recent research indicated that simultaneous feeding of leaf and root insect will reduce the tritrophic interaction in both above-ground and under-ground, which indicated the interference between the two systems (Rasmann and Turlings, 2007).

V. Identifying Active Signals In Indirect Defense

The qualitative and quantitative nature of the mixture volatile compound can first tell the parasitoids the presence of herbivorous insects. Wounding damage is also known to be able to induce volatile compounds, in particular, the green leaf volatiles. The systemic signal induced by wounding and herbivore damage are shown to be different (Rose and Tumlinson, 2005), and these differences could account for the different foraging behavior. Not only that volatiles can help parasitoids to locate their forage, they can also help parasitoids to best use the forage and avoid interspecific competition (Tamo et al., 2006). Besides the plant emitted signal, parasitoid and predators can also exploit the smell from herbivorous insects to locate the forage. It has been considered that plant emitted volatiles mainly provide long distance signal for locating the forage (Turlings et al., 1991; Steinberg et al., 1993).

Testing Pure, Synthetic Compounds

Many compounds induced by herbivorous insects can lead to response in the parasitoid antennal lobe, and a mixture of compounds may give an advantage to

induce synergic strong signals leading to the parasitoid locating herbivorous insects (Gouinguene et al., 2005). The parasitoid response to volatile compounds is characterized both at the physiological and behavioral levels. In one study, the positive EAG (Electroantennogram) results were found for green leaf volatile 3(Z)-hexen-1-ol, terpenoid linalool, caryophyllene, and homoterpene (3E,7E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene for the parasitoid *Opius dissitus*. Most but not every of the EAG positive compounds also showed the positive results in Y-tube assay, which highlights the correlation between physiology and behavior studies (Carroll et al., 2006). The mixture of insect induced plant volatiles thus can confer the information regarding the presence of herbivorous insects with certain specificity.

Genetic Engineering

Genetic engineering has provided the most solid evidence for the involvement of a certain terpene synthase gene in the indirect defense, though this kind of research has been limited. Recent research indicated that switching the subcellular location of sesquiterpene synthase into mitochondria resulted in the new volatile profile with stronger capacity to attract the parasitoids (Kappers et al., 2005). Moreover, overexpressing of a single maize sesquiterpene synthase, TPS10, can result in transgenic plants with greater capacity for parasitoid attraction (Schnee et al., 2006). The genetic engineering work indicated the potential for using terpene synthase as a potential way to protect plants against herbivorous insects.

Volatile Content and Composition in Relevance to Herbivorous insect Presence

The ability for natural enemies to distinguish the different types of herbivorous insects by volatile compounds depends on the ecosystem. In most of the volatile-mediated indirect defense systems, damage caused by different insects will lead to different qualitative or quantitative features of the volatile compounds (Blaakmeer et al., 1994; Himanen et al., 2005; Takabayashi et al., 2006). The ability for the parasitoids to distinguish different volatile mixtures relevant to the host species largely defines the specificity of the system. There are differences when plants are damaged by specialist and generalist insects, which lead to no differences of parasitoid foraging behavior (Blaakmeer et al., 1994). For example, when strawberries were damaged by the herbivorous insects *Phytonemus pallidus* or the mite *Galerucella tenella*, substantially different volatile compounds are emitted, yet these different volatile mixtures do not seem to be able to make a difference in the choice of predators (Himanen et al., 2005). However, in several other cases, the different content in the volatile mixtures can be found when plants are damaged by different type of insects and such differences can lead to different foraging behavior of parasitoids or predators (Takabayashi et al., 2006). The specialist parasitic wasp *Cardiochiles nigriceps* was shown to be able to exploit the different qualitative and quantitative features of volatile mixtures to distinguish their specific host *H. virescens* from by *H. zea* (De Moraes et al., 1998). The predatory mite *Phytoseiulus persimilis* has been shown to be able to distinguish the volatile

between prey and non-prey herbivores (De Boer et al., 2004). Actually, the reorganization of proper host insects by specialist parasitoids may also be enhanced by previous learning experience (Rose et al., 1997).

Quantity of Volatile Compounds as a Message

Quantity is another consideration of the volatile compound release. As shown in Table 1.2, predominant volatiles from the same species turn to be more likely to be major components among different treatments for different insect species and elicitors. We define a major component as approximately more than 5% of the total amount of the total volatile. However, it does not mean major component is always high amount. The phenomena are explainable if biosynthesis of volatile compounds and the regulation are taken into consideration. For example, in terpenoid biosynthesis, a mixture of compounds are normally regulated by only a few terpene synthase genes and the enzyme activity of terpene synthase largely determined which peak will be the major compounds as long as the gene is induced (Kollner et al., 2004).

Despite the fact that no volatiles serve as a common major component across the species, there are some compounds that are more often to be major compound across different systems. Among these compounds are linalool and beta-carophyllene. Since the generalist parasitoids may use similar signals to locate herbivorous insects across a variety of plants, more attention should be paid to

the terpenoids appearing more frequently in the ecosystems and their roles in indirect defense.

Is the quantity of volatile level an important aspect of the signal? The question can be answered from two perspectives based on current research. First, the level of green leaf volatiles and some monoterpenes can serve as an indicator of level of damage by herbivorous insects (Mithofer et al., 2005). Second, experiments have shown different compound levels induce differential physiological and behavioral effects in parasitoid insects. For some compounds, the effect is strictly in a dose dependent pattern, where too low level compounds may be below the detection limit for parasitoid and too high level of compounds may lose its function for attracting parasitoids, too (Ramachandran et al., 1991; Turlings et al., 1995). Regardless the possible importance of volatile compound levels, the herbivore induced volatiles always exist in a mixture in which several major compounds may exist, even though it is more likely for the combination of qualitative and quantitative signature of these volatile compounds to render a meaning for information for parasitoids and predators to make decisions regarding the forage.

What can the qualitative and quantitative features tell the natural enemies about the herbivorous insects and plants? Obviously, the most important information is the existence and the location of the herbivorous insects. As discussed before, many volatiles alone have been proved to be able to attract the parasitoids and

predators of herbivorous insects if the level of the compound is high enough. Then why do plants strive to evolve complex enzyme systems to produce a mixture compounds? Researches have shown that the effectiveness of the indirect defense largely depends on the qualitative and quantitative differences of the mixture of volatile organic compounds, which confers a broad spectrum of information including the age of the plants, the type of the host and such. Such mixture of volatiles can help the natural enemies best locate their proper forage. We hereby review the information that might be conferred by the features of volatile compounds released by the insect damaged plants.

Volatiles from Natural Enemies' Perspective

Another level of complexity added to the infochemical-food web of the indirect defense is the perception of volatile compounds by natural enemies of herbivorous insects. Previous experiments indicated the rearing history, diet, sex, previous sexual experience and oviposition experience can all influence the perceiving of the volatile signals (Li et al., 1992; Takabayashi and Dicke, 1992; Whitman and Eller, 1992; Perezlachaud and Campan, 1995; Vaughn et al., 1996; Gandolfi et al., 2003)

Among all the features of natural enemies, the learning capability is the most important one to consider in the volatile induced indirect defense (Lewis et al., 1991; Du et al., 1997; Powell et al., 1998). Learning is a common theme among the parasitoids and predators of herbivorous insects. Considering the complexity

and dynamics of the infochemical-food web, the learning ability is very important for species to adapt to constantly changing environments (De Boer and Dicke, 2006). Parasitoids and predators are known to have a strong learning ability to distinguish different mixtures of volatile compounds. Previous experiments showed that naïve *Cotesia* prefers fresh damaged tissue, however, they can quickly learned to adapt to the old damaged tissue if forage is available (Hoballah and Turlings, 2005). Considering the complexity of the volatile reflecting the situation of the infestation by herbivorous insects, the strong learning allows natural enemies to promptly develop adaptive behavior toward the most efficient foraging. In short, the qualitative and quantitative differences of the herbivore-induced volatile are important in the ecosystem context, and the information conferred by the mixture can be exploited by the natural enemies to increase their fitness.

VI. Evolution Of Indirect Defense

Theories about evolution of plant defense systems appear sequentially in the mid-1970s, however, most of the current theories largely ignore the importance of indirect defense and the role of volatiles in the evolution. More systems level studies need to be developed to addressed the issue of how volatile-mediated indirect defense originated and what are the driving forces for the evolution of volatile-mediated indirect defense (van Veen et al., 2006). We hereby attempt to propose the theories regarding the origination of volatile-mediated indirect defense and the driving forces for it.

Insect-induced Plant Volatiles: Multiple Functions

1. Volatiles in direct defense

Besides the role in indirect defense, volatile compounds emitted upon insect damage can also serve as direct defense components to reduce the oviposition rate of herbivorous insects (Binder et al., 1995; Binder and Robbins, 1997; Kessler and Baldwin, 2001) or change the feeding behavior of herbivorous insects (Wilson et al., 1996; Bernasconi et al., 1998).

2. Volatiles in plant-plant communication

Exposure of plants to volatile compounds from herbivore damaged plants can help plant to potentiate to adapt to incoming danger (Bruin et al., 1992; Stowe et al., 1995). Herbivore damaged plants can induce ethylene emission in neighboring plant (Arimura et al., 2002), and the volatile exposed plants can also help the neighboring plants to emit volatiles to be more attractive to the natural enemies of herbivorous insects, which can both benefit the neighboring plants for potential danger and increase the distance of volatile signaling attracting the natural enemies of herbivorous insects (Choh and Takabayashi, 2006). Recent genomic level study has revealed molecular level changes of such adaptation resulted from volatile exposure (Ton et al., 2007).

3. Volatiles as intra-plant signals

Volatile compounds like methyl salicylate and methyl jasmonate are believed to be able to serve as signal compounds for the same plant when damaged by

herbivorous insects (Heil and Silva Bueno, 2007). The methyl ester of defense hormone jasmonic acid and salicylic acid were suggested to play similar signal roles as the original compounds. On one hand, these methyl esters are emitted by plants during the defense process; on the other hand, these methyl esters can induce plant defense response (Martin et al., 2002; Martin et al., 2003; Miller et al., 2005). Their role as intra-plant signaling is therefore proposed (Martin et al., 2003).

The Origin of Indirect Defense Mediated by Plant Volatiles

Several scenarios have been proposed to explain the origin of volatile-mediated indirect defense. One theory indicates that the plant emits volatiles during herbivore damage just in response to the wounding, and once the parasitoids or predators began to respond, co-evolution starts. An alternative theory suggested that the plant emits volatile to attract the parasitoids. (Godfray, 1995; Agrawal, 1998; Walker and Jones, 2001; Dicke and Hilker, 2003) The first theory seems to fit more into the situation. However, regardless of the theories, it should be noted that volatiles are important components for plant reproduction and growth. Before the co-evolution of volatile-mediated indirect defense, volatile compounds may already exist for plant growth and reproduction. Many volatile compounds such as terpenoids and benzenoids involved in indirect defense also show up in the flower as signal for pollinators and some components such as methyl jasmonate and methyl salicylate are important plant hormones in direct defense. Other compounds like green leaf volatiles are known to have protective

roles when plant tissues are damaged. The release of these compounds may be just part of the plant direct defense, reproduction, or damage repair process in the beginning. Once these volatile compounds can be recognized by the parasitoids or predators, the co-evolution begins. It should be pointed out that evolution of olfactory system in insects is so well developed that many insects have quick learning ability in adapting to the new environment. Once the volatile signals are being exploited for locating the herbivorous insects, the co-evolution can be quite rapid on the natural enemies' side.

The Presence of Natural Enemies as a Driving Force for Co-evolution

The fact that different plant cultivars often emit quite different volatile components when treated with same insect species indicates the evolution of indirect defense is a highly dynamic and versatile process or that specific enzymes exist in specific plants. From a top-down view, natural enemies are important in the co-evolution of volatile-mediated defense (Walker and Jones, 2001). The presence of natural enemies for the herbivorous insects is a prerequisite for co-evolution of the volatile-mediated indirect defense, and the co-evolution of plant-insect-natural enemy can take different routes in different environments. If the natural enemies can effectively control the population of herbivorous insects, plants will develop more sophisticated indirect defense to increase the fitness of the parasitoids or predators and thus increase their fitness. However, if the population of herbivorous insects cannot be contained by natural

enemies, plants developing a stronger direct defense may survive better in the environment.

The Resource Availability as a Driving Force for Evolving the Volatile-mediated Indirect Defense

As any defense mechanism, the volatile production is associated with a cost. Cost of volatile production in indirect defense can be counterbalanced by the benefit when the parasitoids are present (Hoballah et al., 2004). However, when the natural resource such as nitrogen and phosphate is limited, resource availability becomes an important consideration in evolving defense strategy (Gouinguene and Turlings, 2002). The cost for volatile production was indicated to be low as compared to direct defense compounds since few volatile compounds contain nitrogen, which more often to be the limited plant resource (Hoballah et al., 2004). In such scenario, if natural enemies exist in the environment and nitrogen resource is limited, the evolution of indirect defense may increase plant fitness.

Plant Biochemistry as a Driving Force for Indirect Defense Evolution

Considering the potential tradeoff between direct and indirect defense, the enzyme activity and gene expansion leading to the different plant biochemistry can be considered as both a result and a driving force for the evolution of indirect defense. Plants with high endogenous toxic compounds are unlikely to evolve indirect defense, since these compounds can be exploited by the

herbivorous insects to reduce the fitness of parasitoids and predators. Once the co-evolution starts, plants need to adapt to the indirect defense by changing the enzyme activity or gene expression to maximize the fitness of both natural enemies and plants.

Herbivorous insect Behavior as a Driving Force for Indirect Defense

The insect induced volatiles cannot only be employed by the natural enemies, but also by the herbivorous insects to locate their food (Birkett et al., 2006). So these volatiles themselves can both increase and reduce the fitness of the plants regardless the cost of producing them. The dynamics of volatile-mediated defense thus will be influenced by the insect behavior at the same time. Once herbivorous insects developed to recognize the signal to parasitoids, these volatile compounds may end up reducing the fitness of plants. Different volatile compounds or defense strategies are needed.

Volatiles Being Exploited: Fitness Measurement

The volatile production does not always increase the fitness of plants, and sometimes these volatiles can be exploited the herbivorous insects or even parasitic plants to decrease the fitness of the hosting plants (Carroll et al., 2006; Runyon et al., 2006). Herbivorous insects can exploit the volatiles to locate host plants or avoid intra species competition by reducing over oviposition (Kessler and Baldwin, 2001; Carroll et al., 2006).

Putting the volatile compounds into the infochemical and food net, the preciseness of volatile-mediated signaling is still not known. The underground herbivore was reported to be able to emit volatiles like farnesene to influence the behavior of above ground parasitoid in the *Cotesia* genus (Soler et al., 2007). Insects and plants seem to be in a never ending battle during the evolution, and volatile compounds are an important components being exploited for the battle.

VI. The *TPS* Gene Discovery With Functional Genomics Approach

Terpenoids represent the largest group of secondary metabolites in plants. Terpenoids can be classified into monoterpenes (10 carbon), sesquiterpenes (15 carbon), diterpenes (20 carbon), triterpenes (30 carbon), and tetraterpenes (40 carbon) according to the number of carbons and isoprene base structures. For example, sesquiterpenes have 15 carbons and three five carbon isoprene structures as shown in Figure 1.1. Most monoterpenes and sesquiterpenes are volatiles, and many of them are important in plant indirect defense (Kessler and Baldwin, 2001; Keeling and Bohlmann, 2006). Many plant diterpenes are involved in direct defense serving as toxins or deterrents. Some diterpenes are part of the plant hormone biosynthesis pathway, for example, the diterpene karuen is an upstream precursor of the plant hormone GA (Prisic et al., 2004; Wilderman et al., 2004).

Even though terpenoids display a diverse range of structures from different folding and modification of the five carbon base units, the biosynthesis of

terpenoids is relatively straightforward. All terpenoids are synthesized by members of terpene synthase (TPS) gene family. The *Arabidopsis* genome contains 40 *TPS* genes (Aubourg et al., 2002), and the rice genome contains 52 *TPS* genes (Joshua S. Yuan and Feng Chen, unpublished data). The members of the *TPS* gene family can be classified into monoterpene synthases, sesquiterpene synthases, and diterpene synthases according to their products (Bohlmann et al., 1998b). Monoterpene synthases use GPP (geranyl pyrophosphate) as substrate to synthesize a wide range of ten carbon monoterpenes such as linalool, pinene, ocimene, and limonene (Bohlmann et al., 1997; Bohlmann et al., 2000; McKay et al., 2003). Sesquiterpene synthases use FPP (farnesyl pyrophosphate) to synthesize the fifteen carbon sesquiterpenes such as carophyllene, farnesene, and germacrene (Bohlmann et al., 1998a; Chen et al., 2003; Chen et al., 2004; Kollner et al., 2004; Martin et al., 2004; Pechous and Whitaker, 2004; Tholl et al., 2005). Diterpene synthases use GGPP (geranylgeranyl pyrophosphate) to synthesize the twenty carbon diterpenes such as kaurene, cembrene, and diene (Prisic et al., 2004; Wilderman et al., 2004; Ro and Bohlmann 2006). Plant terpene synthase genes are members of a diverse gene family with very dynamic evolution, assumingly because of the interactive evolution between insects and plants (Keeling and Bohlmann 2006; Pichersky et al., 2006; Schnee et al., 2006). Even though the biochemical function of many terpene synthases have been characterized, the biological and ecological functions of many TPS genes are still not clear, especially for their role in plant defense.

Functional genomics and comparative genomics were shown to be powerful approaches in gene discovery. In a functional genomics approach, microarray gene profiling often can help to identify the candidate genes for further analysis. In a comparative genomic study, candidate genes are identified based on sequence similarity and further analyzed for their biochemical and biological function. Both approaches have been used for gene discovery for terpene synthase (Ralph et al., 2006; Ro et al., 2006). Microarray analysis and other global gene expression profiling techniques can also help to derive the overall picture of transcriptome changes during the insect treatment, and thereby help to better understand the coordinative changes in direct and indirect defense, which can explain the molecular mechanisms of indirect defense not only at the biochemical level, but also at the signal transduction and gene regulation level (Reymond et al., 2004; Ehlting et al., 2005; Ralph et al., 2006; Ro et al., 2006; Wang et al., 2006).

In this thesis, rice (*Oryza sativa*) will be used as a model to study the *TPS* gene family and the biological functions of *TPS* genes in plant indirect defense. Rice has several advantages as a model system. First, rice is an important crop, with more than 600 million tons of annual production; second, rice is the only monocot plant with complete genome sequence available. The recent completion of the genome sequencing for both indica and japonica biotypes of rice has also enabled the establishment of a variety of functional genomics tools including the whole genome long-oligo arrays, the proteomics database, the predicted gene

sequences, and the insertional mutant database. Moreover, traditional rice genetics work has established many genetic resources including the well-defined genetic map and comparative physical map between different rice biotypes. Rice also has a wide range of biotypes, which will allow in depth study of the evolution of secondary metabolism genes through a comparative genomics approach. The rich genetic and genomic resources available to rice allow us to design an integrated genomics approach to study the involvement of *TPS* genes in plant indirect defense in this model plant.

VII. Summary and Overview of Research Objectives

Overall, the volatile-mediated plant indirect defense has been studied intensively during the past two decades, and the complex nature is just being unfolded. There are still numerous questions remaining in the field. First, many genes have been discovered for producing these volatiles, however, the relative importance of each gene and volatile compound is still largely unknown. Second, the complex nature of the volatile-mediated indirect defense still need to be better explained. Are there any common compounds for the defense? Which compounds are more effective? What do the qualitative and quantitative features of compound mixture mean in a certain system? Third, the evolution of volatile-mediated indirect defense needs to be better studied. Few of the current theories have been well tested in a systemic level due to the complexity of designing experiments to test theories in multitrophic systems. With these questions remaining, more work needs to be carried out to further unveil the nature of

volatile-mediated indirect defense. Specifically, the genes involved in terpenoid biosynthesis need to be defined and characterized to allow genetic and gene expression studies.

I propose to identify and characterize the rice candidate *TPS* genes involved in indirect defense against insects using an integrative functional and comparative genomics approaches. The main objective of the thesis is gene discovery and the study of gene regulation in rice. Parasitoid attraction experiments demonstrated that relevant tritrophic interactions exist in our experimental system. Volatile profiling results indicate that the terpenoid volatiles are involved in rice indirect defense by attracting parasitoids to damaging insects of rice. The proposed thesis has six specific objectives. In objective 1, microarray global gene expression profiling was carried out to compare transcriptomes of rice subjected to fall armyworm and control rice to identify potential genes involved in induced defense. My focus is on the genes of terpene biosynthesis pathways, including the terpene synthases and upstream enzymes. For gene function characterization, I focused on three objectives, the characterization of monoterpene synthase(s), the characterization of sesquiterpene synthases, and the characterization of two to three functionally unknown terpene synthases that are localized in a cluster on rice chromosome four. The integrated information from genome analysis, gene expression profiling and volatile profiling helped us identify candidate genes involved in rice indirect defense. These genes were cloned and characterized to understand

their biochemical functions. The next objective of the proposed research was to study regulation of *TPS* gene expression under different conditions. Jasmonic acid is an important plant defense hormone, and the gene expression profiling of early response of jasmonic acid induced response constitutes objective 6. Since terpenoids were shown to be regulated by the diurnal cycle, the volatile and gene expression changes for the induced responses during diurnal cycle were studied as objective 7. The objective 8 compared the global gene expression profiling in dark and that under light conditions to further understand the molecular mechanism of diurnal cycle controlled differential terpenoid emission. In objective 9, genome sequence analysis of the rice *TPS* gene family was performed using genomic sequences, predicted open reading frames, and cDNA sequences to study the function, relatedness, and evolution of the *TPS* gene family. Objective 10 identified and characterize a conserved poplar *TPS* gene.

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Appendix

Table 1.1 Previously described volatile-mediated indirect defense. Only the systems with both well characterized volatile profiling and tritrophic interaction are included.

Host Plant	Herbivorous insect	Insect Predator	Reference
<i>Arabidopsis</i>	<i>Pieris rapae</i>	<i>Cotesia Rubecula</i>	(Van Poecke et al., 2001)
Brussels sprouts	<i>Pieris brassicae</i>	<i>Cotesia glomerata</i>	(Mattiacci et al., 1994)
Cabbage	<i>Pieris brassicae</i> or <i>Pieris rapae</i>	<i>Cotesia glomerata</i> or <i>C. rubecula</i>	(Blaakmeer et al., 1994)
Cabbage	<i>Pieris brassicae</i> and <i>Pieris rapae</i>	<i>Costesia</i>	(Geervliet et al., 1997)
Cotton	<i>Heliothis virescens</i> or <i>Heliothis zea</i>	<i>Cotesia</i>	(De Moraes et al., 1998)
Lima bean	<i>Tetranychus urticae</i>	<i>P. persimilis</i>	(De Boer et al., 2004)
Lima bean	<i>Spodoptera exigua</i>	<i>P. persimilis</i>	(De Boer et al., 2004)
Lima Bean	<i>Tetranychus urticae</i>	<i>Phytoseiulus persimilis</i>	(Dicke et al., 1990)
Maize	<i>Spodoptera exigua</i>	<i>Cotesia marginiventris</i>	(Turlings et al., 1990)
Maize	<i>Spodoptera exigua</i>	<i>Cotesia marginiventris</i>	(Turlings et al., 1991)
Maize/sorghum/napier grass	<i>Liriomyza trifolii</i>	<i>Diglyphus isaea</i>	(NgiSong et al., 1996)
Maize	<i>Heliothis virescens</i> or <i>Heliothis zea</i>	<i>Cotesia</i>	(De Moraes et al., 1998)
Maize	<i>Diabrotica virgifera virgifera</i>	entomopathogenic nematode	(Rasmann et al., 2005)
Maize	<i>Spodoptera exigua</i>	<i>Cotesia Kariya</i>	(Takabayashi et al., 1995)
Pear Tree	<i>Psylla. pyricola</i>	<i>Anthocoris nemorum</i>	(Scutareanu et al., 1997)
Potato	<i>Myzus persicae</i>	<i>Episyrphus balteatus</i>	(Harmel et al., 2007)
Rice	<i>Alotartessus iambe</i>	<i>Anagrus nilaparvatae</i>	(Lou et al., 2005)
Soybean/lima bean	<i>Pseudoplusia includens</i>	<i>Microplitis demolitor</i>	(Ramachandran et al., 1991)
Tobacco	<i>Heliothis virescens</i> or <i>Heliothis zea</i>	<i>Cotesia</i>	(De Moraes et al., 1998)
<i>Vicia faba</i> (bean)	<i>Acyrtosiphon pisum</i>	<i>Aphidius ervi</i>	
<i>Vicia faba</i> (bean)	<i>Nezara viridula</i>	<i>Trissolcus basalis</i>	(Colazza et al., 2004)
Strawberry	<i>Phytonemus pallidus</i>	<i>Neoseiulus cucumeris</i> , <i>N. californicus</i> , and <i>Euseius finlandicus</i>	(Himanen et al., 2005)
Strawberry	<i>Galerucella tenella</i>	<i>Neoseiulus cucumeris</i> , <i>N. californicus</i> , and <i>Euseius finlandicus</i>	(Himanen et al., 2005)

Table 1.2. Insect or Elicitor Induced Compounds. Only volatile compounds characterized in a system with well characterized indirect defense are included. Quantitative information are not available for most studies. For those with quantitative information, major compounds are defined as estimated more than 5% of total emission in any treatment or cultivar.

Volatile	System	Level	Ref
Green Leaf Volatiles			
Hexanal	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgent or glucosidase		(Mattiacci et al., 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Z-3-hexenal	Maize – armyworm	Major	(Turlings et al., 1991)
	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
E-2-hexenal	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize – armyworm		(Turlings et al., 1991)
	Maize –armyworm, or wounding		(Takabayashi et al., 1995)
	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgent or glucosidase		(Mattiacci et al., 1995)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
	Rice-brown leaf hopper		(Lou et al., 2005)
Nananal	Cabbage – <i>Pieris brassicae</i> regurgent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Decanal	Cabbage – <i>Pieris brassicae</i> regurgent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Dodecanal	Rice-brown leaf hopper		(Lou et al., 2005)
Octanal	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
3-Octanone	Soybean-soybean looper	Major	(Ramachandran et al., 1991)
3-pentanone,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
2-pentanone	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
1-pentanol	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
2-penten-1-ol	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
1-Penten-3-one	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
2.4-Hepladienal	Bean - <i>Liriomyza trifolii</i> - <i>Diglyphus isaea</i>	Major	(NgiSong et al., 1996)
2-pentanal	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)

Table 1.2, continued

Volatile	System	Level	Ref
3-pentanol,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize –armyworm, or wounding		(Takabayashi et al., 1995)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
1-penten-3-ol,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize –armyworm, or wounding		(Takabayashi et al., 1995)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
	Cabbage – P brassicae/P Rapae – <i>Cotesia</i>		(Geervliet et al., 1997)
	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
1-hexanol,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize –armyworm, or wounding		(Takabayashi et al., 1995)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cotton-armyworm		(Perezlachaud and Campan, 1995)
	Cabbage – P brassicae/P Rapae – <i>Cotesia</i>		(Geervliet et al., 1997)
	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
E-2-hexen-1-ol	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cotton – armyworm		(Perezlachaud and Campan, 1995)
Z-3-hexen-1-ol,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize - armyworm		(Turlings et al., 1991)
	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cabbage - <i>Pieris brassicae</i> or <i>P. rapae</i>	Major	(Blaakmeer et al., 1994)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Bean - <i>Liriomyza trifolii</i> - <i>Diglyphus isaea</i>	Major	(NgiSong et al., 1996)
	Cotton – Beet Armyworm Systemic	Major	(Rose et al., 1996)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum	Major	(Scutareanu et al., 1997)
	Cabbage – P brassicae/P Rapae – <i>Cotesia</i>		(Geervliet et al., 1997)
	Tobacco - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Cotton - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Maize - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	<i>Vicia faba</i> - <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
	<i>Arabidopsis</i> - <i>Pieris rapae</i>		(Van Poecke et al., 2001)
2.4-Hepladienal	Bean - <i>Liriomyza trifolii</i> - <i>Diglyphus isaea</i>	Major	(NgiSong et al., 1996)
1-hexen-1-yl acetate,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Maize – armyworm	Major	(Turlings et al., 1991)
	Maize – Armyworm Regurigent	Major	(Turlings and Tumlinson, 1992)
	Maize – Armyworm Regurigent Systemic		(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
E-2-hexen-1-yl acetate,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
Z-3-hexen-1-yl acetate,	Brussels Sprouts - <i>Pieris brassicae</i> - <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cotton – armyworm	Major	(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cabbage - <i>Pieris brassicae</i> or <i>P. rapae</i>	Major	(Blaakmeer et al., 1994)

Table 1.2. continued

Volatile	System	Level	Ref
	Cotton – earworm	Major	(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgitant or glucosidase		(Mattiacci et al., 1995)
	Cotton – Beet Armyworm Systemic	Major	(Rose et al., 1996)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum	Major	(Scutareanu et al., 1997)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Cotton – <i>H. virescens</i> or <i>H. zea</i>	Major	(De Moraes et al., 1998)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	<i>Vicia faba</i> – <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
	Lima bean – <i>S. exigea</i>		(De Boer et al., 2004)
Hexyl acetate	Cotton – earworm		(McCall et al., 1994)
	Cotton – armyworm		(Perezlachaud and Campan, 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin		(Alborn et al., 1997)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
3-hexen-1-yl propanoate,	Brussels Sprouts – <i>Pieris brassicae</i> – <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
Z-3-hexen-1-yl butyrate,	Brussels Sprouts – <i>Pieris brassicae</i> – <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgitant or glucosidase		(Mattiacci et al., 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
E-2-hexenyl-butyrate	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
Z-e-hexen-1-yl isovalerate	Brussels Sprouts – <i>Pieris brassicae</i> – <i>Cotesia glomerata</i> ; wounding, and elicitor		(Mattiacci et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgitant or glucosidase		(Mattiacci et al., 1995)
Z-3-hexenyl 2-methylbutyrate	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
E-2-hexenyl 2-methylbutyrate	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
Z-Jasmone	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Tabacco- <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
6-methyl-5-hepten-2-one	<i>Vicia faba</i> – <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
Terpenes			
Alpha-Pinene	Cotton – armyworm	Major	(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Cotton – <i>H. virescens</i> or <i>H. zea</i>	Major	(De Moraes et al., 1998)
Camphene	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
Beta-Pinene	Cotton – armyworm	Major	(Loughrin et al., 1994; Perezlachaud and Campan, 1995; Pare and Tumlinson, 1997)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurgitant or glucosidase		(Mattiacci et al., 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)

Table 1.2. continued.

Volatile	System	Level	Ref
α -thujene	Cabbage – <i>Pieris brassicae</i> or <i>P. rapae</i>	Major	(Blaakmeer et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
Myrcene	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995) (Pare and Tumlinson, 1997)
	Cabbage – <i>Pieris brassicae</i> or <i>P. rapae</i>	Major	(Blaakmeer et al., 1994)
	Cotton – earworm	Major	(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
	<i>Arabidopsis</i> – <i>Pieris rapae</i>		(Van Poecke et al., 2001)
δ -ionone	<i>Arabidopsis</i> – <i>Pieris rapae</i>		(Van Poecke et al., 2001)
Limonene	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cabbage – <i>Pieris brassicae</i> or <i>P. rapae</i>	Major	(Blaakmeer et al., 1994)
	Cotton – earworm		(McCall et al., 1994)
	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
Sabinene	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
1-8-cineol	Cabbage – <i>Pieris brassicae</i> regurigent or glucosidase		(Mattiacci et al., 1995)
	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
β -(E)- β -ocimene	Lima bean – Spidermite	N/A	(Takabayashi et al., 1991)
	Lima bean – Spider mite damaged leaves	N/A	(Takabayashi et al., 1991)
	Cotton – armyworm	Major	(Loughrin et al., 1994; Perezlachaud and Campan, 1995)
	Cotton – earworm	Major	(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Tobacco – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Cotton – <i>H. virescens</i> or <i>H. zea</i>	Major	(De Moraes et al., 1998)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	<i>Vicia faba</i> – <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
	Lima bean-T. Urticae		(De Boer et al., 2004)
	Lima bean – S. exigea	Major	(De Boer et al., 2004)
α Terpinene	Cotton-armyworm		(Perezlachaud and Campan, 1995)
γ Terpinene	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
limonene	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
Beta-Phellandrene	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
linalool	Maize – armyworm	Major	(Takabayashi et al., 1995)
	Maize – armyworm		(Turlings et al., 1991)
	Soybean – Soybean Looper	Major	(Ramachandran et al., 1991)
	Maize – Armyworm Regurigent	Major	(Turlings and Tumlinson, 1992)
	Maize – Armyworm Regurigent Systemic	Major	(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Cotton – armyworm		(Loughrin et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin	Major	(Alborn et al., 1997)
	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
	Tobacco – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	<i>Vicia faba</i> – <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
	Rice-brown leaf hopper	Major	(Lou et al., 2005)
	<i>Vicia faba</i> (bean)-Nezara viridula		(Colazza et al., 2004)
p-Cymene	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)
Alpha-Copaene	Cabbage – <i>P. brassicae</i> /P Rapae – Cotesia		(Geervliet et al., 1997)

Table 1.2. continued.

Volatile	System	Level	Ref
Trans-Sabinene Hydrate	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Alpha-Cubenene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Beta-Cubenene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Sesquiterpene			
[alpha]-trans-bergamotene,	Maize-beet Armyworm – Cotesia marginiventris		(Turlings et al., 1990)
	Maize – armyworm	Major	(Turlings et al., 1991)
	Maize – Armyworm Regurigent	Major	(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin		(Alborn et al., 1997)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
Longifolene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Beta-Elementene	Cabbage – <i>Pieris brassicae regurigent</i> or <i>glucosidase</i>		(Mattiacci et al., 1995)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
E-Beta-Caryophyllene	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaund and Campan, 1995)
	Cotton – earworm	Major	(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic	Major	(Rose et al., 1996)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Tobacco – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Cotton – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
	Mazie – <i>M.v. virgifera</i>		(Rasmann et al., 2005)
	Lima bean – <i>S. exigea</i>		(De Boer et al., 2004)
	Vicia faba (bean)- <i>Nezara viridula</i>		(Colazza et al., 2004)
α -guaiene	Cotton – earworm		(McCall et al., 1994)
Alpha-humulene	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaund and Campan, 1995; Pare and Tumlinson, 1997)
	Cotton – earworm	Major	(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Tobacco – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
γ -Murolene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Alpha-Murolene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
b-Selinene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
a-Selinene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Gamma-Bisabolene	Cotton – armyworm		(Loughrin et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
Germacrene-D	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
δ -guaiene	Cotton – earworm	Major	(McCall et al., 1994)
7-Cadinene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
j-Cadinene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
(E)-[beta]-farnesene,	Maize-beet Armyworm – Cotesia marginiventris		(Turlings et al., 1990; Pare and Tumlinson, 1997)
	Maize – armyworm	Major	(Turlings et al., 1991)
	Maize – Armyworm Regurigent	Major	(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Cotton – armyworm		(Loughrin et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin	Major	(Alborn et al., 1997)
	Cotton – <i>H. virescens</i> or <i>H. zea</i>	Major	(De Moraes et al., 1998)
	Maize – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	<i>Vicia faba</i> – <i>Acyrtosiphon pisum</i>		(Du et al., 1998)
	Potato-Myzus persicae		(Harmel et al., 2007)
(E,E)-alpha-farnesene	Cotton – armyworm		(Loughrin et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)

Table 1.2. continued.

Volatile	System	Level	Ref
	Tobacco – <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Cotton - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
Calamenene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Alpha-Calacorene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
Beta-Calacorene	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
(E)-nerolidol,	Maize-beet Armyworm - Cotesia marginiventris		(Turlings et al., 1990)
	Maize - armyworm	Major	(Turlings et al., 1991)
	Maize – Armyworm Regurigent		(Turlings and Tumlinson, 1992; Turlings et al., 1993)
Homoterpene			
(3E,7E)-4,8,12-dimethyl-1,3,7,11-tridecatetraene	Lima bean –Spider mite- <i>Phytoseiulus persimilis</i>	N/A	(Dicke et al., 1990)
	Maize – Armyworm Regurigent		(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Maize – JA or beta-glucosidase	Major	(Hopke et al., 1994)
	Cotton – armyworm		(Loughrin et al., 1994; Perezlachaoud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin		(Alborn et al., 1997)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Cotton - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Maize - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Lima bean-T. Urticae		(De Boer et al., 2004)
	Lima bean – S. exigea		(De Boer et al., 2004)
	Vicia faba (bean)-Nezara viridula		(Colazza et al., 2004)
DMNT	Lima bean –Spider mite- <i>Phytoseiulus persimilis</i>	N/A	(Dicke et al., 1990)
	Maize - armyworm		(Turlings et al., 1991)
	Maize – Armyworm Regurigent	Major	(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Maize – Armyworm Regurigent Systemic	Major	(Turlings and Tumlinson, 1992; Turlings et al., 1993)
	Maize – JA or beta-glucosidase	Major	(Hopke et al., 1994)
	Cotton – armyworm	Major	(Loughrin et al., 1994; Perezlachaoud and Campan, 1995)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – Beet Armyworm Systemic		(Rose et al., 1996)
	Maize – Volicitin	Major	(Alborn et al., 1997)
	Cabbage – P brassicae/P Rapae – Cotesia		(Geervliet et al., 1997)
	Cotton - <i>H. virescens</i> or <i>H. zea</i>	Major	(De Moraes et al., 1998)
	Maize - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Lima bean-T. Urticae		(De Boer et al., 2004)
	Lima bean – S. exigea		(De Boer et al., 2004)
	Vicia faba (bean)-Nezara viridula		(Colazza et al., 2004)
Indolic			
Indole	Maize-beet Armyworm - Cotesia marginiventris		(Turlings et al., 1990)
	Maize - armyworm	Major	(Turlings et al., 1991)
	Cotton – earworm		(McCall et al., 1994)
	Cotton – armyworm	Major	(Perezlachaoud and Campan, 1995)
	Maize – Volicitin	Major	(Alborn et al., 1997)
	Maize - <i>H. virescens</i> or <i>H. zea</i>		(De Moraes et al., 1998)
	Lima bean – S. exigea		(De Boer et al., 2004)
Phenolic			
methyl salicylate	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
	Strawberry- <i>P. pallidus</i> or <i>Galerucella tenella</i>		(Himanen et al., 2005)
	Lima bean – S. exigea		(De Boer et al., 2004)
	Lima bean-T. Urticae		(De Boer et al., 2004)

Table 1.2. continued.

Volatile	System	Level	Ref
	Lima bean – <i>S. exigea</i>		(De Boer et al., 2004)
	<i>Arabidopsis - Pieris rapae</i>		(Van Poecke et al., 2001)
benzyl alcohol,	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
Others			
3-hexan-1-yl benzoate	Pear tree- Psylla. Pyricola-Anthocoris nemorum		(Scutareanu et al., 1997)
2- and 3-methylbutanal- <i>o</i> -methyl	Lima bean – <i>S. exigea</i>		(De Boer et al., 2004)
oxime <i>p</i> -mentha-1,3,8-triene	Lima bean – <i>S. exigea</i>		(De Boer et al., 2004)
3-Methylbutanenitrile,	Cabbage – <i>P brassicae/P Rapae – Cotesia</i>		(Geervliet et al., 1997)
Benzonitrile	Cabbage – <i>P brassicae/P Rapae – Cotesia</i>		(Geervliet et al., 1997)
Dimethyl disulfide	Cabbage – <i>P brassicae/P Rapae – Cotesia</i>		(Geervliet et al., 1997)
	<i>Arabidopsis - Pieris rapae</i>		(Van Poecke et al., 2001)
Methyl(iso)thiocyanate	Cabbage – <i>P brassicae/P Rapae – Cotesia</i>		(Geervliet et al., 1997)
5-(methylthio)-pentanenitrile,	<i>Arabidopsis Pieris rapae</i>		<i>Arabidopsis</i>
2-butanone	Lima bean-T. Urticae		(De Boer et al., 2004)
6,7-dithiooctanenitrile	<i>Arabidopsis Pieris rapae</i>		<i>Arabidopsis</i>
guaiacol	Soybean- soybean looper	Major	(Ramachandran et al., 1991)

TABLE 1.3. In Vitro Volatile Attraction Experiments. Both field experiments and Y tube assay are included.

Volatile	Predator	Comments	Ref
Linalool	<i>Phytoseiulus persimilis</i>		(Dicke et al., 1990)
	<i>Microplitis demolitor</i>	Repelling	(Ramachandran et al., 1991)
E- β -Ocimene	<i>Phytoseiulus persimilis</i>		(Dicke et al., 1990)
(E,E)- α -Farnesene	<i>Anthocoris nemorum</i>		(Scutareanu et al., 1997)
6-methyl-5-hepten-2-one	<i>Aphidius ervi</i>		(Du et al., 1998)
linalool	<i>Aphidius ervi</i>		(Du et al., 1998)
(Z)-3-hexen-1-yl acetate	<i>Aphidius ervi</i>		(Du et al., 1998)
(E)-beta-ocimene	<i>Aphidius ervi</i>		(Du et al., 1998)
(Z)-3-hexen-1-ol	<i>Aphidius ervi</i>		(Du et al., 1998)
	<i>Micro-Hymenoptera</i>	field	(James, 2005)
(E)-beta-farnesene	<i>Aphidius ervi</i>		(Du et al., 1998)
DMNT	<i>Phytoseiulus persimilis</i>		(Dicke et al., 1990)
Methyl salicylate	<i>Phytoseiulus persimilis</i>		(Dicke et al., 1990)
	<i>Anthocoris nemorum</i>		(Scutareanu et al., 1997)
	<i>Micro-Hymenoptera</i>	field	(James, 2005)
Indole	<i>Micro-Hymenoptera</i>	field	(James, 2005)
3-Octonone	<i>Microplitis demolitor</i>		(Ramachandran et al., 1991)
Borneol	<i>Cyzenisis albicans</i>		(Roland et al., 1995)
guaiacol	<i>Microplitis demolitor</i>		(Ramachandran et al., 1991)

Chapter II. Molecular Basis of Volatile-mediated Indirect Defense against Insects in Rice

Adapted from:

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Abstract

Many plant species respond to insect herbivory with an elevated emission of volatile organic compounds. These volatiles can attract natural enemies of the feeding insects and therefore function as an indirect plant defense. Here we report on investigation of molecular basis of indirect defense in rice using an integrated genomic approach. Rice plants damaged by fall armyworm (*Spodoptera frugiperda*, FAW) larvae were found to be highly attractive to females of parasitoid *Cotesia marginiventris*. Rice volatiles potentially responsible for parasitoid attraction were determined to be a complex mixture of compounds including terpenes, methyl salicylate, indole and fatty-acid derived metabolites. Candidate genes for making FAW-induced rice volatiles were identified using microarray experiments. Three FAW-induced rice terpene synthase (*TPS*) genes, Os02g02930, Os08g07100 and Os08g04500, were biochemically characterized. Os02g02930 was determined to encode a monoterpene synthase making a single product *S*-linalool. Both Os08g07100 and Os08g04500 were determined to encode sesquiterpene synthase with each producing multiple products. These three enzymes were responsible for virtually all terpenes released from FAW-damaged rice plants. The *in silico* promoter analysis identified different *cis*-elements with varying frequencies of occurrence in promoters of Os02g02930, Os08g07100 and Os08g04500, suggesting that regulation of production of insect-induced terpenes involves different mechanisms. In addition to *TPS* genes, genes in upstream terpenoid pathways were also found to be up-regulated by FAW feeding, indicating that regulation

of production of herbivore-induced terpenoids occurs at not only the terpene synthase level but also the pathway level.

Key words: indirect defense, volatiles, terpene, terpene synthase, rice, genomics.

Introduction

Nearly half of 800,000 existing insect species are herbivores (Schoonhoven, 2005). The earth is still green, because plants have evolved various mechanisms to defend themselves. Plants may use toxic metabolites to directly defend themselves. They may also defend themselves indirectly by enhancing the effectiveness of natural enemies of the herbivores. For instance, many plant species release an elevated level of volatile organic compounds after herbivory. These volatiles can serve as cues to attract predators or parasitoids, the natural enemy of the herbivores (Turlings et al., 1990; De Moraes et al., 1998). Such plant volatile-mediated recruitment of natural enemies of the herbivores can have a fitness benefit to the host plant by increasing selection pressure on the herbivores. It is therefore believed to often function as an indirect defense mechanism (Turlings et al., 1995; Takabayashi and Dicke, 1996).

Volatiles emitted from insect-damaged plants are often a complex mixture of small molecular weight secondary metabolites. For instance, *Heliothis virescens*-damaged tobacco plants emit more than 15 volatile compounds that include terpenes, green leaf volatiles and shikimic acid-derived products (De Moraes et al., 2001). The high number of compounds in insect-induced plant volatiles has made it difficult to demonstrate the role of individual volatiles in indirect defense. Some studies using synthetic compounds in behavioral assays showed that specific plant volatiles, such as methyl salicylate and monoterpene linalool, are active in attracting parasitoids (Dicke et al., 1990). Other studies,

however, suggested that mixtures of volatiles as a whole constitute the active signal (Turlings et al., 1991). Recently, genetic engineering was used to manipulate herbivory-induced plant volatiles for understanding the relevance of individual components of volatile mixtures in indirect defense. By over-expressing terpene synthase (*TPS*) genes in *Arabidopsis*, constitutive emission of novel terpenes in *Arabidopsis* plants were shown to attract predators (Kappers et al., 2005) and parasitoids (Schnee et al., 2006), demonstrating that the role of individual volatiles in indirect defense can be dissected using genetic engineering. Such information will be more biologically and ecologically relevant if manipulation of volatile production is conducted with the host plant (Degenhardt *et al.*, 2003).

A paucity of information on the specific role of individual compounds in indirect defense is partly due to our poor understanding of the biosynthesis of insect-induced volatiles. Some progress has been made in this area lately. A number of genes for producing insect-induced volatiles has been isolated and characterized from maize (Degenhardt and Gershenzon, 2000; Shen et al., 2000; Schnee et al., 2002; Lou et al., 2006), lotus (Arimura et al., 2004), tomato (van Schie et al., 2007) and *Arabidopsis* (Chen *et al.*, 2003a). Nonetheless, the number of genes responsible for biosynthesis of routinely more than ten volatiles emitted from insect-damaged plants in most species is unknown. The lack of knowledge about specific genes and enzymes for making insect-induced plant volatiles has also hindered our understanding of other important aspects of

indirect plant defense. For instance, we know little about how production of insect-induced plant volatiles is regulated at the molecular level. We also know little about mechanisms that have driven the evolution of the trait of indirect defense. Studying biosynthesis of insect-induced plant volatiles at the molecular level is therefore an important area of indirect defense that needs much more emphasis (Kessler and Baldwin, 2002; D'Alessandro and Turlings, 2006; Dudareva et al., 2006).

Indirect defense has been observed in more than 20 plant species (Turlings, 2004), among which are important agricultural crops such as maize (Turlings et al., 1990; Turlings et al., 1991; Turlings et al., 1998; Hoballah et al., 2002), tomato (Kant et al., 2004) and tobacco (De Moraes et al., 1998), and the model plant *Arabidopsis* (Van Poecke et al., 2001). We have undertaken a project to study the molecular basis of indirect defense in rice. There are a number of reasons why rice is chosen as model. First, the rice genome has been fully sequenced (International_Rice_Genome_Sequencing_Project, 2005), which makes it possible to employ various genomic approaches to study indirect defense. Second, there are numerous genetic resources available for this species. For example, various mutant rice lines have been or are being generated (Hirochika et al., 2004). Third, a better understanding of the molecular basis of indirect defense in rice will facilitate cross-species studies. For example, comparative analysis of indirect defense in rice and maize will provide novel insights into the evolution of this important trait in monocots.

In this report, we first show that lepidopteran larvae-damaged rice plants significantly attract parasitic wasps. The volatiles potentially responsible for parasitoid attraction were identified using headspace analysis. We then conducted microarray experiments to identify candidate genes for producing insect-induced volatiles. Next, we provide biochemical evidence that three *TPS* genes are responsible for virtually all volatile terpenes emitted from insect-damaged rice plants. Finally, we present information on the mechanisms involved in regulating production of insect-induced volatile terpenes.

Material and Methods

Plants, insects and plant treatments

Rice (*Oryza sativa* ssp. *Japonica* cv. *Nipponbare*) seeds were dehulled and germinated at 30 °C in the dark for five days. The seedlings were planted with eight plants per 60mL fisher glass jars and grown at 26 °C with 14 hours of light for two weeks. Fall armyworm (*Spodoptera frugiperda*) was used as the herbivore model. FAW eggs were incubated on moist filter paper, and emerged FAW larvae were reared on an artificial diet. Second-instar FAW were used for herbivore treatment. Two larvae were placed on the leaves of a single two-week-old rice seedling at 18:00 PM in the afternoon. After 18 hours, about 20% of leaf area was consumed. Insects were removed and the rice plants were subject to tissue collection for RNA extraction, volatile collection, or Y-tube olfactometer bioassay.

A generalist parasitic wasp *Cotesia marginiventris*, the natural enemy of FAW, was used as the carnivore model. Eggs of *C. marginiventris* were obtained from the USDA-ARS Southern Grain Insects Research Laboratory (Tifton, GA, USA). To rear parasitoids, 25 young FAW caterpillars (3-4 days old) were offered to a single mated female (4-7 days old) for 3 hours in a plastic box. The caterpillars were further reared on an artificial diet in an incubator (24°C and 16h light:8h dark) until cocoon formation. Cocoons were kept in Petri dish until adult emergence. Emerging adults were sexed and kept in cages (30 cm x 30 cm x 40 cm), with honey drops as a food source. The cages were kept in the lab under

ambient light and temperature conditions. Four-day-old naïve female wasps were used for Y-tube olfactometer bioassay.

Two-choice Y-tube olfactometer bioassay

To determine whether FAW-damaged rice plants preferentially attract parasitic wasps, we performed Y-tube olfactometer bioassay. The Y-tube olfactometer was purchased from Analytic Research Systems (Gainesville, FL, USA). The system consists of a Y-shaped glass body, a pair of odor source adapters with two glass chambers, an insect inlet adapter, and a regulated air delivery system. The dimensions of the olfactometer are 2.8 cm diameter, 15.25 cm of main body length, and 8.89 cm of branch length. The air flow was maintained at 0.8 L/min. Inexperienced female wasps (4 days old) were released individually at the base of the olfactometer and observed for five minutes. If a wasp did not make a choice during this period, it was removed from the olfactometer and recorded as a “no choice”. Wasps that flew or walked to the end of one of the arms and stayed there for at least 10 seconds were recorded as having made a choice for the odor offered through that arm. After 10 individuals were tested, treatment and control arms were alternated to avoid directional bias. The apparatus was washed with acetone and air-dried after each trail. The bioassays were performed between 12:00PM and 3:00PM.

Volatile analysis

Volatiles emitted from FAW-damaged rice plants and control rice plants were collected in an open headspace sampling system (Analytical Research Systems, Gainesville, FL, USA). Eight plants grown in a single glass jar wrapped with aluminum foil were placed in glass chamber of 3 inch in diameter and 10 inch in height that consisted of a removable O-ring snap lid with an air outlet port. Charcoal-purified air entered the chamber at a flow rate of 0.8 L/min from the top through a Teflon hose. Volatiles were collected for 4 h by pumping air from the chamber through a SuperQ volatile collection trap (Analytical Research Systems, Gainesville, FL, USA). Volatiles were eluted with 40 μ L of CH_2Cl_2 , and 1-Octanol was added as an internal standard as previously described (Mitchell and McCashin, 1994).

Samples from volatile collections were analyzed on a Shimadzu 17A gas chromatograph coupled to a Shimadzu QP5050A quadrupole mass selective detector. Separation was performed on a DB5 column of 30 m x 0.25 mm i.d. x 0.25 μ m thickness. Helium was the carrier gas (flow rate of 5 mL/min), a splitless injection (injection volume of 5 μ L) was used, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 240°C was applied. The identities of compounds were determined by comparison of retention times and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology and Wiley libraries (Agilent Technologies, Palo Alto, CA, USA).

RNA isolation

Total RNA was isolated from appropriate rice tissues using Plant RNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA contamination was removed with an on-column DNase (Qiagen, Hilden, Germany) treatment. Isolated total RNA was used for real-time PCR analysis, gene cloning, and microarray experiments.

Microarray experimentation

The NSF rice half genome oligonucleotide array (Version 2.0) provided by UC Davis microarray core facility was used for global gene expression profiling. Messenger RNA was isolated from total RNA using Oligotex mRNA kit (Qiagen, Hilden, Germany). One microgram of mRNA was labeled with Superscript III direct Labeling Kit (Invitrogen, Carlsbad, CA, USA) according to the instruction of the manufacturer. The purified probes were mixed and hybridized with the long-oligo microarrays using the Microarray Hybridization Kit (Corning, Corning, NY, USA) according to the manufacturer's instruction and the protocol provided by UC, Davis (<http://www.ricearray.org>). Reverse labeling experiments were included to eliminate dye-specific bias. For each sample set of FAW-treated rice versus control, the treated mRNA was first labeled with Cy5 and the control with Cy3. In the reverse experiment, the labeling dyes were swapped. The labeling reactions and dye swapped microarray hybridizations were performed in parallel. Considering the reverse

labeling experiments, a total of three biological replicates and two technical replicates are included.

After hybridization, the microarray slides were washed and scanned in GenePix 4000 scanner (Axon Instrument, Union City, CA, USA), and the image was processed by GenePix Pro software (Axon Instrument, Union City, CA, USA). The microarray gpr files obtained were analyzed with R-based open source software Bioconductor (<http://www.bioconductor.org>), where local background subtraction and Lowess normalization were performed for each microarray slide. Linear models from the limma package of Bioconductor were applied to derive a p value and average of logarithm 2-based ratio across six slides. Changes in gene expression pattern were considered statistically significant at $p < 0.01^*$. A ratio cutoff of 2 and degree of freedom higher or equal to three were included as quality controls

Quantitative real-time PCR

Quantitative real-time PCR experiments were carried out as previously described (Yang et al., 2006). The primers for target genes were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA) and the primer sequences were as shown in Table 2.4.

Full length cDNA cloning

Full length cDNAs of rice *TPS* genes were cloned from FAW-damaged rice leaves using RT-PCR as previously described (Chen *et al.*, 2003b). The primers used were 5'-ATGGTTTGCCACGTCTTCTCG-3' (forward) and 5'-CGCCATTATGCATGGACGA-3' (reverse) for Os02g02930, 5'-ATGTCATCGACACCTGCAGCTAA-3' (forward) and 5'-TTAAATGCTATATGGCTCAACGTAAA-3' (reverse) for Os08g07100, 5'-ATGTCGTCGCCACCTGCAGC-3' (forward) and 5'-TCTTGCCACGATTTTTGGT-3' (reverse) for Os08g07080, and 5'-ATGGCAACCTCTGTTCCGAGTGTACT-3' (forward) and 5'-TTAAACAGAGAGGATGTAGATGGAGTGT-3' (reverse) for Os08g04500. In addition, a forward primer 5'-ATGGCCACCGTCGACCACCT-3' and the same reverse primer were used to amplify the truncated form of Os02g02930.

Terpene synthase biochemical assay

Protein expression in *E. coli* and terpene synthase assay were performed as previously described (Köllner *et al.* 2004).

Promoter Analysis

Conserved *cis*-element sequences were searched against sequences for 1.5kb upstream of Os02g02930, Os08g04500, and Os08g07100, respectively. The frequency of the *cis*-elements was recorded and compared with the expected

frequency calculated based on the chances of randomly appearing element
sequence in any 1.5 kb sequence.

Results

Fall armyworm-damaged rice plants significantly attract Cotesia marginiventris

During its life cycle, a rice plant may encounter many different types of insects (Litsinger et al., 2005). These include brown plant hopper, water weevil, fall armyworm (*Spodoptera frugiperda*, FAW), and rice stink bug, to name a few. Brown planthopper is a sap-sucking insect. It has been extensively studied for rice-insect interactions (Zhang et al., 2004; Yang et al., 2005; Yuan et al., 2005), including studies of indirect defense (Lou et al., 2005; Lou et al., 2005; Lou et al., 2006). In this study, we chose FAW as the herbivore model because it is a generalist chewing insect and a natural pest of rice. The response of *Cotesia marginiventris*, a generalist parasitic wasp and natural enemy of FAW (Loke and Ashley, 1984), to FAW-induced volatiles emitted from rice plants was tested using a Y-tube two choice olfactometer bioassay. Naive female parasitic wasps were given a choice between the odor of rice plants damaged by FAW and the odor of untreated control rice plants. Of all wasps tested, 89.7% walked to the arm of the Y-tube that carried the odor of FAW-damaged rice plants. The remaining 10.3% either did not make a choice or chose the arm that carried the odor of untreated rice plants (Figure 2.1). These assay results indicate that *C. marginiventris* could clearly discriminate between the odor from FAW-treated rice plants and that from control rice plants.

Rice plants emit a blend of volatiles when damaged by FAW larvae

Volatile constituents emitted from rice plants were sampled by passing purified air over FAW-damaged rice plants or control plants through a passive chemical filter. After desorption of the filter with organic solvent, volatile constituents were analyzed using GC-MS. As shown in Figure 2.2a, control rice plants emitted only trace amounts of volatiles, which include limonene and nonanal. The volatile profile of FAW-damaged rice plants was dramatically different from that of control plants. While some volatiles such as limonene and nonanal showed an elevated level of emission, a total of 28 novel volatiles were detected (Figure 2.2). Similar to volatiles emitted from other plant species after insect herbivory, insect-induced volatiles from rice can be categorized into three major groups: terpenes, shikimic acid-derived metabolites and fatty acid-derived metabolites. The terpene group contains two monoterpenes *S*-linalool and limonene and 19 sesquiterpenes. *S*-linalool is the most abundant compound in FAW-induced volatiles. Zingiberene is the most abundant sesquiterpene. Two compounds are derived from the shikimate pathway: methyl salicylate and indole. Decanal is a representative of fatty acid-derived volatiles.

Rice genes induced by FAW infestation revealed by microarray analysis

To identify candidate genes for the production of FAW-induced volatiles, we performed microarray experiments to analyze gene expression changes in FAW-damaged rice plants vs. control plants. Three biological samples and two technical samples were analyzed. Quality control analysis showed that dye-

dependent effects were effectively removed by performing Lowess normalization (Figure 2.3a). In addition, the genes displayed a normal distribution based on their expression levels (Figure 2.3b). Using two-fold change as cut-off value, 196 rice genes were found to be significantly up-regulated by FAW feeding (Table 2.3). In this table and in the reminder of this paper, gene identities were based on the TIGR rice genome pseudomolecules (<http://www.tigr.org/tdb/e2k1/osa1/>) except that “LOC_” was omitted. Abundance of genes in different functional groups is shown in Figure 2.4.

Genes encoding enzymes compose the largest group of up-regulated genes with known or putative functions (Figure 2.4). More than half of the genes in the metabolism category appear to be involved in secondary metabolism. These include *TPS* genes, P450 genes, lipoxygenase genes, methyltransferase genes and *BAHD* acyltransferase genes (Table 2.1). Many secondary metabolites produced by the action of the above-mentioned enzymes have roles in plant defense against insects and pathogens (Reymond et al., 2004; D'Auria, 2006; Qi et al., 2006; Schnee et al., 2006; Vellosillo et al., 2007). Transcription factors are the second largest group of induced genes that are annotated with known function (Figure 2.3). Up-regulation of transcription factors represents a general shift in transcriptional regulation in response to insect damage (Reymond et al., 2004). Notably, three WRKY transcription factor genes were up-regulated. Some WRKY genes have been shown to be involved in plant defense (Wang et al., 2006). Protein phosphorylation plays an important role in many plant signal

transduction pathways involved in plant defense responses (Mishra et al., 2006; Zheng et al., 2006). Eleven genes that contain a kinase domain or LRR repeat were induced by FAW herbivory. In addition, two phosphatase genes were found to be up-regulated by FAW herbivory. Membrane transport is important for many physiological processes (Higgins, 1995). Several genes encoding sugar transporters and amino acid transporters were induced by herbivory. Additionally, three ABC transporter genes were induced. The active involvement of ABC transporters in plant defense has been previously described (Jasinski et al., 2001; Xiong et al., 2001; Fleissner et al., 2002; Sasabe et al., 2002; Campbell et al., 2003). A number of genes encoding proteins with functions in insect and pathogen resistance were also induced by FAW. Four Bowman-Birk serine protease inhibitor genes belong to this group. Twenty six genes with putative functions that do not belong to any of the above described functional categories were grouped together (Figure 2.4). Many of these genes, such as those encoding later embryogenesis abundant proteins and senescence-associated proteins (Table 2.3), have roles in general stress response.

Candidate genes for making FAW-induced rice volatiles

Volatile terpenes are the most abundant in FAW-induced volatiles in rice (Figure 2.2). In plants, terpenes are synthesized by the action of *TPS*, which converts geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), to monoterpenes, sesquiterpenes and diterpenes, respectively (Tholl, 2006). A large number of *TPS* genes have been

cloned and characterized from various plant species (Chen et al., 2003; Tholl, 2006). They are related at the protein sequence level. Phylogenetic analysis suggests that plant *TPS* genes share a common origin (Bohlmann et al., 1998; Aubourg et al., 2002). Ten rice *TPS* genes were found to be significantly up-regulated by FAW feeding (Table 2.1). Os08g04500 is another rice *TPS* gene. It showed 1.5 fold induction by FAW herbivory in the microarray analysis. Because this gene is closely related to Os08g07100 and Os08g07080 (see Figure 2.7), we included it in our later analysis.

Methyl salicylate and indole are synthesized from the shikimate pathway. Methyl salicylate is a methyl ester of salicylic acid synthesized in plants from salicylic acid by the action of salicylic acid methyltransferase (SAMT) (Zubieta et al., 2003). SAMTs isolated from a number of plant species (Ross et al., 1999; Negre et al., 2002; Pott et al., 2002; Chen et al., 2003) all belong to the protein family called SABATH (Chen et al., 2003). Two rice SABATH genes, Os02g48770 and Os05g01140 were found to be significantly up-regulated by FAW herbivory (Table 2.1). Our preliminary study showed that Os02g48770 had SAMT activity (N. Zhao and F. Chen, unpublished). Indole is a product of the tryptophan branch of the shikimate pathway (Hansen and Halkier, 2005; D'Alessandro et al., 2006). Indole-3-glycerol phosphate lyase and anthranilate phosphoribosyl transferase are two key enzymes involved in indole biosynthesis (Hansen and Halkier, 2005). Genes encoding these two enzymes, Os03g58300

and Os03g03450 respectively, were found to be significantly up-regulated by FAW feeding (Table 2.1).

Fatty-acid derived products are generally synthesized from fatty acids such as α -linolenic acid and linoleic acid via their respective hydroperoxides (Noordermeer et al., 2001). The enzymes catalyzing fatty acid oxidation are lipoxygenases (Feussner and Wasternack, 2002; Kessler et al., 2004), and three rice lipoxygenase genes Os12g37320, Os12g37260 and Os03g52860 were found to be significantly up-regulated by FAW herbivory (Table 2.1).

Expression validation of FAW-induced rice TPS genes

Relevance of some individual terpenes (e.g., linalool) or a group of terpenes (e.g., sesquiterpene products of maize TPS10) in indirect defense has been previously demonstrated (Kessler and Baldwin, 2001; Schnee et al., 2006). Because terpenes are the most abundant among FAW-induced volatiles in rice (Figure 2.2), we attempted to identify all rice *TPS* genes involved in making FAW-induced volatile terpenes. Microarray experiments showed that ten *TPS* genes were up-regulated by FAW feeding (Table 2.1). False positive results could be produced in microarray experiments due to cross-hybridization (Xu et al., 2001). To validate the induction of the ten rice *TPS* genes plus Os08g04500, we performed real time-PCR experiments using gene-specific primers. Up-regulation for seven of the 11 *TPS* genes, including Os02g02930, Os03g22634,

Os04g27190, Os04g27670, Os08g07110, Os08g04500, Os08g07080, was confirmed (Figure 2.5).

Biochemical characterization of three rice TPS genes

Monoterpenes and diterpenes are synthesized in plastids and sesquiterpenes are synthesized in the cytosol (Tholl, 2006). Thus, monoterpene- and diterpene synthases contain a transit peptide while sesquiterpene synthases do not (Bohlmann et al., 1998; Aubourg et al., 2002; Tholl, 2006). Protein sequence analysis using TargetP revealed that proteins encoded by three of the seven *TPS* genes, Os08g07100, Os08g04500 and Os08g07080, do not contain a transit peptide (Figure 2.6). This suggests that these genes code for sesquiterpene synthase. Os08g07080 was determined to be a pseudogene (Figure 2.8). The remaining four *TPS* proteins contain a transit peptide. They are therefore either monoterpene synthase or diterpene synthase. From phylogenetic analysis, Os08g04500 and Os08g07100 were found to be most closely related to the maize sesquiterpene synthase TPS10 (Figure 2.7). Os02g02930 was clustered with a group of monoterpene synthases that include linalool synthase from *Arabidopsis* (Figure 2.7). In this paper, we chose to study the biochemical activity of Os02g02930, Os08g07100 and Os08g04500.

Full-length cDNAs of Os02g02930, Os08g07100 and Os08g04500 were cloned from FAW-damaged rice leaves using RT-PCR. *E. coli*-expressed recombinant Os02g02930, Os08g07100 and Os08g04500 proteins were assayed for TPS

activity. Previous analysis has shown that the pseudomature protein of a monoterpene synthase (i.e., without transit peptide) is more active than the protein encoded by full-length cDNA (Chen et al., 2003). We made two constructs with and without the N-terminal sequence containing the transit peptide of Os02g02930 (Figure 2.6). Both full-length and truncated form of Os02g02930 catalyzed the formation of a single product *S*-linalool using GPP as substrate (Figure 2.9), with the truncated form more active.

E. coli-expressed recombinant Os08g07100 catalyzed the formation of 14 sesquiterpenes using FPP as substrate, with zingiberene and β -sesquiphellandrene as the major products (Figure 2.10a). Recombinant Os08g04500 catalyzed the formation of five sesquiterpenes using FPP as substrate, with (*E*)- β -caryophyllene as the major product (Figure 2.10b).

The in silico analysis of promoters of Os02g02930, Os08g07100 and Os08g04500

One of the key determinants of gene expression is the type and number of *cis*-elements present in the promoter region of a gene. As Os02g02930, Os08g07100 and Os08g04500 all are involved in making FAW-induced volatiles in rice, we would like to understand whether they are coordinately regulated. To gain insight into this question, we performed *in silico* promoter analysis of the three genes for occurrence of five previously described stress-related *cis*-elements: W-box, H-box, G-box, TGA and DRE element (Lam and

Lam, 1995; Faktor et al., 1997; Stockinger et al., 1997; Eulgem et al., 1999; Eulgem et al., 2000; Niggeweg et al., 2000; Rocher et al., 2005). All five *cis*-elements except a G-box were found to be present in the promoter of Os02g02930 (Table 2.2) with H-box, DRE and TGA being overrepresented. In contrast, the majority of these *cis*-elements are underrepresented in the promoters of Os08g07100 and Os08g04500 (Table 2.2).

Expression analysis of selected terpenoid pathway genes

In plants, the precursors for terpenes, GPP, FPP and GGPP, are synthesized through two separate biochemical pathways (Tholl, 2006): a cytosol-localized mevalonate pathway leading to the formation of FPP and a plastid-localized non-mevalonate pathway leading to the formation of GPP and GGPP. In the mevalonate pathway, 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase (HMGR) catalyzes the first committed step by converting HMG-CoA to mevalonic acid (Learned and Fink, 1989). In the non-mevalonate pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) catalyzes the first committed step by converting D-glyceraldehyde-3-phosphate and pyruvate to 1-deoxy-D-xylulose-5-phosphate (Eisenreich et al., 2001). Microarray analysis showed that one putative *HMGR* gene, Os05g02990, was up-regulated by FAW herbivory by 1.5 fold and one putative *DXPS* gene, Os07g09190, was up-regulated by 5.3 fold. In addition to the above two genes, other terpenoid pathway genes that were up-regulated by FAW herbivory include: a putative 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene Os01g01710 (*DXR*, 2.1 fold) of the non-

mevalonate pathway and a putative isopentenyl diphosphate isomerase gene Os02g55030 (*IPPS*, 1.4 fold) and a putative FPP synthase gene Os01g50760 (*FPPS*, 1.8 fold) of the mevalonate pathway: To validate expression changes of these pathway genes, we performed real-time PCR analysis. Induction by FAW feeding for all selected genes was verified (Figure 2.11).

Linalool applied rice plants significantly attract Cotesia marginiventris

The response of *Cotesia marginiventris* toward linalool was tested using a Y-tube two choice olfactometer bioassay (Figure 2.12). Naive female parasitic wasps were given a choice between rice plants applied with linalool and control plants. The assay results indicate that *C. marginiventris* could clearly discriminate between the odor of linalool applied plants and control plants.

Discussion

Studying the molecular basis of rice indirect defense using an integrated genomic approach

Indirect plant defense against insects is a complex trait due to its association with a high number of plant volatiles. FAW-damaged rice plants emitted more than 30 volatile compounds derived from secondary metabolic pathways (Figure 2.2). Genes for production of secondary metabolites in plants often belong to large gene families. Individual members of such families are often highly similar to each other at the protein sequence level but have distinct substrate specificities or product variation (Pichersky and Gang, 2000). It is therefore a challenging task to identify specific genes for production of specific insect-induced volatiles. Most genes for producing insect-induced volatiles that have been isolated so far are *TPS* genes. Most of these *TPS* genes were isolated individually based on their homology to known *TPS* genes followed by biochemical characterization (Degenhardt and Gershenzon, 2000, Schnee *et al.*, 2002, Van Schie *et al.*, 2007). In this report, we employ an integrated genomic approach that combines metabolic profiling, expression profiling and biochemical analysis to systematically identify candidate genes for production of insect-induced volatiles in rice.

Such an analysis relies first on accurate metabolic profiling. Quality and relative quantity of rice volatiles induced by herbivory were measured using headspace collection coupled with GC-MS analysis. Thirty one volatiles were detected

from FAW-damaged rice plants, twenty of them were terpenes (2.2). Our preliminary analysis showed that the rice genome contains more than 50 *TPS* genes (J. Yuan and F. Chen, unpublished). To determine which *TPS* genes are responsible for production of the 20 volatile terpenes, a brute-force approach would be to individually characterize at least 50 *TPS* genes. Our approach was to narrow down the number of candidate genes by correlating volatile production with gene expression changes. With the help of metabolic profiling, microarray experiments were conducted using tissues collected from the plants that showed abundant volatile emission. A comprehensive catalog of candidate genes for production of insect-induced volatiles in rice was identified through this integrated genomic approach (Table 2.1). Such an approach has been successfully employed in elucidating the biosynthesis of a large number of plant secondary metabolites (Tholl et al., 2007). It will be equally useful in studying the molecular basis of indirect defense in other plant species as genomic resources for those plants are being built up.

Three TPS enzymes produce virtually all terpenes emitted by FAW-damaged rice plants

Our integrated genomic analysis led to the identification of seven *TPS* genes potentially involved in production of 20 volatile terpenes (Figure 2.5). Which genes are indeed responsible for production of which terpenes can not be determined simply based on the sequence relatedness of the *TPS* genes to known *TPS* genes. This is because many characterized TPSs catalyze the formation of

multiple products from a single substrate (Steele et al., 1998; Chen et al., 2003; Kollner et al., 2004, 2004). In addition, different TPS enzymes may synthesize partially overlapping mixtures of compounds (Chen et al., 2003).

The *in vitro* assays and product analyses of recombinant enzymes encoded by the two FAW-induced genes Os08g07100 and Os08g04500 indicated that virtually all sesquiterpene volatiles emitted from FAW-damaged rice plants are produced by these two TPS enzymes. Os08g07100 makes 14 sesquiterpenes and Os08g04500 makes five (Figure 2.10). While the products of Os08g07100 and Os08g04500 do not overlap, all of them were present in FAW-induced rice volatiles (Figure 2.2). A recent paper reported on cloning and biochemical characterization of several sesquiterpene synthase genes in rice (Cheng et al., 2007). In that report, Os08g04500 was shown to be a sesquiterpene synthase producing 14 products using FPP as substrate, three of which was detected in sesquiterpene profiles of rice plants. Both our study and the study of Cheng et al. (2007) showed that Os08g04500 produces three major sesquiterpenes: β -elemene, (*E*)- β -caryophyllene and α -humulene. In the same paper, Os08g07100 was reported to have no activity with FPP (Cheng et al., 2007). This discrepancy may be due to different rice cultivars used in their and our studies. In our analysis, the relative portions of individual sesquiterpenes in the product profile of Os08g07100 and Os08g04500 match well with those in FAW-induced rice volatiles (Figures 2.2 and 2.10). Although genetic evidence is still needed, our

results strongly suggest that Os08g07100 and Os08g04500 are the two major, if not the only, TPSs that are responsible for FAW-induced sesquiterpenes.

Os02g02930 was determined to be an *S*-linalool synthase (Figure 2.9). Whether it is the only rice TPS responsible for production of *S*-linalool is not yet clear.

As described in the previous section, FAW feeding induced expression of seven *TPS* genes (Figure 2.5). Besides Os08g07100, Os08g04500, Os08g07080 and

Os02g02930, Os03g22634, Os04g27190 and Os04g27670 were also up-

regulated by FAW herbivory. Whether these genes have *S*-linalool synthase

activity remains to be determined. It should also be noted that the rice

oligonucleotide array we used in the microarray study did not contain all rice

putative *TPS* genes. Whether the rice *TPS* genes not present on the

oligonucleotide array contribute to the FAW-induced terpenes also remains to be determined.

Regulatory mechanisms for production of insect-induced volatile terpenes

How the biosynthesis of a complex mixture of plant volatiles induced by insects

is regulated is an intriguing question. If multiple insect-induced plant volatiles

contribute to indirect defense as previously suggested, it will be reasonable to

speculate that production of these volatiles has been co-selected during the

evolutionary process. Then production of some of these volatiles may be

coordinately regulated. Identification of multiple key volatile producing genes in

rice permits novel opportunities to test this hypothesis. In this paper, we

performed *in silico* analysis of promoters of three *TPS* genes Os02g02930, Os08g07100 and Os08g04500 for the presence/absence of five *cis*-elements that have been previously characterized and shown to be involved in stress response. Interestingly, the majority of these *cis*-elements were present in the promoter of Os02g02930 but underrepresented in the promoters of Os08g07100 and Os08g04500. This suggests that regulation of insect-induced monoterpene production may be somewhat independent from regulation of insect-induced sesquiterpene production. While certain *cis*-elements such as W-box, DRE elements and TGA are present in the promoter of Os08g04500, none of them is present in the promoter of Os08g07100. On the contrary, the H-box is present in the promoter of Os08g07100 but not in the promoter of Os08g04500. This observation implies that both common and distinct mechanisms are involved in regulating the expression of the two sesquiterpene synthase genes.

Terpenes are synthesized through complex biochemical pathways, and therefore could be regulated at multiple steps. A number of studies have suggested that terpene production is regulated at the pathway level. For example, snapdragon flowers emit the monoterpene myrcene as a component of its floral scent (Dudareva et al., 2003). The emission of myrcene was correlated with the induced expression of both myrcene synthase gene and the key pathway genes including *DXPS* and *DXR* (Dudareva et al., 2005). Our analysis in rice provides strong evidence that regulation of insect-induced plant volatiles also occur at the

pathway level. Key genes in the mevalonate and non-mevalonate pathways were found to be up-regulated in rice plants damaged by FAW (Figure 2.11).

Evolution of the genetic basis of indirect defense trait

Volatiles induced by herbivory from different plant species are often qualitatively and quantitatively different (Pare and Tumlinson, 1999). This is sometimes true even for plant species that are closely related or different cultivars of a same species (Degen et al., 2004; Lou et al., 2006). Upon herbivory by lepidopteran larvae, both maize and rice plants emit a complex mixture of volatiles (Figure 2.2; Hoballah *et al.*, 2002). Some of these volatiles are identical and some are species-specific. It is intriguing to notice that there is a strong similarity in responses of parasitic wasps to rice and maize volatiles induced by herbivory (Figure 2.1; Hoballah *et al.*, 2002). It remains to be determined whether the insect-induced volatiles common to maize and rice play more important roles in attracting parasitoids.

It is equally intriguing to ponder what has caused the huge chemical diversity associated with indirect defense. In maize, it has been shown that allele variation is responsible for volatile variation in different cultivars (Kollner et al., 2004). Our study provides important evidence on possible mechanisms that lead to genetic variation related to indirect defense. Among rice *TPS* genes, Os08g07100, Os08g04500 and Os08g07080 are most related, suggesting that they have a common origin and were derived from gene duplication.

Os08g07100 and Os08g07080 likely have resulted from a recent gene duplication. Os08g07080 became a pseudogene. On the contrary, Os08g04500 and Os08g07100, which produce distinct products, have undergone functional divergence after duplication.

TPS10 from maize was shown to be an important gene for producing insect-induced sesquiterpenes that can attract parasitic wasps (Schnee et al., 2006). *TPS10* catalyzes the formation of a group of six sesquiterpenes (Schnee et al., 2006), all of which are also the products of Os08g07100. However, proportions of individual sesquiterpenes in product profiles of Os08g07100 and *TPS10* are different. For example, the two major products of *TPS10*, (*E*)- α -bergamotene and (*E*)- β -farnesene, are not major products of Os08g07100. Maize *TPS10* is most related to Os08g07100 (Figure 2.7), suggesting that they are likely orthologous genes. If true, it would suggest that lineage-specific evolution of a sesquiterpene synthase gene for indirect defense had occurred since the divergence of rice and maize lineages. Novel insight into evolution of indirect defense will itself evolve as the molecular basis of volatile-mediated indirect defense in rice, maize and other plant species is being elucidated.

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Appendix

Table 2.1. Candidate rice genes for making FAW-induced volatiles.

Gene Function	Gene ID	Probe ID	Log2R	STD	P Value	Ratio
<i>Terpene Synthase Genes</i>						
Putative terpene synthase	Os04g27720	TR009879	3.68	0.32	<0.001	12.80
Putative terpene synthase	Os04g27670	TR009877	3.23	0.65	<0.001	9.41
Putative terpene synthase	Os02g02930	TR003904	2.53	1.56	0.003	5.77
Putative terpene synthase	Os04g27760	TR009881	2.47	0.75	<0.001	5.54
Putative terpene synthase	Os08g07100	TR015140	1.87	0.23	<0.001	3.67
Terpene synthase family	Os03g22620	TR007427	1.73	0.22	<0.001	3.31
Putative terpene synthase	Os04g27190	TR009868	1.64	0.31	<0.001	3.13
Putative terpene synthase	Os08g07080	TR015138	1.63	0.75	<0.001	3.10
Putative terpene synthase	Os03g22634	TR007429	1.52	0.36	<0.001	2.87
Similar to sesquiterpene synthase 1	Os04g01810	TR009269	1.51	0.83	0.002	2.84
Terpene synthase family	Os08g04500	TR015023	0.77	0.24	<0.001	1.70
<i>SABATH Methyltransferase genes</i>						
SAM dependent carboxyl methyltransferase	Os02g48770	TR005327	1.43	0.48	<0.001	2.69
SAM dependent carboxyl methyltransferase	Os05g01140	TR011519	1.12	0.20	<0.001	2.18
<i>Indole Pathway Genes</i>						
Putative indole-3-glycerol phosphate lyase	Os03g58300	TR008831	2.14	0.23	<0.001	4.40
Anthranilate phosphoribosyltransferase	Os03g03450	TR006173	1.82	0.24	<0.001	3.52
<i>Lipoxygenase Pathway Genes</i>						
Similar to lipoxygenase	Os12g37320	TR019991	3.55	0.33	<0.001	11.69
Lipoxygenase	Os12g37260	TR019987	3.35	0.58	<0.001	10.19
Lipoxygenase L-2; lipoxygenase	Os03g52860	TR008522	2.12	0.79	<0.001	4.36

Table 2.2. Observed and expected frequencies of motifs in the promoters of three rice *TPS* genes

<i>cis</i> -elements	sequences	Os02g02930	Os08g04500	Os08g07100	Expected
W-box	TTGAC	3	1	0	2.9
H-box	CCTACC	1	0	1	0.2
G-box	CACGTG	0	0	0	0.2
DRE	CCGAC	2	3	0	0.5
TGA	TGACG	5	2	0	1.2

Table 2.3. Rice genes whose expression was significantly up-regulated by FAW

herbivory.

Gene Function	Gene ID	Probe ID	Log2R	STD	P Value	Ratio
Metabolic Enzymes						
Putative terpene synthase	Os04g27720.1	TR009879	3.68	0.32	<0.001	12.80
Similar to lipoxigenase	Os12g37320.1	TR019991	3.55	0.33	<0.001	11.69
Lipoxygenase	Os12g37260.1	TR019987	3.35	0.58	<0.001	10.19
Putative terpene synthase	Os04g27670.1	TR009877	3.23	0.65	<0.001	9.41
Diaminopimelate epimerase	Os12g37960.1	TR020035	3.10	0.30	<0.001	8.56
Leucoanthocyanidin dioxygenase-like protein	Os03g18030.1	TR007127	2.86	0.84	<0.001	7.28
Putative terpene synthase	Os02g02930.1	TR003904	2.53	1.56	0.003	5.77
Putative terpene synthase	Os04g27760.1	TR009881	2.47	0.75	<0.001	5.54
1-deoxy-D-xylulose-5-phosphate synthase	Os07g09190.1	TR013099	2.41	0.45	<0.001	5.32
Chloroplastic quinone-oxidoreductase	Os04g29030.1	TR009923	2.31	0.83	0.001	4.96
Plant neutral invertase	Os01g22900.1	TR001141	2.28	0.54	<0.001	4.86
O-methyltransferase	Os10g02880.1	TR016885	2.20	0.27	<0.001	4.58
BAHD Transferase	Os01g63480.1	TR003124	2.19	0.82	<0.001	4.55
Putative indole-3-glycerol phosphate lyase	Os03g58300.1	TR008831	2.14	0.23	<0.001	4.40
Lipoxygenase L-2; lipoxygenase	Os03g52860.1	TR008522	2.12	0.79	<0.001	4.36
Oxidoreductase, 2OG-Fe(II) oxygenase	Os04g10350.1	TR009496	2.03	0.39	<0.001	4.08
Cytochrome P450	Os05g12040.1	TR011774	2.00	0.29	<0.001	4.00
Oxidoreductase	Os04g41960.1	TR010504	1.95	0.78	<0.001	3.86
Similar to saccharopin dehydrogenase-like protein	Os02g54254.1	TR005699	1.88	0.40	<0.001	3.67
Putative terpene synthase	Os08g07100.1	TR015140	1.87	0.23	<0.001	3.67
Peroxidase, putative	Os07g48020.1	TR014741	1.87	0.57	<0.001	3.64
Anthranilate phosphoribosyltransferase	Os03g03450.1	TR006173	1.82	0.24	<0.001	3.52
Terpene synthase family	Os03g22620.1	TR007427	1.73	0.22	<0.001	3.31
Axidoreductase, aldo/keto reductase	Os07g04990.1	TR012860	1.72	0.40	<0.001	3.30
Monoxygenase	Os03g05880.1	TR006344	1.72	0.28	<0.001	3.30
Putative flavanone 3-hydroxylase	Os10g39140.1	TR018142	1.70	0.29	<0.001	3.26
Similar to beta-glucosidase-like protein	Os04g43390.1	TR010579	1.68	0.14	<0.001	3.21
Putative roteinase inhibitor	Os03g52390.1	TR008492	1.68	0.15	<0.001	3.21
Putative terpene synthase	Os04g27190.1	TR009868	1.64	0.31	<0.001	3.13
Putative terpene synthase	Os08g07080.1	TR015138	1.63	0.75	<0.001	3.10
Plant-specific FAD-dependent oxidoreductase	Os04g29090.1	TR009924	1.56	0.22	<0.001	2.94
Putative terpene synthase	Os03g22634.1	TR007429	1.52	0.36	<0.001	2.87
Sulfotransferase domain	Os08g20130.1	TR015594	1.52	0.53	0.001	2.87
Similar to sesquiterpene synthase 1	Os04g01810.1	TR009269	1.51	0.83	0.002	2.84
Oxidoreductase	Os04g15920.1	TR009650	1.49	0.15	<0.001	2.81
Lipase, putative	Os03g22670.1	TR007430	1.48	0.20	<0.001	2.78
Oxidoreductase	Os07g46930.1	TR014677	1.45	0.53	<0.001	2.73
SAM dependent carboxyl methyltransferase	Os02g48770.1	TR005327	1.43	0.48	<0.001	2.69
UDP-glucosyl transferase	Os04g37820.1	TR010268	1.41	0.58	0.001	2.66
Sucrose synthase	Os03g22120.1	TR007405	1.40	0.30	<0.001	2.64
Prenylcysteine oxidase	Os04g59630.1	TR011495	1.38	0.52	<0.001	2.61
Putative lipase	Os03g51010.1	TR008410	1.37	0.49	<0.001	2.59
Putative alpha-galactosidase	Os10g35070.1	TR017926	1.37	0.45	0.001	2.59
Hydrolase	Os05g46460.1	TR012193	1.37	0.22	<0.001	2.59
Alcohol dehydrogenase	Os03g08999.1	TR006550	1.36	0.42	<0.001	2.57
Ribose-phosphate pyrophosphokinase	Os01g52530.1	TR002438	1.34	0.35	<0.001	2.54
Serine carboxypeptidase	Os10g01110.1	TR016826	1.31	0.33	<0.001	2.49
Cytochrome P450-like protein	Os10g38090.1	TR018094	1.27	0.26	<0.001	2.41
Peroxidase	Os10g39170.1	TR018144	1.24	0.45	<0.001	2.37
Malate dehydrogenase	Os08g33720.1	TR016056	1.20	0.17	<0.001	2.29
Lysine-ketoglutarate reductase 2	Os02g54254.1	TR005700	1.18	0.70	0.002	2.26
Phosphoenolpyruvate carboxylase	Os08g27840.1	TR015787	1.16	0.17	<0.001	2.24
Cytidine deaminase	Os07g14150.1	TR013329	1.15	0.17	<0.001	2.21
Adenosylmethionine decarboxylase	Os02g39790.1	TR005040	1.14	0.44	<0.001	2.21
O-methyltransferase	Os08g38910.1	TR016300	1.14	0.20	<0.001	2.20
Aminotransferase	Os04g52440.1	TR011087	1.13	0.22	<0.001	2.19
Transketolase	Os07g07470.1	TR012995	1.13	0.35	<0.001	2.18
SAM dependent carboxyl methyltransferase	Os05g01140.1	TR011519	1.12	0.20	<0.001	2.18
Hydrolase	Os03g61360.1	TR009029	1.11	0.36	<0.001	2.16
Putative thiolase	Os10g31950.1	TR017765	1.10	0.10	<0.001	2.14
Possible lysine decarboxylase	Os03g01880.1	TR006071	1.09	0.65	0.002	2.13
Putative dioxygenase	Os10g40934.10	TR018258	1.09	0.33	<0.001	2.13
Mandelate racemase	Os01g04630.1	TR000218	1.08	0.27	<0.001	2.12
Putative pyruvate kinase	Os10g42100.1	TR018336	1.08	0.51	0.001	2.11
Lysine decarboxylase-like protein	Os01g40630.1	TR001771	1.07	0.66	0.002	2.10

Table 2.3. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P Value	Ratio
Probable adenylate kinase 1	Os04g57540.1	TR011360	1.06	0.11	<0.001	2.09
Glycosyl hydrolase family 1	Os07g46280.1	TR014624	1.06	0.41	0.001	2.09
1-deoxy-d-xylulose-5-phosphate reductoisomerase	Os01g01710.1	TR000040	1.06	0.34	<0.001	2.08
Pyridine nucleotide-disulphide oxidoreductase	Os08g04630.1	TR015031	1.03	0.65	0.003	2.04
Cytochrome P450	Os01g52790.1	TR002458	1.01	0.55	0.006	2.02
Transcription Factors						
probable wrky transcription factor 62	Os11g02520.1	TR018424	2.337	0.490	<0.001	5.053
Clp amino terminal domain, putative	Os02g32520.1	TR004753	2.004	0.253	<0.001	4.011
No apical meristem (NAM) protein, putative	Os01g60020.1	TR002912	1.834	0.194	<0.001	3.565
CAF1 family ribonuclease, putative	Os04g58810.1	TR011447	1.614	0.901	0.001	3.060
putative Myb-like DNA-binding protein	Os03g55590.1	TR008678	1.441	0.291	<0.001	2.714
AP2 domain, putative	Os06g07030.1	TR012540	1.382	0.245	<0.001	2.606
zinc finger protein	Os12g18120.1	TR019539	1.318	0.369	<0.001	2.493
WRKY DNA -binding domain, putative	Os11g02470.1	TR018422	1.273	0.967	0.006	2.417
Zinc finger, C3HC4 type (RING finger)	Os03g20870.1	TR007324	1.233	0.211	<0.001	2.350
Zinc finger, C2H2 type, putative	Os05g37190.1	TR011962	1.151	0.386	0.001	2.221
Zn-finger in Ran binding protein and others	Os02g10920.1	TR004149	1.140	0.207	<0.001	2.203
CHY zinc finger, putative	Os01g52110.1	TR002406	1.134	0.191	<0.001	2.195
Myb-like DNA-binding domain	Os08g06240.1	TR015090	1.117	0.324	<0.001	2.169
Helix-loop-helix DNA-binding domain	Os01g01870.1	TR000050	1.103	0.240	<0.001	2.148
putative Cys2/His2 zinc-finger protein	Os03g60570.1	TR008976	1.088	0.401	0.001	2.126
WRKY-type DNA binding protein	Os11g02540.1	TR018425	1.069	0.329	0.001	2.098
putative transcription factor	Os03g48450.1	TR008266	1.002	0.228	<0.001	2.003
Protein Kinases and Phosphatases						
Leucine Rich Repeat, putative	Os01g66760.1	TR003332	2.026	0.752	0.001	4.072
Protein kinase domain, putative	Os01g50410.1	TR002302	1.920	0.481	<0.001	3.784
Protein kinase domain, putative	Os12g16520.1	TR019493	1.827	0.407	<0.001	3.549
Protein kinase domain, putative	Os12g37980.1	TR020037	1.693	0.491	<0.001	3.234
Ser/Thr protein phosphatase family protein	Os08g41880.1	TR016479	1.687	0.330	<0.001	3.221
Protein kinase domain, putative	Os08g28710.1	TR015823	1.622	0.499	<0.001	3.079
protein kinase Xa21 (EC 2.7.1.-) A1	Os11g36200.1	TR018698	1.328	0.629	<0.001	2.510
putative protein kinase	Os10g33040.1	TR017812	1.251	0.644	0.002	2.381
Protein kinase domain, putative	Os03g03510.1	TR006178	1.219	0.101	<0.001	2.328
putative Serine/threonine phosphatases	Os03g16170.1	TR007005	1.167	0.138	<0.001	2.246
putative inositol 1,3,4-trisphosphate 5/6-kinase	Os03g51610.1	TR008444	1.080	0.287	<0.001	2.114
Protein kinase domain, putative	Os01g50370.1	TR002300	1.073	0.498	<0.001	2.104
Protein kinase domain, putative	Os10g10130.1	TR017123	1.027	0.283	<0.001	2.038
Transporters and Aquaporins						
Transmembrane amino acid transporter protein	Os01g41420.1	TR001822	2.840	0.417	<0.001	7.162
Sugar transporter	Os07g37320.1	TR014131	1.950	0.544	<0.001	3.865
Mitochondrial carrier protein, putative	Os08g40850.1	TR016408	1.629	0.278	<0.001	3.093
monosaccharide transporter 4	Os03g11900.1	TR006722	1.601	0.536	<0.001	3.034
phosphate:H ⁺ symporter	Os10g30790.1	TR017712	1.312	0.263	<0.001	2.483
ABC transporter, putative	Os09g16330.1	TR016714	1.305	1.001	0.006	2.471
ABC transporter, putative	Os01g42380.1	TR001879	1.213	0.728	0.003	2.319
ABC transporter, putative	Os01g50160.1	TR002288	1.059	0.472	0.003	2.083
Defense Proteins						
Bowman-Birk serine protease inhibitor family, putative	Os01g04050.1	TR000179	3.359	1.231	<0.001	10.260
Probenazole-induced protein - rice	Os12g36880.1	TR019971	2.648	0.215	<0.001	6.270
jacalin homolog - barley	Os12g14440.1	TR019445	2.605	1.014	<0.001	6.085
Bowman-Birk serine protease inhibitor family, putative	Os01g03330.1	TR000145	2.219	0.762	<0.001	4.654
Bowman-Birk serine protease inhibitor family, putative	Os01g03320.1	TR000144	2.136	0.592	<0.001	4.395
Bowman-Birk serine protease inhibitor family, putative	Os01g03680.1	TR000159	2.007	0.596	<0.001	4.020
Potato inhibitor I family	Os01g42860.1	TR001909	1.932	0.848	<0.001	3.816
jacalin homolog - barley	Os12g09700.1	TR019320	1.802	0.784	<0.001	3.486
Similar to disease resistance-like protein	Os12g39620.1	TR020120	1.555	0.182	<0.001	2.938
putative disease resistance protein	Os10g33130.1	TR017817	1.245	0.437	0.001	2.371
NB-ARC domain, putative	Os10g17690.1	TR017275	1.223	0.928	0.010	2.335
Others						
Similar to plant metallothionein-like protein	Os12g38010.1	TR020039	3.323	0.307	<0.001	10.007
Similar to Pib	Os12g37280.1	TR019989	2.886	0.435	<0.001	7.392
putative actin-binding protein	Os03g60580.1	TR008977	2.771	0.145	<0.001	6.827
selenium-binding protein, putative	Os12g13110.1	TR019393	2.695	0.247	<0.001	6.478
contains similarity to hedgehog-interacting protein	Os12g37200.1	TR019985	2.620	0.096	<0.001	6.146
WSI18 protein	Os01g50910.1	TR002328	2.586	0.975	<0.001	6.006
putative antifungal zeamatin-like protein	Os03g46070.1	TR008151	2.410	0.840	<0.001	5.315
Common central domain of tyrosinase, putative	Os01g58100.1	TR002800	1.821	0.817	<0.001	3.534
late embryogenesis abundant protein	Os03g20680.1	TR007313	1.794	0.284	<0.001	3.467
ACT domain, putative	Os04g32110.1	TR010044	1.737	0.848	0.003	3.332
Senescence-associated protein-like	Os12g24020.1	TR019624	1.501	0.190	<0.001	2.830
putative root-specific protein	Os03g50960.1	TR008405	1.475	1.063	0.007	2.781

Table 2.3. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P Value	Ratio
Late embryogenesis abundant protein	Os01g21250.1	TR001082	1.432	0.145	<0.001	2.699
Retinal pigment epithelial membrane protein	Os08g28240.1	TR015802	1.432	0.287	<0.001	2.698
p21 protein, putative	Os12g38170.1	TR020047	1.343	0.449	<0.001	2.536
transposon protein, putative	Os04g51150.1	TR010999	1.269	0.916	0.008	2.410
cold regulated protein	Os05g39250.1	TR012002	1.255	0.256	<0.001	2.387
Similar to mutT domain protein	Os04g46280.1	TR010742	1.218	0.211	<0.001	2.327
probable dormancy-associated protein	Os11g44810.1	TR018913	1.207	0.216	<0.001	2.308
putative sugar-starvation induced protein	Os03g49440.1	TR008314	1.205	0.954	0.007	2.306
transposon protein, putative	Os12g35940.1	TR019933	1.155	0.178	<0.001	2.227
Cyclin, N-terminal domain, putative	Os02g04010.1	TR003964	1.153	0.380	<0.001	2.224
EF hand, putative	Os01g72080.1	TR003599	1.148	0.211	<0.001	2.217
S-locus glycoprotein family, putative	Os07g36590.1	TR014087	1.123	0.167	<0.001	2.177
MatE, putative	Os03g08910.1	TR006545	1.118	0.648	0.003	2.170
universal stress protein family	Os01g32780.1	TR001429	1.115			
Unknown						
expressed protein	Os07g34280.1	TR013974	3.152	1.732	0.001	8.892
expressed protein	Os12g39840.1	TR020131	2.804	0.335	0.000	6.982
expressed protein	Os08g30510.1	TR015896	2.584	0.328	0.000	5.998
expressed protein	Os11g10470.1	TR018554	2.583	0.370	0.000	5.993
expressed protein	Os12g16540.1	TR019495	2.582	0.408	0.000	5.988
expressed protein	Os03g15270.1	TR006937	2.463	0.871	0.000	5.514
expressed protein	Os03g52410.1	TR008494	2.281	0.295	0.000	4.861
expressed protein	Os04g32480.1	TR010053	2.246	0.205	0.000	4.744
expressed protein	Os01g66530.1	TR003312	2.169	1.099	0.001	4.496
expressed protein	Os03g28940.1	TR007632	2.130	0.286	0.000	4.378
expressed protein	Os03g51920.1	TR008464	2.067	0.408	0.000	4.190
expressed protein	Os03g13600.1	TR006836	2.006	1.076	0.002	4.018
expressed protein	Os03g47280.1	TR008206	1.975	0.397	0.000	3.931
expressed protein	Os04g58280.1	TR011415	1.968	0.348	0.000	3.911
expressed protein	Os04g05650.1	TR009388	1.837	0.347	0.000	3.572
expressed protein	Os01g51670.1	TR002374	1.817	0.650	0.000	3.522
expressed protein	Os01g53730.1	TR002519	1.799	0.315	0.000	3.480
expressed protein	Os03g53540.1	TR008567	1.750	0.256	0.000	3.364
expressed protein	Os11g33394.1	TR018682	1.745	0.640	0.000	3.352
expressed protein	Os12g26290.1	TR019676	1.713	0.127	0.000	3.279
expressed protein	Os07g12800.1	TR013277	1.605	0.143	0.000	3.042
expressed protein	Os03g32420.1	TR007758	1.576	0.956	0.003	2.982
expressed protein	Os01g10640.1	TR000566	1.558	0.351	0.000	2.944
expressed protein	Os11g10800.1	TR018575	1.511	0.520	0.000	2.851
expressed protein	Os07g47720.1	TR014727	1.485	0.128	0.000	2.799
expressed protein	Os10g36550.1	TR018013	1.386	0.398	0.000	2.614
expressed protein	Os10g32680.1	TR017789	1.314	0.195	0.000	2.487
expressed protein	AK120651	TR003315	1.291	0.601	0.000	2.446
expressed protein	Os04g50120.1	TR010978	1.284	0.351	0.000	2.434
expressed protein	Os03g12500.1	TR006764	1.273	0.940	0.005	2.417
expressed protein	Os01g54340.1	TR002556	1.244	0.249	0.000	2.368
expressed protein	Os10g20470.1	TR017333	1.207	0.242	0.000	2.309
expressed protein	Os04g49370.1	TR010933	1.188	0.521	0.000	2.278
expressed protein	Os05g44060.1	TR012111	1.185	0.286	0.000	2.273
expressed protein	Os01g54670.1	TR002576	1.174	0.848	0.004	2.256
expressed protein	Os08g01940.1	TR014904	1.109	0.365	0.000	2.156
expressed protein	Os03g09900.1	TR006577	1.082	0.276	0.000	2.117
expressed protein	Os07g06850.1	TR012952	1.071	0.732	0.004	2.101
expressed protein	Os01g53090.1	TR002476	1.046	0.560	0.001	2.065
expressed protein	Os07g44910.1	TR014568	1.039	0.301	0.001	2.055
expressed protein	Os10g14180.1	TR017227	1.034	0.459	0.003	2.048
expressed protein	Os01g34790.1	TR001521	1.021	0.675	0.006	2.030
expressed protein	Os10g36180.1	TR017992	1.004	0.575	0.003	2.006
expressed protein	Os01g14850.1	TR000824	-1.004	0.446	0.000	0.498
hypothetical protein	AK059202	TR018561	4.828	0.689	0.000	28.403
hypothetical protein	Os12g38990.1	TR020087	3.208	0.507	0.000	9.239
hypothetical protein	Os12g14320.1	TR019443	3.068	1.067	0.000	8.387
hypothetical protein	chr12:23925573	TR020085	2.585	0.334	0.000	5.999
hypothetical protein	Os04g29310.1	TR009925	2.571	0.911	0.000	5.944
hypothetical protein	Os02g30190.1	TR004676	2.197	0.915	0.001	4.585
hypothetical protein	Os12g16570.1	TR019497	2.088	0.816	0.000	4.251
hypothetical protein	Os01g18120.1	TR000965	2.072	0.971	0.000	4.206
hypothetical protein	Os12g29500.1	TR019724	1.336	0.439	0.000	2.525
hypothetical protein	Os12g37150.1	TR019983	1.318	0.611	0.000	2.494
hypothetical protein	Os04g30510.1	TR009973	1.261	0.753	0.002	2.397
hypothetical protein	Os03g12820.1	TR006790	1.255	0.497	0.001	2.387
hypothetical protein	Os08g41570.1	TR016456	1.207	0.327	0.000	2.308
hypothetical protein	Os08g42590.1	TR016511	1.167	0.283	0.000	2.245
hypothetical protein	Os03g61500.1	TR009032	1.110	0.573	0.004	2.159
hypothetical protein	Os08g07160.1	TR015142	1.088	0.380	0.000	2.126

Table 2.3. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P Value	Ratio
hypothetical protein	Os04g42680.1	TR010551	1.086	0.132	0.000	2.123
hypothetical protein	AK105524	TR007623	1.075	0.279	0.000	2.106
hypothetical protein	Os10g28200.1	TR018400	1.059	0.301	0.000	2.083
hypothetical protein	Os01g60640.1	TR002950	1.138	0.418	0.000	2.201
Unknown protein	Os03g08310.1	TR006501	2.331	1.516	0.004	5.031
Unknown protein	Os03g08320.1	TR006502	2.546	0.567	0.000	5.839
Unknown protein	Os03g08330.1	TR006503	1.228	0.606	0.001	2.343
unknown protein	Os02g32580.1	TR004757	1.273	0.225	0.000	2.416
unknown protein	Os01g50940.1	TR002330	1.549	1.106	0.004	2.926
unknown protein	Os01g45640.1	TR002025	1.072	0.207	0.000	2.103

Table 2.4. Primers used for real-time PCR.

TIGR Gene ID	Forward Primer	Reverse Primer
TPS Genes		
Os02g02930	5'-TTTGATGGATTCATGACAGAGA-3'	5'-CAACAAACTCTGCTGCATTTT-3'
Os03g22620	5'-CGATGCTGTCAAGGCTTGT-3'	5'-GAATGCAGCCAAGTCATCCT-3'
Os03g22634	5'-GTCGAAGATGCATGGAAAACC-3'	5'-CCGATGTACAAATGGCAACAGTT-3'
Os04g01810	5'-GAGGAGCAGTGGAGAAGCA-3'	5'-GACTGGGACCGTCGTGTT-3'
Os04g27190	5'-CCATGTGGTGCAACGAGTTA-3'	5'-CATTTCCCTGATGATACTTCGA-3'
Os04g27670	5'-TTTCAAAGGCAGTCTACTTATTACCTA-3'	5'-AATGCTTCTGTCGTCACCTGCAT-3'
Os04g27720	5'-CCTTTGCGAAGATTAATGCACTA-3'	5'-TGGAAACGGTAAAGTTCACAAT-3'
Os04g27760	5'-AGACTTCGACGGTGCAATTG-3'	5'-CCACTGGAATGCTTCACTTGA-3'
Os08g04500	5'-CGCTACGAGATGCTTTTACAAC-3'	5'-CACCGTAGCAGCTACCTGATC-3'
Os08g07080	5'-GGATAAAGGAGTTGATAGAAGACTCA-3'	5'-TCTTGCCACGATTTTGGT-3'
Os08g07100	5'-CCAAAAATCGTGGCAAGAACA-3'	5'-TGTATGTGAAAAGGTGAATGAATCTG-3'
Terpenoid Pathway Genes		
Os07g09190	5'-AAAAGCCCATGTGTATGTGTTTCTT-3'	5'-TGTACACAAACACCTCCATTATCATTT-3'
Os01g01710	5'-GCAAGCTGGGTTCACGTACA-3'	5'-GCTGCATAGGCGAGATCCAT-3'
Os01g50760	5'-GCTCATGGTTAGTTGTGCAAGCT-3'	5'-TCTGGCTTCCCATATTTTCAAA-3'
Os05g02990	5'-TCAGCTCTGGCGTTTGCA-3'	5'-GATGTTGTTGCTTCGGTGAAGA-3'
Os05g55030	5'-GGATGTGAAAGGCCACAAA-3'	5'-GGTCCCTCGTTCATTCACAATC-3'
Control Gene		
OsUBQ5	5'-ACCACTTCGACCGCCACTACT-3'	5'-ACGCCTAAGCCTGCTGGTT-3'

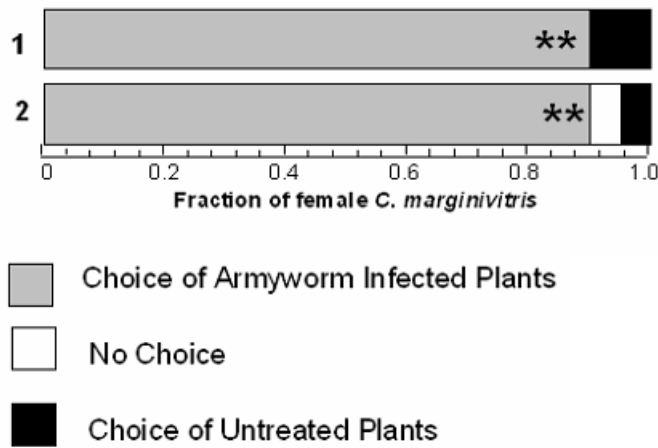


Figure 2.1. Attraction of female *C. marginiventris* to FAW-damaged and control rice plants. The olfactory response of female *C. marginiventris* to FAW-damaged rice plants and untreated control plants was measured in a Y-tube two-choice experiment. The figure shows the fraction of wasps that selected the odors of damaged plants (gray bars), control plants (black bars) or that did not make a choice (white bars). Two replicates choice tests (n=20) are presented. An asterisk (*) denotes significant deviation from H0 (wasps chose insect-damaged plants and control plant in a ratio of 50%:50%) according to a replicate test for goodness-of-fit at $\alpha=0.01$.

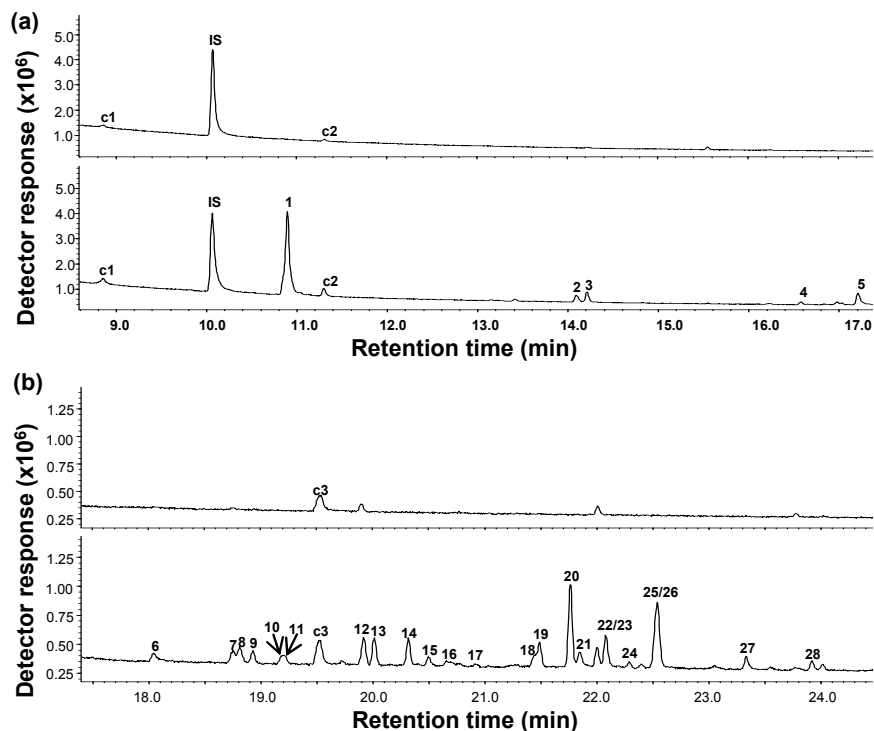


Figure 2.2. The volatile profiles of FAW-damaged rice plants. The upper panel shows a GC chromatogram of the volatiles from control plants and the lower panel from FAW-treated plants (A) from 9 to 17 minutes, (B) from 17 to 24 minutes. IS represents the internal standard. In (A) and (B), C1 to C3 represent the compounds present in both control and treated plants. C1, limonene; C2, nonanol; C3, unknown. Peak 1 to 28 are novel compounds induced by FAW herbivory. 1, linalool; 2, methyl salicylate; 3, decanal; 4, indole; 5, unknown; 6, unknown; 7, unknown; 8, 7-*epi*-sesquithujene; 9, sesquithujene; 10, β -elemene; 11, (*Z*)- α -bergamotene; 12, (*E*)- β -caryophyllene; 13, (*E*)- α -bergamotene; 14, sesquisabinene A; 15, (*E*)- β -farnesene; 16, Sesquisabinene B; 17, α -humulene; 18, γ -curcumene; 19, unknown sesquiterpene; 20, zingiberene; 21, unknown; 22, β -bisabolene; 23, β -curcumene; 24, germacrene A; 25, β -sesquiphellandrene; 26, (*E*)- γ -bisabolene; 27, nerolidol; 28, undecane.

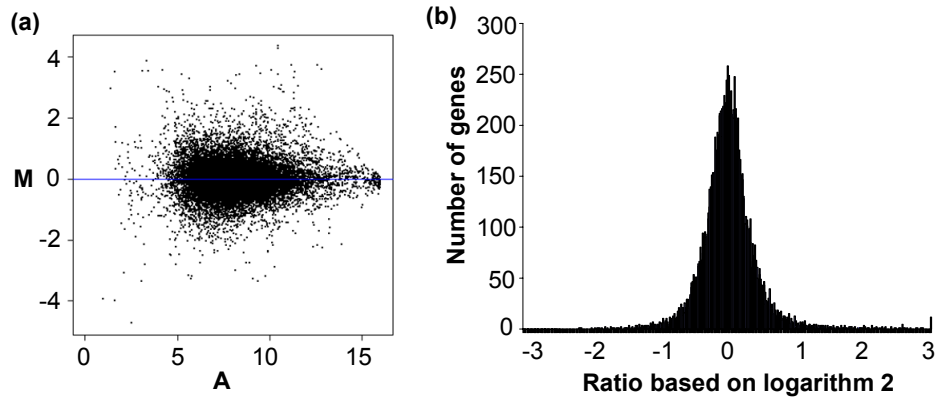


Figure 2.3. Microarray quality control. (A) M vs. A plot. The plot was generated as a scatter plot of logarithm transformed ratios of fluorescence intensities $M = \log_2 (R/G)$ versus logarithm transformed multiples of intensities $A = \log_2 (R*G)/2$, where R and G represent the fluorescence intensities in the red (R) and green (G) channels, respectively (Yang and Speed, 2002). (B) A histogram for the distribution of logarithm 2 based transformed ratios shows the typical shape of normal distribution.

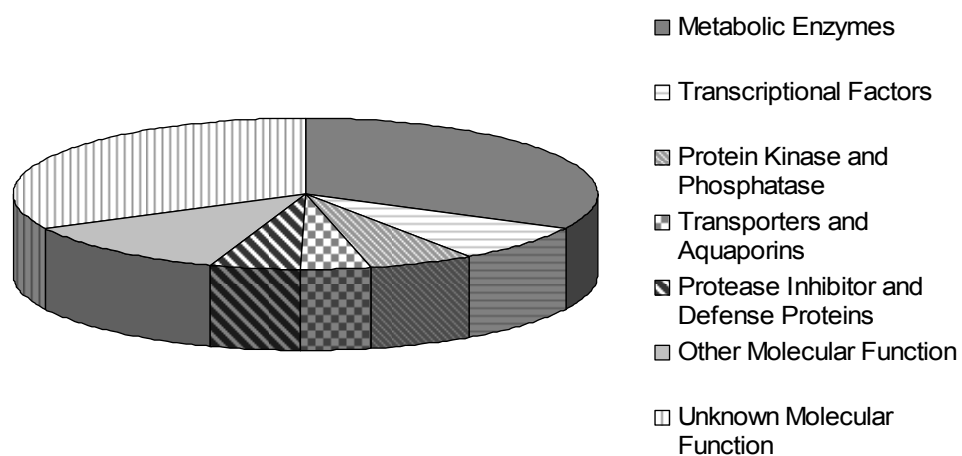


Figure 2.4. Abundance of functional classes among the significantly induced 196 rice genes by FAW herbivory.

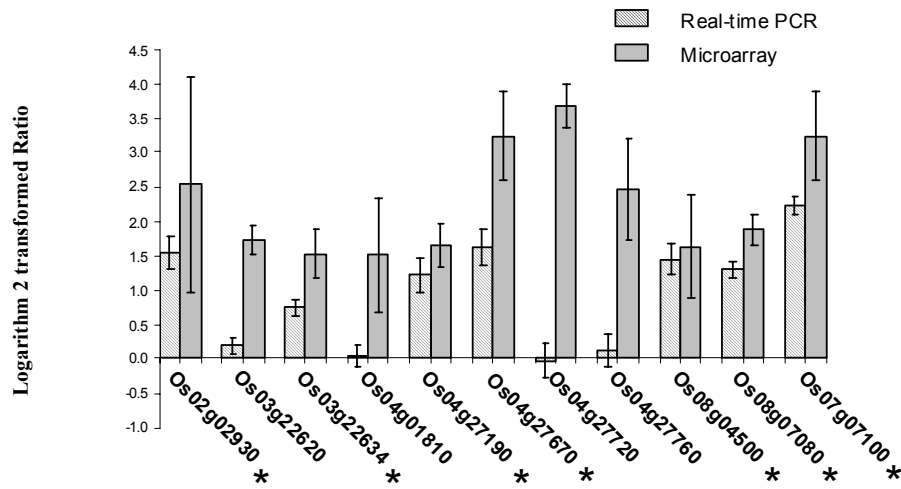


Figure 2.5. Expression validation of 11 *TPS* genes. Expression of seven *TPS* genes (marked with *) including Os02g02930, Os03g22634, Os04g27190, Os04g27670, Os08g07110, Os08g04500, Os08g07080 is consistent in real time-PCR and microarray analyses.


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Os08g07100 : -----MSTPAANFSNEDDERKAPTGFHSTWGTFPISYQPTAPKH--AYMKERAEVLKE : 54
ZmTPS10 : -----MATAFHSWGFFVKYKPLAPKR--GMTERAEVLKE : 38
Os08g04500 : -----MATSVPSVLLLPVPTCTDMLSPVEGGDLLHCKPHFDHNNWGDYELTESCPMSML--LMMKRAHMSSE : 70
Os02g02930 : MVCHVFSSFSSSLIRVLEAPLLPAASASSSSSSSPASRSGGRARRAAHVRSPIAYGRQELASHSMLEDFDFI--KVLIERHAAITD : 88
AT1G61680 : -----MALIATKISSRSCFVSAYPNNSPTFISKFPNTVDSLSPANTAKRSLLRNVHASVSNKQFHNKTSLEYLHNNIK : 77

Os08g07100 : EVNKKVKGSG-----NEVPEIDDTITLRLSDSYKASIDELCTVYN-TDYN--KDIHLVSGSVLLRKNSVDVSS-DTQHK : 132
ZmTPS10 : EVNKKVKAAG-----NQTNAIDDTITLRLSDHYENELSELRFYSSSDYD--KDIYVSGSVLLRKNSVDVSS-DVTSK : 118
Os08g04500 : QVRKLTLECSG-----GNLHVKDEVDITLRLSDYHVEKELENVRRHEEEDDTNHYDDHTTARSVLLRKNSYVSP-DVQRR : 154
Os02g02930 : DVQELQHQRRRHQKTASGGREHATVDHRLSDMDHYFDDEVDADACLLEELAHG--GDLLDADLRRLMREAGHHVSADEVLGRIT : 176
AT1G61680 : KLNKLNLSAN-----VDVPSENPDTVDVLSLSDLHRLQELQETLHMYKEGLQFN--GDLHETALRRLRLQESHYQE-IKKNIL : 157

Os08g07100 : DKEGSEVADG-----TRSLLELYNAAYVTHGEKVLEAVVPTNRRRSEVKKH-KSPVADVSLADTDLFRVRIITETQNYPIYESAT : 217
ZmTPS10 : DEEGNFVVDG-----TVCLLELYNAAYVTHGEKVLEAVVPTNRRRSEVKKH-LDLPSPADVSLADTDLFRVRIIEAINVPIYGKEA : 204
Os08g04500 : DEEGNFTRDNNNGRRSMLELYNAAYVTHGEKVLEAVVPTNRRRSEVKKH-LDLPSPADVSLADTDLFRVRIIEAINVPIYGKEA : 243
Os02g02930 : DDNGSRLLVYR-KDTRGGLQQLQISHNITGQASCKAKESPRNLESAANY--LPNLRVYVROSLDHVHVSINQYKARHHSYLQTLTP : 264
AT1G61680 : DKKSGKDVVK-NDVKGCTPLHPESELNVEESTLDCRPFPTSRNLECSG-RSHQKQDMKMLACRHHITVGGTTSKRRTSIIKIAG : 245

Os08g07100 : TRN----TALTEPKLVNVLQQLYCBELKLTTRMKKLVNVSNSFIRPRIVECHFWTGACSEPHVSLLEITLTKTAFTHITDDIT : 303
ZmTPS10 : GRN----TALTEPKLVNVLQQLYCBELKLTTRMKKLVNVSNSFIRPRIVECHFWTGACSEPHVSLLEITLTKTAFTHITDDIT : 290
Os08g04500 : TRN----TALTEPKLVNVLQQLYCBELKLTTRMKKLVNVSNSFIRPRIVECHFWTGACSEPHVSLLEITLTKTAFTHITDDIT : 329
Os02g02930 : IIC----TALTEPKLVNVLQQLYCBELKLTTRMKKLVNVSNSFIRPRIVECHFWTGACSEPHVSLLEITLTKTAFTHITDDIT : 350
AT1G61680 : QEPPEWLSLRVAPTDSIRLKSITQGSNSQTFKNTLGLKDEKARSQPKWHTVSMKILQDTLRCRDITKPSISLVVITDDIT : 335

Os08g07100 : TTAETESMLAKLYMCESTVILPEKMDPFLYYLTKTFDSPEERGPNKSRYRLVPRKELFKITFGISEEKKRDD--HYTPK : 390
ZmTPS10 : TTSPTESMLAKLYMCESTVILPEKMDPFLYYLTKTFDSPEERGPNKSRYRLVPRKELFKITFGISEEKKRDD--HYTPK : 377
Os08g04500 : NLSPTESMLAKLYMCESTVILPEKMDPFLYYLTKTFDSPEERGPNKSRYRLVPRKELFKITFGISEEKKRDD--HYTPK : 418
Os02g02930 : LVGPTESMLAKLYMCESTVILPEKMDPFLYYLTKTFDSPEERGPNKSRYRLVPRKELFKITFGISEEKKRDD--HYTPK : 437
AT1G61680 : VVSELPTPTTRVVERVHKGLKTLERPRVCEALDMITTEISMRVYKSHGNPTYARQSWASCTALVLEKPFNSG---YLPT : 422

Os08g07100 : EDELSEMTVAFOACASLVGMCDFTEEDTLDLTLYEKLEKSYTTCTVRLSNDVASTREQ-AGDHYASTVOCVLEHGTTHHEACI : 477
ZmTPS10 : EDELSEMTVAFOACASLVGMCDFTEEDTLDLTLYEKLEKSYTTCTVRLSNDVASTREQ-AGDHYASTVOCVLEHGTTHHEACI : 464
Os08g04500 : EDELSEMTVAFOACASLVGMCDFTEEDTLDLTLYEKLEKSYTTCTVRLSNDVASTREQ-AGDHYASTVOCVLEHGTTHHEACI : 508
Os02g02930 : EDELSEMTVAFOACASLVGMCDFTEEDTLDLTLYEKLEKSYTTCTVRLSNDVASTREQ-AGDHYASTVOCVLEHGTTHHEACI : 525
AT1G61680 : EDELSEMTVAFOACASLVGMCDFTEEDTLDLTLYEKLEKSYTTCTVRLSNDVASTREQ-AGDHYASTVOCVLEHGTTHHEACI : 510

Os08g07100 : GTELETSKMDMKETVATNLOPKIVARTVIDATCGDYTVKQADSESHITIDMNASLYTEPYSI : 546
ZmTPS10 : GTELETSKMDMKETVATNLOPKIVARTVIDATCGDYTVKQADSESHITIDMNASLYTEPYSI : 533
Os08g04500 : GTELETSKMDMKETVATNLOPKIVARTVIDATCGDYTVKQADSESHITIDMNASLYTEPYSI : 576
Os02g02930 : HVRLETSKMDMKETVATNLOPKIVARTVIDATCGDYTVKQADSESHITIDMNASLYTEPYSI : 595
AT1G61680 : HVRLETSKMDMKETVATNLOPKIVARTVIDATCGDYTVKQADSESHITIDMNASLYTEPYSI : 569

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Figure 2.6. Protein sequence alignment of three rice TPS genes, Os02g02930, Os08g07100, and Os08g04500, with exemplary terpene synthases, including ZmTPS10 (a sesquiterpene synthase from maize), and At1g61680 (an *S*-linalool synthase from *Arabidopsis*). The alignment was made with ClustalX. Amino acid residues conserved in three or more sequences are shaded. The R residue in the Os02g02930 sequence that is bold and italicized indicates the position of the first amino acid in the truncated Os02g02930 construct described in the text (replaced by a Met). The frame marks the conserved DDXXD motif.

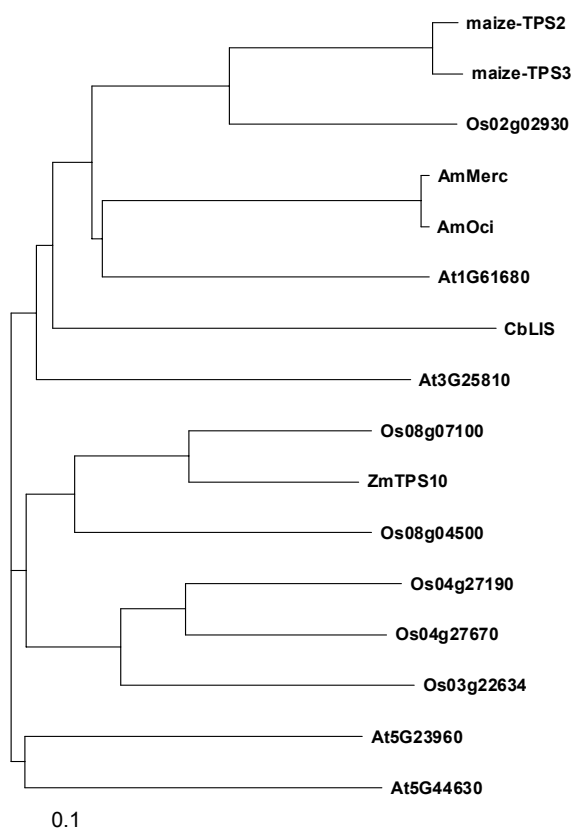


Figure 2.7. A neighbor-joining dendrogram analysis based on the degree of sequence similarity between rice TPSs and selected TPSs from other plants. Six FAW-induced rice TPS, Os02g02930, Os03g22634, Os04g27190, Os04g27670, Os08g07110, and Os08g04500 were included in the analysis. For other selected terpene synthase, ZmTPS10 is a sesquiterpene synthase from maize (Schnee *et al.*, 2006), maize-TPS2 and maize-TPS3 are two uncharacterized terpene synthases from maize, At5g23960 and At5g44630 are two sesquiterpene synthases from *Arabidopsis* (Chen *et al.*, 2003b), At1g61680 and At3g25810 are two monoterpene synthases from *Arabidopsis*, AmMerc and AmOci are two monoterpene synthases from snapdragon (Dudareva *et al.*, 2003). CbLIS is *S*-linalool synthase isolated from *Clarkia breweri* (Dudareva *et al.*, 1996).

		Thr	Pro			Leu	Gly	Gln	Phe	Leu	Pro	Gln	Leu	Pro	Ala	Thr	Tyr	Gly	Ala	Stop
<i>Os08g07080</i>	73	ACC	CC		T	CTG	GGG	CAA	TTT	CTT	CCT	CAG	TTA	CCA	GCC	ACC	TAC	TGC	GCC	TAA
<i>Os08g07100</i>	67	ACC	CCA	GCC	TCT	GGG	GCG	ATT	TCT	TCA	TCA	GTT	ACC	AGC	CAC	CTA	CTG	CAC	CTA	
		Thr	Pro	Ala	Ser	Gly	Ala	Ile	Ser	Ser	Ser	Val	Thr	Ser	His	Leu	Leu	His	Leu	

Figure 2.8. Os08g07080 is a pseudogene.

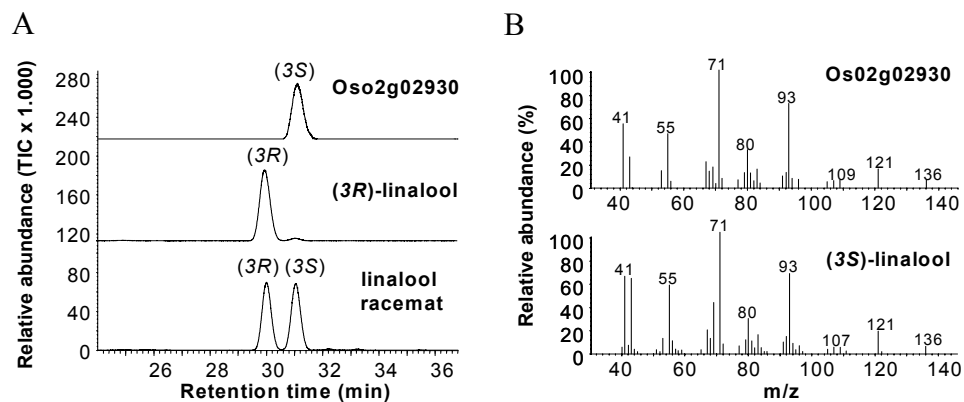


Figure 2.9. Monoterpene products of Os02g02930. The enzyme was expressed in *E.coli*, extracted, and incubated with the substrate GPP. The resulting terpene product was analyzed by GC-MS using a chiral column. (A) The traces of the MS detector are shown for the active enzyme Os02g02930, an authentic (3R)-linalool standard, and a racemic linalool mixture. The product was identified as (3S)-linalool by comparison of their retention time and mass spectrum to those of authentic standards. (B) Mass spectra of the enzyme product and reference substance (3S)-linalool.

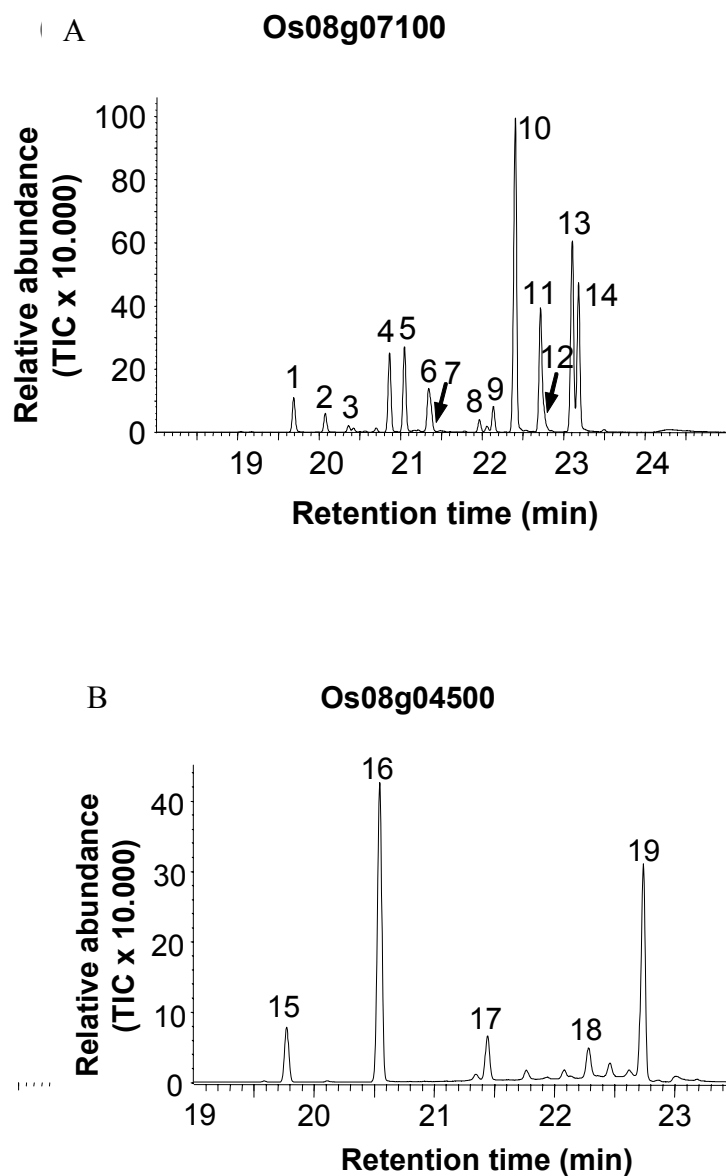


Figure 2.10. Sesquiterpene products of the herbivore inducible rice enzymes Os08g07100 and Os08g04500. The enzymes were expressed in *E.coli*, extracted, and incubated with the substrate FPP. The resulting terpene products were separated by GC-MS. The traces of the MS detector are shown for the active enzyme Os08g07100 (A) and Os08g04500 (B). Products were identified

Figure 2.10. continued.

as 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)- α -bergamotene; 4, (*E*)- α -bergamotene; 5, sesquisabinene A; 6, (*E*)- β -farnesene; 7, sesquisabinene B; 8, γ -curcumene; 9, unknown sesquiterpene; 10, zingiberene; 11, β -bisabolene; 12, β -curcumene; 13, β -sesquiphellandrene; 14, (*E*)- γ -bisabolene; 15, β -elemene; 16, (*E*)- β -caryophyllene; 17, α -humulene; 18, unknown sesquiterpene; 19, germacrene A by comparison of their retention times and mass spectra to those of authentic standards.

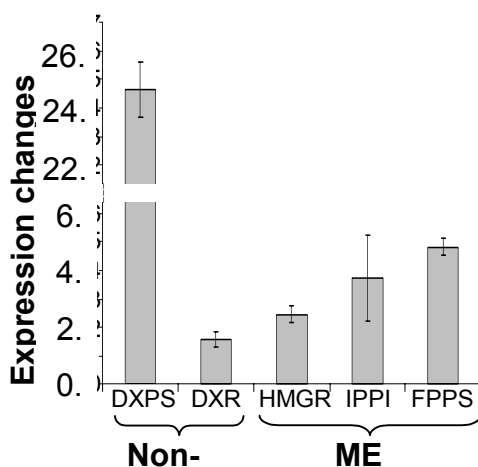


Figure 2.11. Expression of *DXPS*, *DXR*, *HMGR*, *IPPI* and *FPPS* genes in leaf tissues after FAW feeding analyzed using real-time quantitative PCR.

Expression values of individual genes were normalized to the levels of ubiquitin expression in respective samples. The level of expression of individual genes in control leaves was arbitrarily set at 1.0. Data were representation of three independent experiments and were plotted as means $\pm$ SD. Non-MEV denotes the non-mevalonate pathway and MEV denotes the mevalonate pathway.

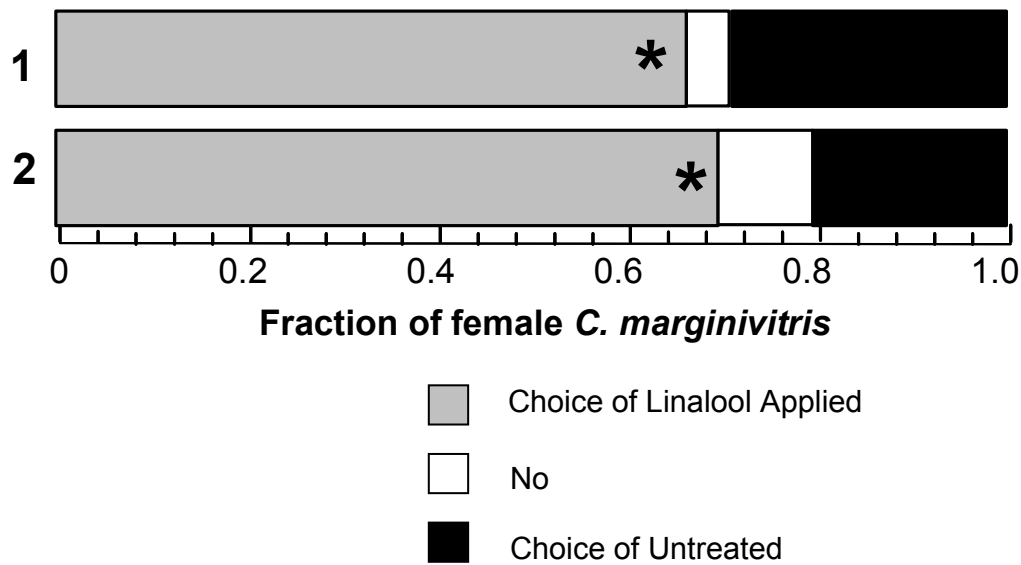


Figure 2.12. Attraction of female *C. marginivitrīs* to linalool applied and control rice plants. The olfactory response of female *C. marginivitrīs* to linalool applied rice plants and untreated control plants was measured in a Y-tube two-choice experiment. The figure shows the fraction of wasps that selected lialool applied plants (gray bars), control plants (black bars) or that did not make a choice (white bars). Two replicates choice tests (n=20) are presented. An asterisk (*) denotes significant deviation from H0 (wasps chose insect-damaged plants and control plant in a ratio of 50%:50%) according to a replicate test for goodness-of-fit at $\alpha=0.01$.

Chapter III. Identification and Characterization of Two Jasmonic Acid-Induced Terpene Synthase Genes with Integrative Genomics Approach

Adapted from:

Yuan J.S. and Chen F., Two monoterpene synthases are responsible from
production of basal level and insect-induced limonene in rice. Drafted

Abstract

Jasmonic acid is a key plant hormone involved in defense against herbivorous insects. Volatile terpenoids are involved in indirect defense of herbivorous insects with tritrophic interaction. The genes involved in jasmonic acid-induced monoterpene synthesis in rice are studied with metabolic and genomic analysis. Volatile profiling revealed time-dependent monoterpene emission for limonene and linalool, the two major monoterpenes induced in *Nipponbare* rice by jasmonic acid treatment. Microarray analysis of early jasmonic acid responses in rice revealed the up-regulation of many defense relevant genes including one annotated terpene synthase gene. The biochemical analysis of the monoterpene synthase gene showed its activity as a limonene synthase (LMS). At the meantime, we cloned and characterized another rice terpene synthase gene highly similar to *OsLMS* genes identified from microarray analysis. The second gene was shown to be another limonene synthase (*OsLMS2*) gene with slightly different activity for product specificity. Gene expression analysis revealed that both terpene synthase genes could be up-regulated by jasmonic acid in a time dependent pattern. Phylogenic analysis suggested that the *OsLMS* genes might evolve from convergent evolution as compared to *LMS* genes from other species. The biological function and enzyme activity mechanisms were further discussed based on MPSS data, sequence alignment and previous publications.

Introduction

Monoterpenes and sesquiterpenes are important volatile organic compounds (VOCs) involved in plant indirect defense against herbivorous insects (Takabayashi and Dicke, 1996). The indirect defense often involves tritrophic interaction, where plant emitted VOCs including monoterpenes and sesquiterpenes serve as infochemicals for natural enemies to better forage or parasitize herbivorous insects (Takabayashi and Dicke, 1996). In order to understand the molecular mechanisms of induced indirect defense in plants, it is important to study the biosynthesis of terpenoid volatiles and its regulation during the plant defense and in response to defense related hormones. Terpenoid volatiles can be induced by herbivore damage, physical wounding, elicitors, or treatment of plant defense hormones like jasmonic acid (Martin et al., 2003; McKay et al., 2003; Lou et al., 2005; Lou et al., 2005). The various contents of terpenoid volatiles in indirect defense depend on a variety of factors including different plant species, cultivar, and treatment (Hoballah et al., 2002; Gouinguene et al., 2003; Degen et al., 2004; Lou et al., 2006). The diverse contents of volatile compounds could help the natural enemies by providing information regarding the herbivore infestation on plants, and thus improve their efficiency in best locating the hosts or forage.

Terpenoids are the most diverse group of secondary metabolites. Both monoterpene and sesquiterpene display diverse structures. Regardless the diverse structure, monoterpenes and sesquiterpenes are both synthesized by

terpene synthase (TPS) using GPP (geranyl diphosphate) and FPP (farnesyl diphosphate) as substrate respectively. Monoterpene synthase uses GPP as substrate to synthesize monoterpenes; whilst sesquiterpene synthase converts FPP substrate to sesquiterpenes (Pichersky et al., 2006; Tholl, 2006). Even though more than one hundred monoterpene synthases and sesquiterpene synthases have been biochemically characterized, many of them have unclear biological functions. The study of terpene synthase gene expression in correlation to the volatile emission during defense related hormone treatment or during the defense process will help us to further understand the biological function of both volatile terpenoids and the TPS genes.

As aforementioned, volatile terpenoids can be induced by jasmonic acid, the key plant hormone involved in plant defense against insects (Lou et al., 2005; Liechti et al., 2006). Jasmonic acid biosynthesis starts with release of α -linolenic acid from chloroplast membranes with phospholipase. The α -linolenic acid is then modified by lipoxygenase to form hydroperoxyoctadecatrienoic acid, which can be further processed to *cis*(+)-12-oxophytodienoic acid (OPDA) by 13-allyl oxide synthase and allyl oxide cyclase. OPDA can then be first reduced and then oxidized via a series of reaction to form jasmonic acid (Browse, 2005; Shah, 2005; Liechti and Farmer, 2006). Jasmonic acid can be further methylated into methyl jasmonate, which can also serve as a signal compound with similar effects as seen for jasmonic acid (Seo et al., 2001). Jasmonic acid serves as a signaling compound during plant growth,

development and stress response (Liechti et al., 2006). Jasmonic acid has been shown to be involved in a variety of wounding response including oxidation, pathogen defense, and particularly defense against herbivorous insects (Gols et al., 2003; Browse, 2005; Liechti et al., 2006). Both physical wounding and jasmonic acid can induce the release of VOCs including monoterpenes and sesquiterpenes, however, the profiles are known to differ (Krumm et al., 1995; Van Poecke et al., 2001; Faldt et al., 2003; Gols et al., 2003; Lou et al., 2005; Liechti et al., 2006). A comprehensive survey of differentially regulated volatile emission and gene expression upon jasmonic treatment as compared to wounding treatment will help to understand the mechanisms of jasmonic acid signaling and the plant defense response (Zhang et al., 2004; Liechti et al., 2006).

We used rice as a monocot model to study jasmonic acid induced responses including the volatile compound biosynthesis and emission. Rice is the only monocot species with a completed genome sequence and numerous genomics resources including whole genome long-oligo microarrays (Project, 2005). Jasmonic acid serves as an important signaling compound for both pathogen and herbivore defense in rice. Jasmonic acid and methyl jasmonate have been shown to induce a variety of volatile compounds in rice (Lou et al., 2005). Several rice terpene synthases have been characterized and shown to be induced by herbivorous insects or methyl jasmonate treatment (Lou et al., 2006; Cheng et al., 2007; Joshua S. Yuan, 2007). Moreover, pathogen and elicitor treatments can also induce terpenoid emission in rice. Despite the progress with

biochemical work, questions still remain. First, a comprehensive gene profiling of jasmonic acid induced response is still lacking. The jasmonic acid induced gene expression profiling will help to understand the coordinative regulation of secondary metabolism and relevant pathways in plant defense. Moreover, it will help to identify the candidate TPS genes whose biochemical activity is still not clear. Second, the enzymes responsible for the biosynthesis of some volatile compounds are still not characterized. For example, limonene can be induced shortly after jasmonic acid treatment, yet the limonene synthase gene is still not characterized.

In this article, we aim to address the mechanisms of jasmonic acid induced response and volatile terpene biosynthesis using four different experimental approaches including jasmonic acid induced monoterpene emission, global gene expression profiling of jasmonic induced response in rice, biochemical characterization of two rice limonene synthase genes, and the evolution of limonene synthase across the species. Our results show that jasmonic acid can greatly induce monoterpene emission, and the time dependent dynamics of different monoterpenes are different. Global gene expression profiling has shown that early jasmonic acid induced response involves many defense relevant genes and the up-regulation of one terpene synthase, Os04g27190. We cloned and characterized both Os04g27190 and its close homolog Os04g27340, both of which are shown to be monoterpene synthase with major activity for limonene biosynthesis. Gene expression analysis has shown that both putative

limonene synthases are inducible by jasmonic acid and insect treatment, which indicated their role in defense. Comparative analysis of limonene synthase sequence across the species indicated that convergent evolution is the main approach for limonene synthase evolution. The biological function of limonene synthase in rice is then discussed.

Material and Methods

Plant Growth and Treatment

Uncoated rice (*Oryza sativa* ssp. *Japonica* cv. *Nipponbare*) seeds were germinated at 30 °C in the dark for five days. The seedlings were grown at 26 °C with 12 hours of light for two weeks. Jasmonic acid (Sigma-Aldrich, St. Louis, MO) were dissolved in 100% ethanol to make 50 µM stock solution. The jasmonic acid working solution is made by one to five dilution of stock solution in water and adding 0.2% of Triton-X100. The jasmonic acid working solution was evenly applied to rice on both side of rice leaves with cotton swab. For control plants, water solution with 20% ethanol and 0.2% Triton-X100 was applied on the leaves.

Volatile Analysis

Volatiles emitted from jasmonic acid treated rice and control rice were collected in close system as previously described (Chen et al., 2003). Six treated or untreated rice plants were wrapped in aluminum foil for the root part and put in a 1-L bell jar, respectively. Emitted volatiles were collected for four hours on activated charcoal traps that had been fitted into a steel column under continuous air circulation. Volatiles were eluted with 50 µL of CH₂Cl₂ (Mitchell and McCashin, 1994).

GC/MS Analysis

Samples from volatile collections were analyzed on a Shimadzu 17A gas chromatograph coupled to a Shimadzu QP5050A quadrupole mass selective detector. Separation was performed on a DB5 column of 30 m x 0.25 mm i.d. x 0.25 m thickness. Helium was the carrier gas (flow rate of 5 mL/min), a splitless injection (injection volume of 5 μ L) was used, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 240°C was applied. The identities of compounds were determined by comparison of retention times and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology and Wiley libraries (Agilent Technologies, Palo Alto, CA).

RNA Isolation

Total RNA was isolated from appropriate rice tissues using Plant RNA Isolation kit (Qiagen, Inc., Hilden , Germany) according to the manufacture's protocol. DNA contamination was removed with an on-column DNase (Qiagen, Inc., Hilden , Germany) treatment. Isolated total RNA was used for real-time PCR analysis, gene cloning, and microarray experiments.

Microarray Experiment

The Version 2.0 NSF long-oligo rice arrays provided by UC Davis microarray core facility were used for global gene expression profiling. One microgram of total RNA was labeled with TargetAmp aRNA amplification kit (Epicentre

Technologies, Madison, WI) according to the instruction of the manufacturer. The purified probes are mixed and hybridized with the long-oligo microarrays using the Microarray Hybridization Kit (Corning, Inc.) according to the manufacturer's instruction and the protocol provided by UC, Davis (<http://www.ricearray.org>). Reverse labeling experiments were included to eliminate dye-specific bias. For each sample set of armyworm treated rice versus control, the treated mRNA was first labeled with Cy5 and the control with Cy3. In the reverse experiment, the labeling dyes were swapped. The labeling reactions and dye swapped microarray hybridizations were performed in parallel. Considering the reverse labeling experiments, a total of three biological replicates and two technical replicates are included.

After hybridization, the microarray slides were washed and scanned with a GenePix 4000 scanner (Axon Instrument, Union City, CA, USA), and the image was processed by GenePix Pro software (Axon Instrument, Union City, CA, USA). The resultant microarray gpr files were analyzed with R-based open source software Bioconductor (<http://www.bioconductor.org>), where local background subtraction and Lowess normalization were performed for each microarray slide. Linear models from limma package of Bioconductor were applied to derive a p value and average of logarithm 2-based ratio across six slides. Changes in gene expression pattern were considered statistically significant at $p < 0.01^*$. A ratio cutoff of 2 and degree of freedom higher or equal to three were included as quality controls. In order to evaluate the microarray

quality, the M vs. A plot was generated as a scatter plot of log intensity ratios $M = \log_2 (R/G)$ versus average log intensities $A = \log_2 (R*G)/2$, where R and G represent the fluorescence intensities in the red (R) and green (G) channels, respectively.

Quantitative Real-time PCR

Quantitative real-time PCR experiments were carried out as previously described (Yang et al., 2006). Basically, complementary DNA was synthesized from 1 µg of total RNA for different treatment and control samples with Iscript cDNA Synthesis Kit (Bio-rad, Inc) according to the protocol provided. Samples were diluted into 20 ng/µl, 4 ng/µl and 0.08 ng/µl concentration series. Three replicates of real-time PCR experiments were performed for each concentration using an ABI 7000 Sequence Detection System from Applied Biosystems (Foster City, CA) with the PowerSYBR mix (Applied Biosystems). The primers for target genes were designed by Primer Express software (Applied Biosystems) with consideration of high similarity between the two genes. The primers are as shown in Table 3.1. After the real-time PCR experiments, Ct numbers were extracted for both reference genes and target genes with auto baseline and manual threshold. Amplification efficiency for the reactions was estimated as described previously, and multiple regression models were used to derive point estimation of $\Delta\Delta Ct$, p value, standard error and 95% confidence intervals with the SAS 9.1 programs provided (SAS institute, Cary, NC) (Yuan et al., 2006).

cDNA Cloning and Protein Expression in E. coli

Full length cDNAs of rice LMS genes were cloned from FAW-damaged rice leaves using RT-PCR. cDNA synthesis was performed in the same way as described for real-time PCR. The primers used were 5'-ATGGTTTGCCACGTCTTCTCG-3' (forward) and 5'-CGCCATTATGCATGGACGA-3' (reverse) for Os02g02930, 5'-ATGTCATCGACACCTGCAGCTAA-3' (forward) and 5'-TTAAATGCTATATGGCTCAACGTAAA-3' (reverse) for Os08g07100. In addition, a forward primer 5'-ATGCGACAAAGCAGTGCGCATC-3' and the same reverse primer were used to amplify the truncated form of Os04g27190, and a forward primer 5'-ATGCGACAAAGCAATGCGCATC-3' and the same reverse primer were used to amplify the truncated form of Os04g27340. PCR was carried out with the BD Advantage 2 Enzyme (BD Biosciences, Rockville, MD) with the following conditions: 95°C for 2 min, followed by 35 cycles at 95°C for 30 sec; 56°C for 30 sec and 68°C for 2 min, and a final elongation step at 70°C for 10 min. The resulting fragments were cloned into the vector pCRT7/CT-TOPO (Invitrogen, Carlsbad, CA). An *E. coli* BL21 Codon Plus strain, transformed with the appropriate expression construct, was used for protein expression. Induction was performed at 25°C overnight with 1 mM isopropyl-1-thio--D-galactopyranoside.

Enzyme Assay

50-mL cells from induced culture were harvested at 4°C and washed with 10-mL enzyme extraction buffer (50 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, 5 mM MgCl₂, 5 mM DTT, 5 mM sodium ascorbate, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were then disrupted by sonication in 3-mL enzyme extraction buffer, and then centrifuged at 13,000g at 4°C for 5 min. Buffer exchange was achieved by passing through a size exclusion Sephadex column (GE Healthcare, Piscataway, NJ), and the elution was collected in 4-mL of assay buffer containing 10 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, and 1 mM DTT. The enzyme assay was carried out in 1-mL containing 300-μL of enzyme extract, and 700-μL assay buffer containing 20 mM MgCl₂, 0.2 mM MnCl₂, 0.2 mM NaWO₄, 0.1 mM NaF, and 40 μM geranyl diphosphate or farnesyl diphosphate (Echelon Research Laboratories, Salt Lake City, UT). The assay was performed in a glass tube for 2 hours at 30°C with SPME fiber to collect the volatiles. The reaction together with SPME was incubated at 45°C at the end to allow volatile terpenoids to emit, and the SPME was then subject to the GC/MS analysis.

Phylogenic Analysis

Limonene synthase gene sequences were collected from NCBI database with both blast search and name search. In fact, reiterative sequence search for all characterized terpene synthase genes were also carried out. The protein

sequences for all known limonene synthase were aligned by AlignX from VectorNTI suite (Invitrogen, Carlsbad, CA) to produce the phylogenetic tree. In the meantime, selected sequences were aligned with same parameter and the MSF file was exported to be visualized in GeneDoc software (<http://www.nrbsc.org/gfx/genedoc/index.html>).

Results

Jasmonic acid induced monoterpene emission in rice

Terpenoid volatiles can be induced by insect and jasmonic acid treatment in rice (Lou et al., 2006; Cheng et al., 2007; Joshua S. Yuan, 2007). Previous experiments have shown that the jasmonic acid induced terpenoid biosynthesis has a time-dependent pattern (Cheng et al., 2007). Among the terpenoid volatiles induced by insect and jasmonic acid, the biosynthesis of limonene is still not clear. Since limonene was previously shown to be inducible by herbivorous insects, we examined the emission of limonene after insect treatment. As shown in Figure 3.1, insect treatment can induce a higher level of limonene emission twenty-four hour after the treatment.

The time-dependent jasmonic acid induced monoterpene emission was also studied as shown in Figure 3.2. Basically, limonene can be induced as early as two hours after treatment and reaches the peak at 2 to 4 hours as shown in Figure 3.2. In Figure 3.2, the volatiles are collected every 2 hours and the end time point were shown in X axis. Y-axis represent the relative level of limonene as compared to internal standard. The 2 to 4 hour time point volatile profiling shows nearly the highest limonene level, trivial linalool emission, and no emission of sesquiterpene (data not shown).

Microarray analysis of early response of jasmonic acid treatment

Microarray experiments were carried out comparing gene expression profiling between four hour jasmonic acid treated rice and control rice with half genome long-oligo rice arrays containing probes for 20,000 genes provided by University of California, Davis. The four hour time point was chosen for two reasons. First, the jasmonic acid induced response was believed to begin in the early stage of treatment. The response could start as early as 15 minutes after the treatment (Gols et al., 2003; Browse, 2005; Liechti et al., 2006). However, according to our volatile profiling data, the monoterpenes limonene and linalool change at two to four hour, where limonene reaches the peak and linalool begins to appear. Considering the important role of linalool in indirect defense, the fourth hour may represent the point where jasmonic acid induced indirect defense begin to initiate (Yuan *et al.*, 2007). This correlates with our previous data with insect treatment that terpenoid volatile production begins at around five to six hours after insect treatment (Yuan and Chen, data not shown). Second, limonene emission nearly reaches the peak at four hours. Our previous data indicated a good volatile-to-gene expression correlation in the induced response (Yuan *et al.*, 2007). The microarray analysis for four hour jasmonic acid treated rice may best help to identify limonene synthase gene. The enzymes for most of the armyworm and jasmonic acid induced terpenoid volatile biosynthesis have already been identified (Yuan *et al.*, 2007). Limonene is one of the very few terpenoid compounds whose biosynthesis is still not clear.

Microarray renders high quality data of a normal distribution of gene expression around ratio of 1. As shown in Table 3.2, microarray analysis reveals 74 up regulated genes with a P value less than 0.05 and ratio cutoff higher than 2. The functional characterization of up-regulated genes is as shown in Figure 3.3, where the major group of genes up-regulated is metabolic enzymes. Other groups of up-regulated genes include transcriptional factors, signal transduction pathway components, protein metabolism, nucleic acid metabolism, defense proteins and such. The pattern is similar to our previous microarray analysis in fall armyworm induced transcriptome response in that the largest functional group is metabolic enzymes (Yuan *et al.*, 2007).

Comprehensive pathway analysis was carried out to examine the details of the metabolic changes. The overall pattern is the induction of defense relevant pathway including phenolic compound pathway and lignin biosynthesis genes, and down-regulation of growth and development pathway such as cytokinin biosynthesis. The pathway analysis result highlights the important role of jasmonic acid in defense response. It has been reported that the defense relevant plant hormone can lead to the down-regulation of genes involved in plant growth and development to relocate the resource during plant defense. Figure 3.4 provides a summary of several metabolic pathways with genes induced. These pathways include mevalonate pathway, non-mevalonate pathway and phenolic compound biosynthesis. The up-stream biosynthesis of terpenoids is therefore slightly up-regulated. Moreover, there is one terpene synthase gene

was up-regulated. The terpene synthase gene is Os04g27190, which was then cloned for biochemical analysis.

Biochemical Analysis of OsLMS

The full length Os 04g27190 were first cloned and expressed. Biochemical assays were carried out as previously described (Chen et al., 2003), and no enzyme activity was found for full length Os04g27190 with GPP, FPP, and GGPP. Analysis of the protein sequence with TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) indicated that Os04g27190 contains the transient peptide leading to the transport of the protein in plastid (data not shown). On one side, the targetP analysis suggests that Os04g27190 should function in plastids, and could be a monoterpene or diterpene synthase. On the other side, targetP analysis results showed that we had to truncate the protein to remove transient peptide for proper analysis of enzyme activity. We therefore cloned and expressed the truncated Os04g27190 gene, and an enzyme assay showed that the truncated Os04g27190 can use both GPP and FPP as substrate for terpenoid biosynthesis. However, considering the plastid location of the gene, the enzyme should be a monoterpene synthase. As shown in Figure 3.5A, Os04g27190 can produce limonene as a major product and several other very minor monoterpene products.

Os04g27190 is highly similar to another candidate rice terpene synthase gene Os04g27340. The nucleic acid sequence between the two genes shared a

similarity as high as 97%, which indicate the two genes are derived from a recent tandem duplication event. We therefore decided to clone and characterize Os04g27340. As in Os04g27190, the product of full-length gene didn't show activity with GPP, GGPP, and FPP as substrate. Nevertheless, the truncated product can use both GPP and FPP as substrates. Neither enzyme reacted with GGPP as substrate. Since the targetP analysis indicated the plastid location of Os04g27340 as well, we therefore expect the enzyme to be a monoterpene synthase. As shown in Figure 3.5B, the product profile of the gene includes limonene as a major product and several other monoterpene as minor products including terpenole and ocimene. As the major product, limonene accounts for more than 80% of the monoterpene produced by the enzyme. Overall, both Os04g27190 and Os04g27340 encode monoterpene synthase with limonene as major product.

Phylogenic analysis of OsLMS

Limonene synthase has been previously characterized in several plant species (Rajaonarivony et al., 1992; Bohlmann et al., 1997; Bohlmann et al., 1999; El Tamer et al., 2003; Katoh et al., 2004; Hyatt et al., 2007). Phylogenic analysis was carried out for most of the previously published limonene synthase genes. As shown in Figure 3.6, twenty-four limonene synthase genes from eleven different species were included in the analysis. Our analysis included limonene synthase genes for all different isoforms of limonene. The analysis showed four

major clades in a species-dependent pattern. The rice limonene synthase genes shared a clade with limonene synthase genes from grand fir (*Abies grandis*). Besides phylogenetic analysis, multiple sequence alignment was also carried out to analyze the evolution and enzyme function of limonene synthase genes. Several limonene synthase genes from each clade of the phylogenetic analysis were selected for the multiple sequence alignment as shown in Figure 3.7. The sequence alignment reveals a limited conservativeness of the gene.

Gene Expression of OsLMSes during Jasmonic Acid Treatment

Real-time PCR experiments were carried out to characterize the gene expression of OsLMSes in response to jasmonic acid treatment. As shown in Figure 3.8, induction of Os04g27190 and Os04g27340 follows a time dependent pattern. Both genes are up regulated at around four hours after the treatment and reach the peak at hour 8. At 12 hours, Os04g27190 induction begins to drop, yet Os04g27340 level remains high. In term of responsiveness, Os04g27340 seems to be more inducible than Os04g27190. Moreover, spectral data indicate that the baseline level of Os04g27190 is about 4 to 8 times higher than Os04g27340 (data not shown). Considering the baseline level, the contribution of each enzyme on the inducible limonene biosynthesis still need to be further studied.

Discussion

Comparing Global Gene Expression Pattern between JA and Insect induced

In our previous publication, we presented a set of microarray data representing transcriptome changes of rice plants in response to 24 hour insect damage. As compared to the insect treated experiments, the microarray experiments shown in the present work revealed fewer numbers of up-regulated genes, which could be due to two reasons. First, the time point after the treatment is still too early. In fact, linalool and other sesquiterpenes are up-regulated normally around 8 hours after the treatment. There are reports indicating that exogenic jasmonic acid begins to act *in planta* shortly after the application, however, it seems the full extent of jasmonic acid induced responses may take hours to reach its peak (Liechti et al., 2006). Therefore, the transcriptional profiling at 4 hours after the treatment may represent a snapshot at the early response of jasmonic acid induced response. Second, plant defense against insects may involve both jasmonic acid dependent and jasmonic acid independent pathways (Liechti et al., 2006). In fact, other plant hormones including ethylene have been shown to be involved in plant defense against insects (Arimura et al., 2002; Schmelz et al., 2003). It is therefore important to carry out more gene expression profiling and gene functional studies to dissect these responses.

Regardless of the differences between gene expression patterns between the two sets of microarray data, there are some significant correlations between the datasets. First, as previously described, metabolic enzymes encompass the

largest group of changed genes, which indicate the importance of metabolism, especially secondary metabolism, in jasmonic induced defense response. Second, several pathways including mevalonate pathway, phenylpropanoid biosynthesis and lignin biosynthesis are up-regulated in both studies. The correlated up-regulation of defense relevant pathways highlighted the importance of jasmonic acid involved in plant defense. Moreover, there are some genes that are up-regulated specifically in the jasmonic acid treated microarray datasets, and the genes include some nucleic acid and protein metabolism genes. These are the candidate genes involved specifically in the early response of jasmonic acid cascade. The biological implication of up-regulation of these genes may reflect the changes in transcriptome and proteome in response to jasmonic acid treatment.

JA Induced Volatile Production

Jasmonic acid can induce the same set of volatiles as herbivorous insect rice fall armyworm. Unlike herbivorous insects, the jasmonic acid treatment can be well controlled for studying the dynamics of jasmonic acid induced volatile emission during a time course. In fact, previous research has shown that jasmonic acid can induce diverse groups of volatiles in different cultivars of rice, and the emission of some volatiles follow a diurnal cycle dependent pattern (Lou et al., 2005; Lou et al., 2005; Lou et al., 2006; Cheng et al., 2007). We found a similar yet more profound pattern. First, jasmonic acid induced volatiles were emitted at different time point after the treatment. At 0 to 2 hours after treatment, the

terpenoid volatile emission only included limonene, which is similar to the baseline level of emission. Despite the large amount of green leaf volatiles produced, no other terpenoid volatiles can be found. At around 2 to 4 hours, linalool and other sesquiterpenes begin to emerge and linalool reaches its peak at around 8 hours after the treatment. The results highlighted that the emission of different terpenoids are regulated differently, and it is therefore important to carry out a comprehensive study on the emission of terpenoid volatiles in rice during a time course in response to jasmonic acid treatment.

Second, the gene expression has shown certain correlation to the volatile production, yet cannot be fully correlated. As shown in Figure 3.8, the gene expression *OsLMSe*s reaches its peak at around eight hours after treatment, and the volatile limonene didn't increase as much at eight hours. Moreover, the degree of increased gene expression is much larger than the degree of induced volatile emission. It should be noted, at 8 hours, linalool has already increased to a very high level, so a substrate competitive theory maybe developed that lower limonene level at 8 hours may be due to the limitation in substrate availability. Basically, our results indicate gene expression regulation as an important mechanism of volatile emission control, but not as the only way. Other factors like substrate availability are also important.

Biological Function of OsLMS

Terpenoid volatiles have a variety of biological functions including plant indirect defense, protection against abiotic stress, defense against pathogens, and others (Dudareva et al., 2006; Pichersky et al., 2006). Our study showed the up-regulation of OsLMS genes and the induction of limonene in response to jasmonic acid, which indicated the defense roles of limonene synthase in rice. Jasmonic acid has been shown to be an important signaling compound involved in plant defense against insects (Liechti and Farmer, 2006; Liechti et al., 2006). The induced gene expression pattern of limonene synthases indicated their potential roles in defense against herbivorous insects. In fact, herbivorous insect treatment can induce limonene synthase genes, too (Yuan, *et al.*, 2007). Moreover, this response seems to be common in many cultivar of rice. Limonene has been shown to be one of the compounds that can attract parasitoids of herbivorous insects (Byun-McKay et al., 2006). Therefore, the role of limonene as one of the signal compounds for indirect defense is expected. However, since multiple terpenoid compounds have been shown to be up-regulated by rice fall armyworm damage, and linalool is the major terpenoid compound induced, the importance of limonene in the process is expected to be limited in japonica rice. However, in some other cultivars of rice, the emission of limonene is higher in response to insect damage, therefore, the importance of limonene in defense against insects may depend on the species of rice and other environmental factors. A survey of OsLMS gene expression in MPSS database also suggests that Os04g27190 is up-regulated by *Xanthamonus* treatment and

blast fungi *M. grisea* treatment. Since terpenoid compounds including limonene have been shown to be able to serve as phytoalexins, the plant produced antibiotic and antifungal compounds. Therefore, the role of OsLMSes in defense against pathogens can also be expected (Dudareva et al., 2006). Overall, the gene expression and volatile emission pattern of LMS and limonene indicated the defense role of OsLMS genes.

Evolution of OsLMS

The role of OsLMS in defense may be one of the driving forces for the evolution of OsLMS genes. The phylogenic analysis of most of the published limonene synthase genes showed several features. First, the limonene synthase genes have diverse structures. Recently, the X ray structure of one of the limonene synthase gene has been resolved. A multiple sequence alignment of limonene synthase genes cannot support clearly the major acting sites predicted by the research because several of the predicted enzyme activation site are not conserved among different limonene synthase as shown by the sequence alignment. Second, the limonene synthase genes do not share deep evolutionary origins. The phylogenic analysis of limonene synthase genes render a more species specific pattern, where the genes from the same or closely related species were clustered together. Among the limonene synthase genes characterized, some of them have been characterized for the product's chirality. However, the phylogenic analysis did not show a product-specific pattern, which indicated limonene synthase genes can easily evolve between the product specificity. Thirdly, the sequence

similarity among the genes in the same species is much higher as compared to those among the species, which indicated tandem duplication as a major way for limonene gene expansion within the family.

By and large, among the limonene synthases characterized, it seems no ancestor limonene synthase genes can be found for all of the limonene synthase genes characterized in different species. The biological function of these limonene synthase genes is also different. Some limonene synthase genes are expressed in flowers and are expected to be involved in pollination, whereas others are expressed in fruit adding flavor to the fruits (Rajaonarivony et al., 1992; Dudareva et al., 2003). Few limonene synthase genes are found in vegetative tissues and express in an inducible manner (Byun-McKay et al., 2006). Considering the functional and structural diversity, it is very likely for limonene synthase genes to evolve from several origins with convergent evolutionary processes.

Overall, our research discovered the first limonene synthase gene in a *Poaceae* species and these genes share weak similarity to the limonene synthase genes in the grand firs as shown by the phylogenic analysis. However, multiple sequence alignment with representative genes from several species indicated no significant conservation between rice and fir limonene synthase genes. Further study of limonene synthase gene structure from different species will help to understand the catalytic mechanisms of limonene synthases and the evolution of the gene.

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Appendix

Table 3.1. Primers for real-time PCR.

TIGR Gene ID	Forward Primer	Reverse Primer
TPS Genes		
Os04g27190	5'-CCATGTGGTGCAACGAGTTA-3'	5'-CATTTCCCTGATGATACTTCGA-3'
Os04g27340	5'-TCCCTGTGGTGCAACGACTTT-3'	5'-GATCCCCAACAATCTCCTGAATATACC-3'
Control Gene		
OsUBQ5	5'-ACCACTTCGACCGCCACTACT-3'	5'-ACGCCTAAGCCTGCTGGTT-3'

Table 3.2. Genes over-expressed upon jasmonic acid treatment. The ratio cut-off is 2 and p value smaller than 0.05.

ArrayID	Gene ID	Annotation	Log2Ratio	STD	Ratio	P Value
TR015338	LOC_Os08g13040	hypothetical protein	3.58	4.11	11.99	0.04
TR014775	LOC_Os07g48450	No apical meristem (NAM) protein	2.46	1.66	5.51	0.00
TR014741	LOC_Os07g48020	Peroxidase	2.08	2.50	4.24	0.03
TR013015	LOC_Os07g07780	hypothetical protein	2.05	0.62	4.14	0.00
TR000179	LOC_Os01g04050	Bowman-Birk serine protease inhibitor family	1.92	1.20	3.78	0.00
TR018966	LOC_Os12g01530	Ferritin-like domain	1.86	1.19	3.62	0.00
TR000164	N/A	S1/P1 Nuclease	1.75	1.96	3.37	0.02
TR002662	LOC_Os01g55940	GH3 auxin-responsive promoter	1.73	2.17	3.32	0.04
TR017642	LOC_Os10g29290	BTB/POZ domain, putative	1.72	0.80	3.30	0.00
TR018986	LOC_Os12g02120	Dual specificity phosphatase	1.71	1.82	3.27	0.04
TR016055	LOC_Os08g33710	Ribonuclease T2 family	1.70	1.75	3.24	0.02
TR004753	LOC_Os02g32520	Clp amino terminal domain	1.68	1.91	3.20	0.03
TR019445	LOC_Os12g14440	jacalin homolog	1.66	1.73	3.16	0.02
TR005327	LOC_Os02g48770	SAM dependent carboxyl methyltransferase	1.48	1.76	2.78	0.03
TR001658	LOC_Os01g38229	peptidyl-prolyl cis-trans isomerase	1.46	0.51	2.76	0.00
TR019142	LOC_Os12g06120	hypothetical protein	1.43	1.49	2.69	0.04
TR002356	LOC_Os01g51390	Insulinase (Peptidase family M16)	1.42	0.25	2.67	0.00
TR006605	LOC_Os03g10340	ribosomal protein S3a	1.41	1.50	2.67	0.02
TR000507	LOC_Os01g09510	Similar to acidic ribosomal protein	1.38	1.26	2.61	0.01
TR002321	LOC_Os01g50760	farnesyl-pyrophosphate synthetase	1.37	1.07	2.59	0.01
TR008303	LOC_Os03g49230	silencing group B protein	1.36	1.45	2.56	0.02
TR016685	LOC_Os09g11460	AP2 domain, putative	1.33	1.57	2.51	0.03
TR012919	LOC_Os07g06050	hypothetical protein	1.32	0.48	2.50	0.01
TR010077	LOC_Os04g32920	potassium uptake protein	1.32	1.71	2.49	0.05
TR013274	LOC_Os07g12730	expressed protein	1.31	1.69	2.48	0.05
TR000923	LOC_Os01g16890	60s ribosomal protein l30	1.28	0.66	2.43	0.00
TR010684	LOC_Os04g45290	vacuolar acid invertase	1.26	1.22	2.39	0.03
TR001300	LOC_Os01g27520	asparaginyl-tRNA synthetase	1.26	0.65	2.39	0.00
TR010660	N/A	oxidoreductase, short chain dehydrogenase/reductase family	1.25	0.80	2.38	0.00
TR008093	AT003629	putative cytochrome P450	1.25	1.53	2.37	0.04
TR006306	LOC_Os03g05310	lethal leaf-spot 1	1.25	1.15	2.37	0.01
TR005321	LOC_Os02g48660	Ribosomal protein L31e, putative	1.24	0.99	2.37	0.01
TR018827	LOC_Os11g40500	expressed protein	1.23	1.54	2.35	0.04
TR011360	LOC_Os04g57540	probable adenylate kinase 1, chloroplast precursor	1.22	0.42	2.32	0.00
TR017342	N/A	hypothetical protein	1.21	1.09	2.32	0.03
TR004380	LOC_Os02g19150	ATP-dependent Clp protease	1.20	0.74	2.30	0.00
TR012716	LOC_Os07g02460	expressed protein	1.19	1.13	2.29	0.02
TR015867	AK070379	Cytochrome b5-like Heme/Steroid binding domain	1.19	1.02	2.29	0.01
TR009868	LOC_Os04g27190	Terpene synthase family, metal binding domain	1.19	0.93	2.28	0.01
TR000494	LOC_Os01g09280	Transposable element protein	1.18	0.74	2.27	0.01

Table 3.2 continued.

ArrayID	Gene ID	Annotation	Log2Ratio	STD	Ratio	P Value
TR008526	LOC_Os03g52910	expressed protein	1.17	0.91	2.25	0.01
TR004430	LOC_Os02g20360	nicotianamine aminotransferase A	1.16	0.54	2.23	0.00
TR019502	LOC_Os12g16690	expressed protein	1.15	0.94	2.22	0.03
TR007247	LOC_Os03g19580	expressed protein	1.14	0.92	2.20	0.01
TR004588	LOC_Os02g27470	importin (nuclear transport factor) protein	1.13	0.67	2.19	0.00
TR011044	N/A	OSJNBa0060N03.3	1.13	1.40	2.19	0.04
TR012440	LOC_Os06g04280	3-phosphoshikimate 1-carboxyvinyltransferase	1.13	1.18	2.18	0.02
TR015988	LOC_Os08g32380	hypothetical protein	1.11	0.95	2.16	0.02
TR016354	LOC_Os08g39730	Cytochrome P450	1.10	1.01	2.15	0.02
TR019430	LOC_Os12g13900	hypothetical protein	1.10	0.93	2.15	0.03
TR008480	LOC_Os03g52170	putative LytB protein	1.08	1.32	2.12	0.04
TR011899	LOC_Os05g34790	expressed protein	1.08	1.02	2.11	0.04
TR006423	LOC_Os03g07190	expressed protein	1.08	0.48	2.11	0.00
TR016357	LOC_Os08g39820	expressed protein	1.07	0.50	2.10	0.00
TR015207	LOC_Os08g08820	Roc1	1.07	1.24	2.10	0.04
TR011861	LOC_Os05g23860	GDP dissociation inhibitor protein	1.07	1.05	2.09	0.02
TR010632	LOC_Os04g44560	expressed protein	1.05	0.42	2.08	0.00
TR013675	LOC_Os07g26880	hypothetical protein	1.05	0.32	2.07	0.01
TR012283	LOC_Os05g51840	histone deacetylase HD2	1.05	0.56	2.07	0.00
TR005097	LOC_Os02g41630	phenylalanine ammonia-lyase	1.05	1.09	2.07	0.03
TR002525	LOC_Os01g53810	Peptidase family M28	1.05	0.78	2.07	0.01
TR006280	LOC_Os03g04930	expressed protein	1.05	1.04	2.07	0.02
TR000675	LOC_Os01g12560	3-methyl-2-oxobutanoate hydroxymethyltransferase	1.04	0.77	2.06	0.02
TR005087	LOC_Os02g40784	expressed protein	1.04	0.77	2.06	0.01
TR005889	LOC_Os02g57160	ABC1 family, putative	1.04	0.93	2.05	0.02
TR004160	LOC_Os02g12340	hypothetical protein	1.03	0.98	2.04	0.03
TR011583	LOC_Os05g03140	Tetraspanin family	1.02	0.42	2.03	0.00
TR005692	LOC_Os02g54160	AP2 domain, putative	1.02	0.56	2.03	0.00
TR000541	LOC_Os01g10140	RNA dependent RNA polymerase	1.02	0.64	2.03	0.02
TR000925	LOC_Os01g16910	mitochondrial import receptor subunit tom40 homolog	1.01	0.90	2.02	0.02
TR019192	LOC_Os12g07010	ribosomal protein L3	1.01	1.00	2.02	0.02
TR005546	LOC_Os02g51930	UDP-glucuronosyl and UDP-glucosyl transferase	1.01	0.75	2.01	0.02
TR000198	LOC_Os01g04350	Hsp20/alpha crystallin family	1.01	1.14	2.01	0.04
TR003301	LOC_Os01g66350	hypothetical protein	1.00	0.66	2.01	0.01

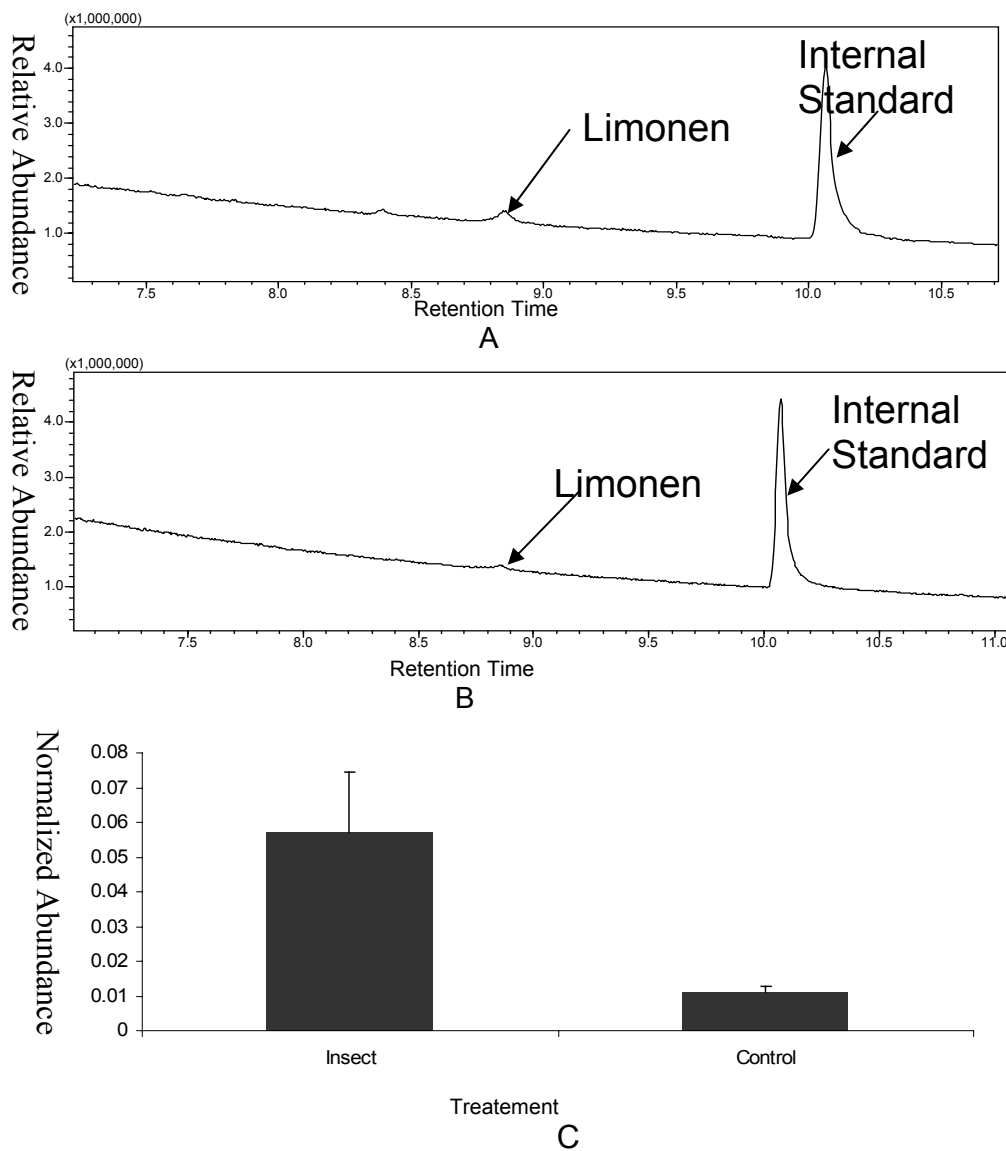


Figure 3.1. Insect induced limonene emission. A showed the volatile emitted from the insect treated plants and B showed the volatile emitted from the control plant. A clear increase in limonene compared with the control plant can be seen. The ratio between treated and control plants is about 5.

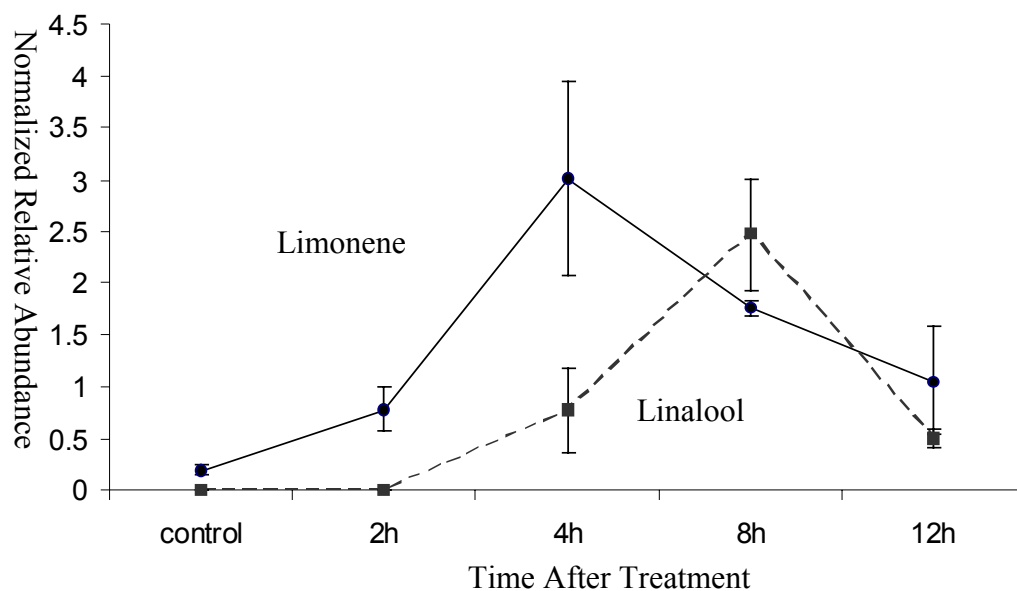


Figure 3.2. Time dependent emission of monoterpenes. The X axis showed the hour after jasmonic acid treatment, the Y axis showed the relative amount of monoterpene emission as compared to the internal standard. The solid line represented the level of emission for limonene and the broken line indicated the level of emission for linalool.

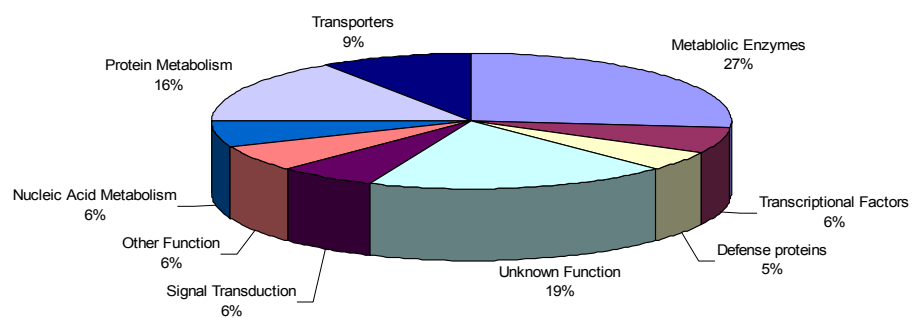
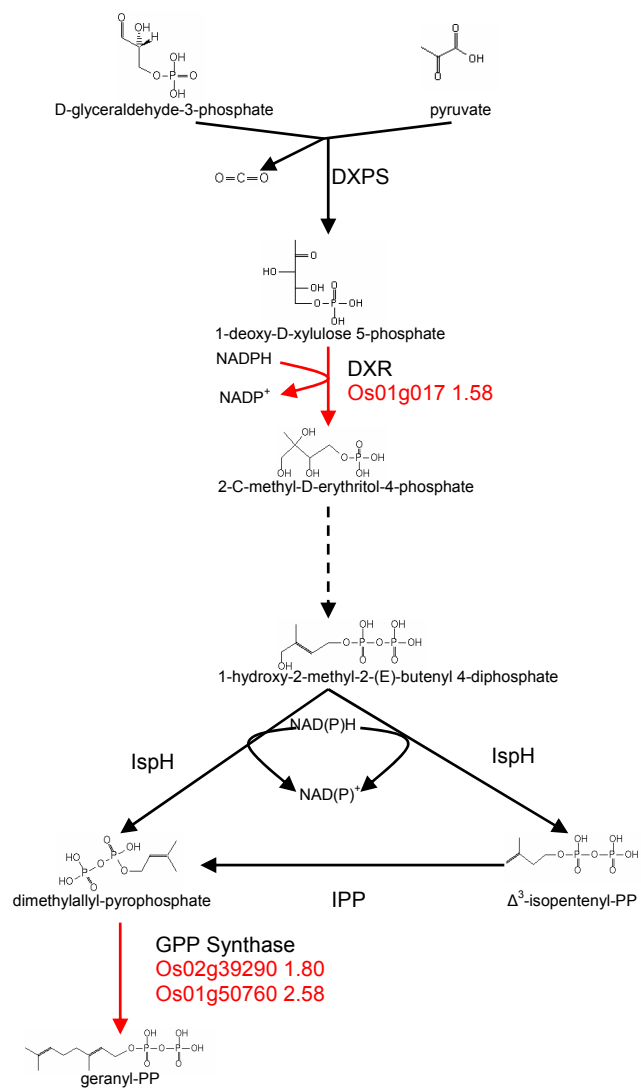
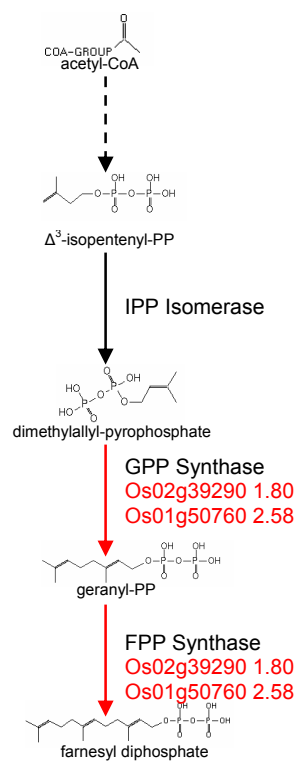


Figure 3.3. The distribution of different categories of genes up-regulated four hour after jasmonic acid treatment.



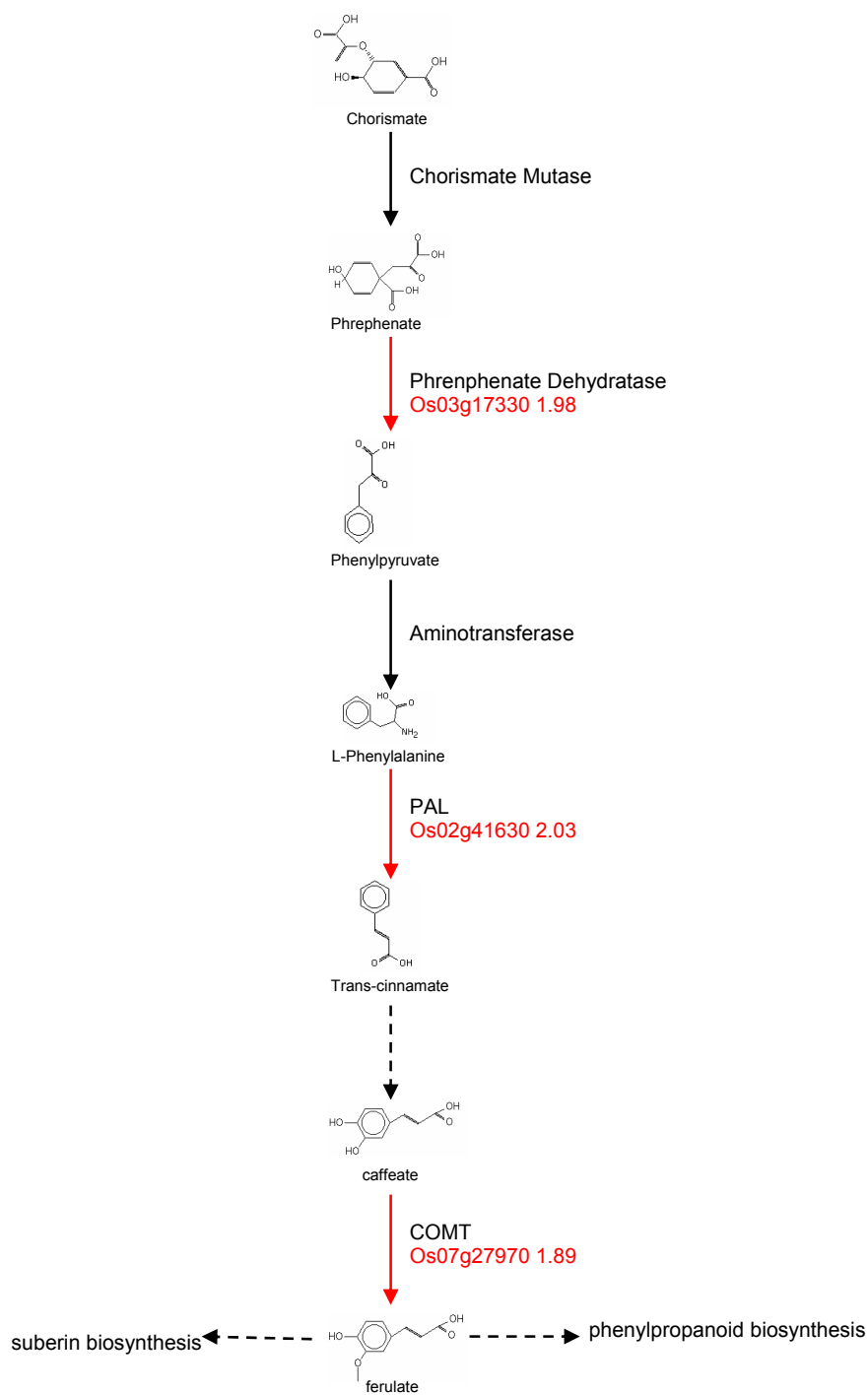
A

Figure 3.4. continued.



B

Figure 3.4.continued.



C

Figure 3.4. Metabolic pathway analysis for genes up-regulated by jasmonic acid treatment. The red lines showed the pathways with genes up-regulated, and the

Figure 3.4. continued.

TIGR gene locus for the up-regulated genes were labeled. A showed the non-mevalonate pathway; B showed the mevalonate pathway; and C showed the upstream pathway for phenolic compound biosynthesis.

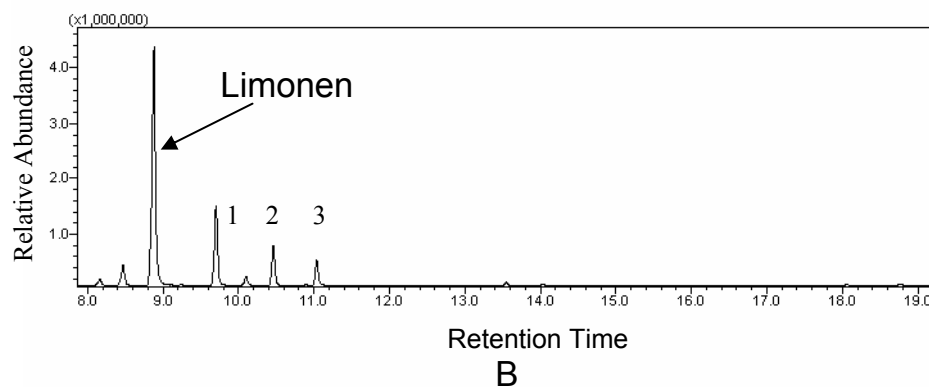
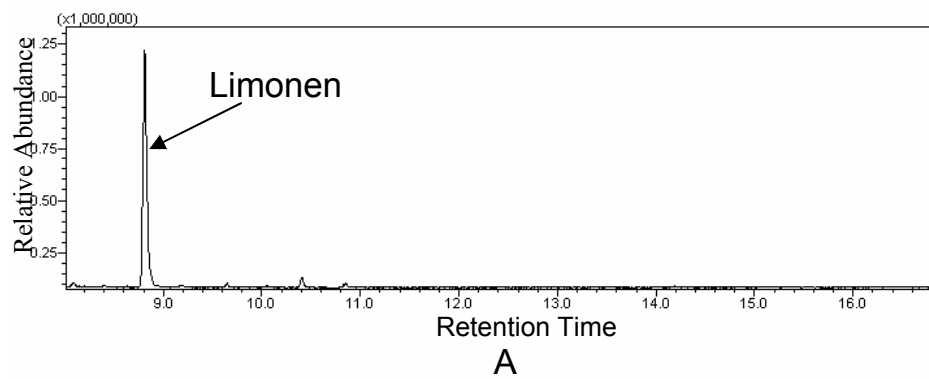


Figure 3.5. The GC/MS chromatograph for enzyme assay of limonene synthase. Besides limonene, the minor peaks were represented by 1, 2, 3, as Terpinene, Terpinolene and, Terpeneol, respectively.

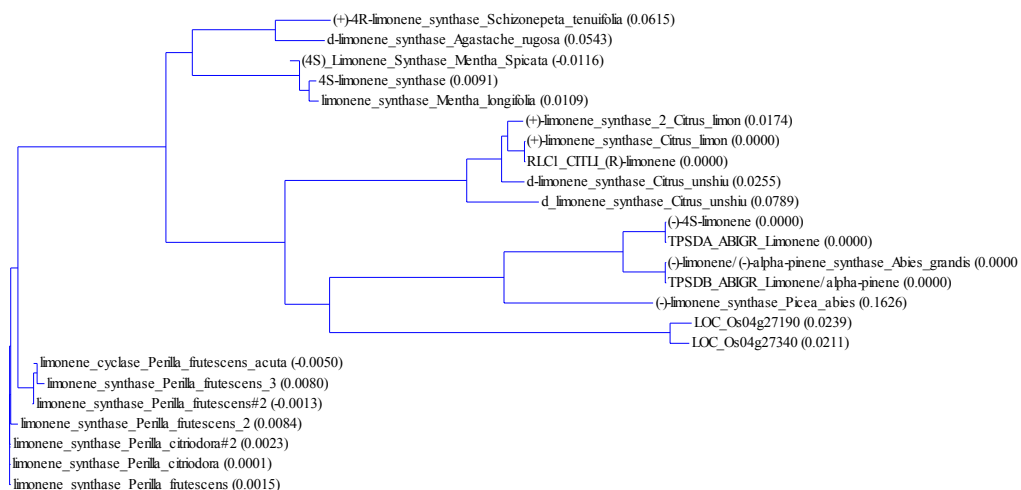


Figure 3.6. Phylogenic analysis of limonene synthase genes across the species.

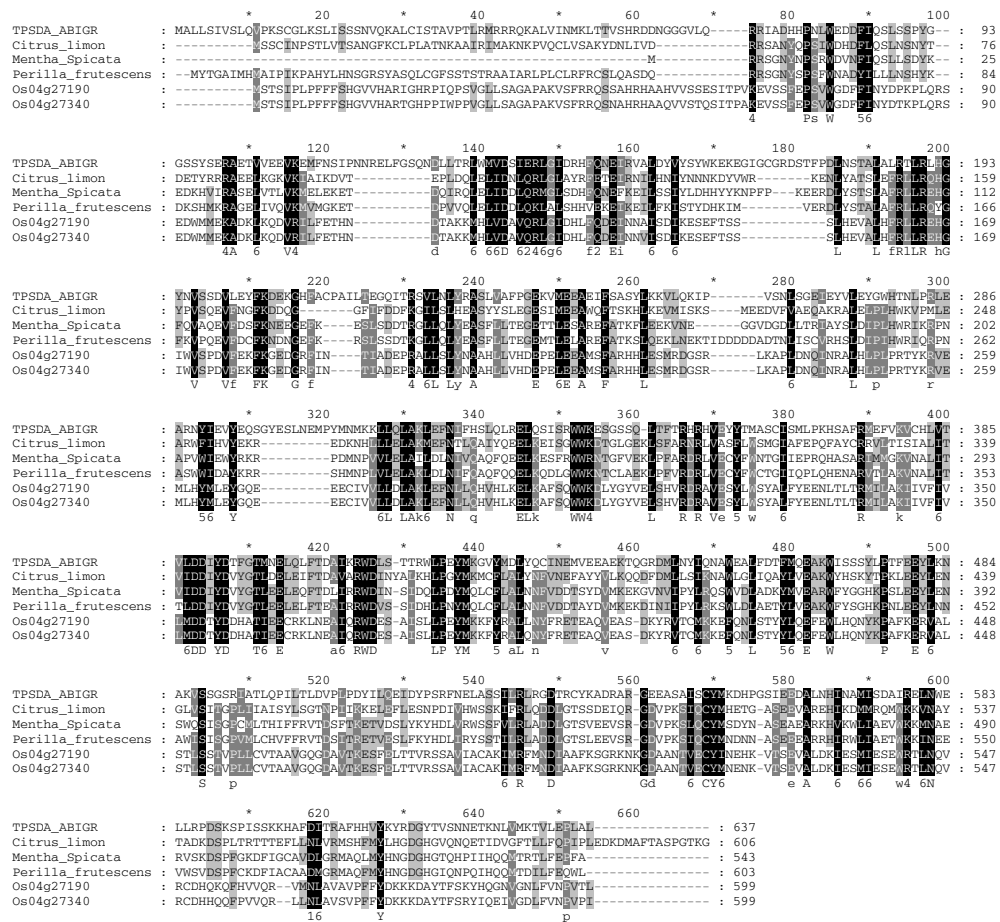


Figure 3.7. Multiple sequence alignment of representative limonene sequence from multiple species.

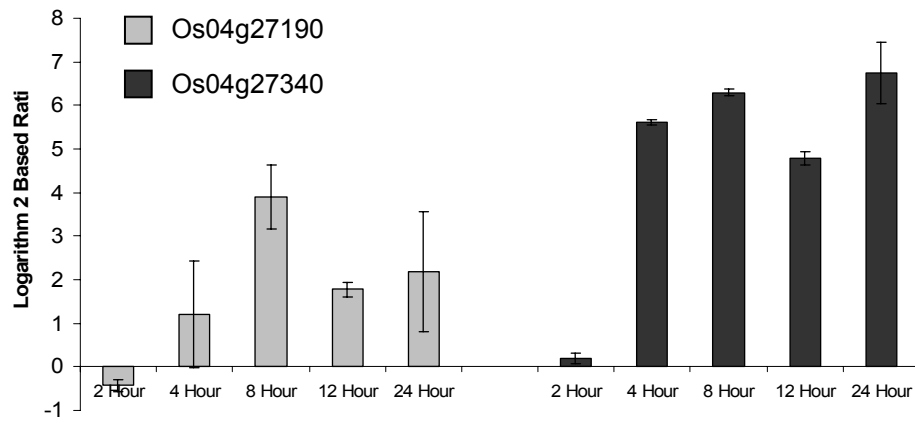


Figure 3.8. Gene expression of Os04g27190 and Os04g27340 under jasmonic acid treatment.

Chapter IV. Variation in the Defense Strategy of Plants during Day and Night: Emission Dynamics of Insect-induced Plant Volatiles and Transcriptomic Changes

Adapted from:

Yuan J.S. and Chen F., Variation in the Defense Strategy of Plants during Day
and Night: Emission Dynamics of Insect-induced Plant Volatiles and
Transcriptomic Changes, Drafted.

Abstract

Volatile terpenoids play important functions in rice indirect defense against herbivorous insects. The regulation of volatile emission and terpene synthase (*TPS*) gene was studied with respect to diurnal cycle. Diurnal cycle dependent volatile emission pattern has been found with both insect and jasmonic acid induced response. The gene expression analysis indicated that *TPS* gene expression regulation only accounts partially for the diurnal cycle dependent terpenoid volatile emission pattern. Global gene expression profiling revealed commonly regulated and differentially regulated insect defense genes during the night as compared to the day time. One key gene in the mevalonate pathway and one in non-mevalonate pathway were found with an on and off gene expression pattern between day time response and night time response. Overall, our results indicated that diurnal cycle dependent volatile emission may be resulted from both substrate level regulation and gene expression level regulation. The evolutionary perspective of the diurnal cycle dependent volatile emission is also discussed.

Introduction

Volatile terpenoids represent an important group of volatile organic compounds involved in the plant indirect defense against herbivorous insects (Pichersky et al., 2006; Schnee et al., 2006). It has been widely accepted that the biosynthesis of terpenoid volatiles can be induced by herbivore damage and the contents and compositions of the synthesized volatiles depend on plant species, herbivorous insect species, herbivorous insect developmental stages, plant developmental stages and such (Chapter I). The function of terpenoid volatiles can be studied for their capacities to attract natural enemies of herbivorous insects (Vet and Dicke, 1992; Agrawal, 1998; Kessler and Baldwin, 2001). Many terpenoid volatiles including linalool and limonene are able to attract predators and parasitoids of herbivorous insects and thereby serve as important components of the plant indirect defense (Kessler and Baldwin, 2001). Most of the volatile terpenoids are either monoterpene (10 carbons) or sesquiterpene (15 carbons). Despite the diverse structure of terpenoid volatiles, almost all of volatile terpenoids are synthesized by terpene synthases (Chen et al., 2003; Tholl, 2006).

Gene expression pattern profiling of terpene synthase genes is a major part of the study of biological functions of terpene synthase in defense (Bede et al., 2006; Ro et al., 2006; Tholl, 2006; van Schie et al., 2007). The gene expression pattern of terpene synthase genes in response to herbivore damage, elicitor and plant hormone treatments can help elucidate the gene and volatile functions in the induced plant defense. Besides herbivorous insect treatments, pathogen

infection, physical wounding, elicitor treatments and jasmonic acid treatments can all induce terpenoid emission (Baldwin et al., 2001; Schmelz et al., 2003; Lou et al., 2005; Keeling and Bohlmann, 2006; Liechti and Farmer, 2006). Moreover, it was also believed that the jasmonic acid induced terpenoid volatile production has a diurnal cycle dependent emission pattern (Cheng et al., 2007). Terpenoid volatiles normally emitted at a much larger amount during the daytime as compared to that of the night time (Martin et al., 2003; Cheng et al., 2007). However, the gene level regulation of such emission pattern has not been well studied.

Plant gene expression changes in diurnal cycles is uniquely entangled with photosensing and circadian rhythm effects, and the change in circadian rhythm and photoperiod can result in developmental consequences such as flowering (Harmer et al., 2000; Alabadi et al., 2001; Forger and Peskin, 2003; Gould et al., 2006). Earlier work employs the global gene expression profiling to describe the coordinative oscillation of photosynthesis, metabolite biosynthesis, and developmental genes which built up a metabolite network changed during diurnal cycle (Gibon et al., 2006). Previous study has shown the dynamic changes of volatile emission during the diurnal cycle in snapdragon flowers (Dudareva et al., 2003; Dudareva et al., 2005). Besides flower volatile production, jasmonic acid and methyl jasmonate induced terpenoid volatile production was also shown to be diurnal cycle dependent in Norway spruce and rice (Martin et al., 2003; Cheng et al., 2007). Insect induced terpenoid volatile

emission was also found to be diurnal cycle dependent, and the pattern was believed to result from photo effects (Gouinguene and Turling, 2002). Other work indicated that the emission of isoprene was controlled by gene expression of isoprene synthase and influenced by both circadian rhythm and photosensing. Regardless of the previous research, the regulation of circadian rhythm dependent insect induced terpenoid volatile emission is still not clear. Few studies have been focused on the gene expression level regulation of terpenoid biosynthesis during diurnal cycle. The diurnal cycle dependent volatile emission could be due to the gene expression of *TPS* genes, substrate availability, enzyme modification and emission control mechanisms. Moreover, a comparison of the gene expression profiling for the induced response at different time points of diurnal cycle is still lacking.

In this article, we use rice as a model to study the mechanisms controlling the diurnal cycle dependent terpenoid volatile emission mainly at the gene expression level with volatile profiling, gene expression study and global gene expression profiling. Our study will answer the question whether *TPS* gene expression is the key in terpenoid volatile emission control, or pathway level regulation is more important. Moreover, we will also study the differential global gene expression changes of day time and night time insect induced response in rice. Although a previous study has indicated the circadian rhythm dependence of the jasmonic acid induced terpenoid volatile emission in rice, the study is limited to two terpenoids only. Moreover, all previous studies of diurnal

cycle dependent induced responses in rice were limited to jasmonic acid or methyl jasmonate induced responses. No insect induced volatile terpenoid emission has been studied at the gene expression level. Our previous study has shown that rice produces more than ten different terpenoid volatiles in response to herbivorous insect damage and jasmonic treatment and these volatiles are mainly produced by five genes. We hereby focus on the volatile emission pattern of all major products of three genes in response to both generalist herbivorous insect rice fall armyworm (*Spodoptera frugiperda*) damage and plant defense hormone jasmonic acid treatments. Furthermore, global gene expression profiling was also carried out to characterize the transcriptomic level response during night as compared to the daytime.

Overall, we have found a diurnal cycle dependent terpenoid volatile production pattern in insect damaged rice. Terpenoid products from various genes respond differently to the diurnal cycle. The changes of terpenoid volatile emission only partially correlated with the changes of *TPS* gene expression pattern. Global gene expression profiling indicated a slightly differential induced metabolic pathway profiling during the night time as compared to the daytime. Key genes in mevalonate and non-mevalonate pathways were found to be a potential switch for the diurnal cycle dependent terpenoid volatile emission. Moreover, jasmonic acid induced volatile emission pattern shows a similar diurnal cycle response, which can also be partially correlated to gene expression pattern. The results indicated a combined regulation of *TPS* gene expression and key up-stream

pathway genes accounted for the regulation of terpenoid volatile emission during the diurnal cycle. The molecular mechanisms and evolutionary context of the diurnal cycle dependent terpenoid volatile emission are discussed.

Material and Methods

Plants, insects and plant treatments

Rice (*Oryza sativa* ssp. *Japonica* cv. *Nipponbare*) seeds were dehulled and germinated at 30 °C in the dark for five days. The seedlings were planted with eight plants per 60 mL fisher glass jars and grown at 26 °C with 12 hours of light for two weeks. The light cycle started at 9:00AM and ended at 9:00PM. Fall armyworm (*Spodoptera frugiperda*) was used as the herbivore model. FAW eggs were incubated on moist filter paper, and emerged FAW larvae were reared on an artificial diet. Second-instar FAW were used for herbivore treatment. Two larvae were placed on the leaves of a single two-week-old rice seedling at 3:00 AM. After 24 hours, about 20% of leaf area was consumed. Insects were removed and the rice plants were subject to tissue collection for RNA extraction. For jasmonic acid treatment, the same developmental stage rice plants were applied with 10mM jasmonic acid at both side of the leaves. The plants were placed in air for ten minutes and then put into chambers for volatile collection.

Volatile analysis

Volatiles emitted from FAW-damaged rice plants, jasmonic acid treated rice plants and control rice plants were collected in an open headspace sampling system (Analytical Research Systems, Gainesville, FL, USA). Eight plants grown in a single glass jar wrapped with aluminum foil were placed in a glass chamber of 3 inches in diameter and 10 inches in height that consisted of a removable O-ring snap lid with an air outlet port. Charcoal-purified air entered

the chamber at a flow rate of 0.8 L/min from the top through a Teflon hose. Volatiles were collected for 4 h by pumping air from the chamber through a Super Q volatile collection trap (Analytical Research Systems, Gainesville, FL, USA). Volatiles were eluted with 40 μ L of CH_2Cl_2 , and 1-Octanol was added as an internal standard as previously described (Mitchell and McCashin, 1994). The volatile collection conditions were set according to the requirements of the experiments as consistent light, or light and dark cycle with 12 h light.

Samples from volatile collections were analyzed on a Shimadzu 17A gas chromatograph coupled to a Shimadzu QP5050A quadrupole mass selective detector. Separation was performed on a DB5 column of 30 mm x 0.25 mm i.d. x 0.25 μ m thickness. Helium was the carrier gas (flow rate of 5 mL/min), a splitless injection (injection volume of 5 μ L) was used, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 240°C was applied. The identities of compounds were determined by comparison of retention times and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology and Wiley libraries (Agilent Technologies, Palo Alto, CA, USA).

RNA isolation

Total RNA was isolated from appropriate rice tissues using Plant RNA Isolation Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA contamination was removed with an on-column DNase (Qiagen,

Hilden, Germany) treatment. Isolated total RNA was used for real-time PCR analysis, gene cloning, and microarray experiments.

Microarray experimentation

The NSF rice half genome oligonucleotide array (Version 2.0) provided by UC Davis microarray core facility was used for global gene expression profiling. Messenger RNA was isolated from total RNA using Oligotex mRNA kit (Qiagen, Hilden, Germany). One microgram of mRNA was labeled with Superscript III direct Labeling Kit (Invitrogen, Carlsbad, CA, USA) according to the instruction of the manufacturer. The purified probes were mixed and hybridized with the long-oligo microarrays using the Microarray Hybridization Kit (Corning, Corning, NY, USA) according to the manufacturer's instruction and the protocol provided by UC, Davis (<http://www.ricearray.org>). Reverse labeling experiments were included to eliminate dye-specific bias. For each sample set of FAW-treated rice versus control, the treated mRNA was first labeled with Cy5 and the control with Cy3. In the reverse experiment, the labeling dyes were swapped. The labeling reactions and dye swapped microarray hybridizations were performed in parallel. Considering the reverse labeling experiments, a total of three biological replicates and two technical replicates are included.

After hybridization, the microarray slides were washed and scanned in GenePix 4000 scanner (Axon Instrument, Union City, CA, USA), and the image was

processed by GenePix Pro software (Axon Instrument, Union City, CA, USA). The microarray gpr files obtained were analyzed with R-based open source software Bioconductor (<http://www.bioconductor.org>), where local background subtraction and Lowess normalization were performed for each microarray slide. Linear models from the limma package of Bioconductor were applied to derive a p value and average of logarithm 2-based ratio across six slides. Changes in gene expression pattern were considered statistically significant at $p < 0.01^*$. A ratio cutoff of 2 and degree of freedom higher or equal to three were included as quality controls

Quantitative real-time PCR

Quantitative real-time PCR experiments were carried out as previously described (Yang et al., 2006; Yuan et al., 2006). The primers for target genes were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA) and the primer sequences were as shown in Chapter II.

Results

Insect Induced Terpenoid Volatile Emission during Diurnal Cycle

As previously described, fall armyworm induces emission of a variety of terpenoid volatile compounds in japonica rice, and these compounds are believed to be important in rice indirect defense (Chapter II). The compound profile includes limonene, linalool, beta-elemene, beta-caryophyllene, Zigerbrene, beta bisabolene, and others. In our previous report, we have shown that limonene is induced in jasmonic acid response in a time-dependent manner. However, the insect induced limonene emission is either trivial or not detected. In the study of diurnal cycle controlled terpenoid volatile emission in response to insect damage, we only included six compounds, linalool, beta-elemene, beta-caryophyllene, Zigerbrene, beta-bisabolene, sesquiphellandrene, which are the major gene products for *OsTPS3*, *OsTPS42* and *OsTPS44*.

As shown in Figure 4.1A, beta-caryophyllene and beta-elemene showed a somehow clear diurnal cycle dependent emission. Both volatile sesquiterpenes are products of enzyme *OsTPS42*. Linalool also showed a diurnal cycle dependent emission with a slightly higher baseline during night. However, the three sesquiterpene products from *OsTPS44* (zigerberene, beta-bisobalene, and sesquiphellandrene) sometimes show up during the night time. Figure 4.1B showed the emission of the volatiles under consistent light. The terpenoid volatile emission patterns were generally preserved with a higher baseline

expression, which indicated that both circadian rhythm and photosensing have an effect on the emission of terpenoid volatiles.

Gene Expression of Terpene Synthase Genes during Diurnal Cycle

Three terpene synthases have been characterized for the biosynthesis of most terpenoid volatiles emitted in japonica rice upon insect treatment as shown in Chapter II. Real-time PCR experiments were carried out to study the gene expression pattern of these three terpene synthases during the diurnal cycle after the insect treatments. As shown in Figure 4.2, essentially all three genes expressed at a lower level at 18 hour, which is the mid-night. However, only one gene, *OsTPS42*, shows strong diurnal cycle pattern, while the other two genes are high at both twilight (12 hours and 24 hours) and mid-day (6 hours). The baseline level expression for both *OsLIS* and *OsTPS44* are high. The diurnal cycle dependent gene expression somehow correlated with volatile emission pattern, where the product of *OsTPS42* shows the strongest diurnal cycle dependent effects.

Global Gene Expression Pattern of Insect Induced Responses at Night

In order to further understand the differential gene expression regulation during the daytime as compared to the night time, global gene expression profiling with half-genome rice long oligo microarray was carried out on plants treated with fall armyworm for 24 hours (Table 4.1). The insect treatment was started during the night time and the biological samples were collected at six hour intervals

after dark during a 12 hour light/dark cycle. The gene profiling results were summarized in Figure 4.3 and 4.4.

As shown in Figure 4.3, a variety of genes including metabolic enzymes, transcriptional factors, defense proteins, signal transduction pathway components were up-regulated. The pattern of up-regulation is similar to that of the daytime insect induced expression as previously described. The cross analysis revealed that 108 genes were shared by both daytime and night induced insect treatments and 225 and 129 genes were night time specific and day time specific, respectively. Moreover, cross analysis of insect defense and early jasmonic acid induced gene expression pattern indicated that early jasmonic acid pathway induces a much weaker response as compared to the insect induced response. Therefore, much fewer genes were shared by early jasmonic acid treatment with the two 24 hour insect treatment experiments. It may be true that a longer jasmonic acid treatment will induce stronger gene expression changes.

Figure 4.5 shows the cluster analysis of genes differentially expressed among the three different treatments. As revealed by the analysis, many of the genes are up-regulated or down-regulated in the similar pattern between daytime and night time treatment. These gene shared two clusters of commonly up-regulated genes. A detailed examination of these genes showed many insect defense specific genes including protease inhibitors, defense pathways, terpene synthase genes and such.

Despite the high similarity between the night and light induced gene expression, there were still two clusters of genes with night or day time specific gene expression pattern. Many of these genes were signal transduction pathway components, which indicated the differential regulatory mechanisms during daytime as compared to night time. Figure 4.6 showed the pathway analysis of one terpenoid relevant gene. As shown by Figure 4.6, two key genes in the upstream terpenoid biosynthesis, DXR and HMG reductase, were differentially regulated during the daytime as compared to the night time. The gene is up-regulated in a daytime specific pattern.

Jasmonic Acid Induced Terpenoid Volatile Emission during Diurnal Cycle

As compared to insect treatments, jasmonic acid treatments are better controlled and have been previously studied for diurnal cycle dependent volatile emission. We have carried out volatile profiling for jasmonic acid induced response as shown in Figure 4.6. The diurnal cycle dependent terpenoid volatile emission is clearer than that of the insect induced pattern for linalool, but not other compounds. The emission patterns were examined in three different settings, consistent light, treatment in the morning for light and dark cycle, and treatment in the evening for light and dark cycle. In both light and night cycle, the diurnal cycle dependent emission of linalool is early, however, in the consistent light condition, the emission of linalool seemed to expand to the supposed night time, which against indicate that the emission of terpenoid is influenced by both

circadian rhythm and photosensing. Real-time PCR was carried out, yet the results are similar to those in the insect treatment, with no strong diurnal dependent pattern seen (data not shown).

Discussion

In this chapter, we first examined the effects of diurnal cycle on the insect induced volatile emission, and then explored the global gene expression profiling of insect induced responses during night. In addition, jasmonic acid induced volatile emission and gene expression profiling during the diurnal cycle were also examined. The features of diurnal cycle dependent terpenoid volatile emission and gene expression were revealed. The implication of the pattern can be viewed from several perspectives.

The Light and Diurnal cycle Dependent Volatile Emission

Although diurnal cycle dependent volatile emission has been reported, most of the previous researches were focused on volatile emission in flower or emission during the jasmonic acid treatments (Kolosova et al., 2001; Dudareva et al., 2003; Martin et al., 2003; Raguso et al., 2003). No research has been carried out to characterize the insect induced volatile emission pattern in plants. In this research, we examined both insect induced and jasmonic acid induced volatile emission during the diurnal cycle. The diurnal cycle dependent volatile emission has been discovered with insect treated rice. The diurnal cycle dependent emission of terpenoid volatile in rice can be slightly influenced by light, where the base level emission is higher in consistent light conditions. However, the pattern of volatile emission can still be observed in consistent light, which indicates that the emission is dependent on both circadian rhythm and photosensing.

It should be noted that not all terpenoid volatiles shared the same pattern of diurnal cycle dependent emission, which indicates that terpenoid biosynthesis regulation and volatile emission are different. Our previous research indicated that the stress response cis-elements distributed differentially among *OsLIS*, *OsTPS42* and *OsTPS44*, which suggested a different regulation of *TPS* genes in the defense process. Diurnal cycle dependent volatile biosynthesis and emission may be different due to the differential gene regulation.

The diurnal cycle dependent terpenoid volatile emission fits into the schema of terpenoid biosynthesis perfectly. In monoterpene synthase, carbon flow from the calvin cycle through glyceraldehyde-3-phosphate (G3P) and pyruvate toward the GPP biosynthesis via non-mevalonate (or MEP) pathway, where GPP serves as the common precursors in the monoterpene synthesis (Tholl, 2006). MEP pathway was also indicated to provide substrate for sesquiterpene synthase through IPP transferring. During the daytime, photosynthesis rate is normally higher, and more G3P will be available from carbon dioxide fixation, which allows more GPP production through the MEP pathway (Tholl, 2006). On the other side, photosynthesis is shut down during night, and inadequate amount of G3P will be available from calvin cycle, which will limit the biosynthesis of GPP and monoterpenes. The diurnal cycle dependent volatile emission may be a reflection of resource availability.

The Differences between Insects and Jasmonic Acid and Insect Induced Volatile Emission

Overall, jasmonic acid and insect induced similar patterns of volatile emission for linalool, but not for the products of *OsTPS42* and *OsTPS44*. The diurnal cycle dependent emission of these products are also different in levels, where insect damage induced much higher volatile emission as well as the baseline level emission. During night, most of the terpenoids cannot be found in jasmonic acid induced emission pattern, yet the products of *OsTPS44* were emitted consistently at relatively high level during the night time. The different pattern indicated a differential regulatory mechanism among the *TPS* genes during the insect defense (Liechti and Farmer, 2006; Liechti et al., 2006). Plant defense against insect has long been speculated to be mainly mediated by jasmonic acid; however, the differences of insect and jasmonic acid induced volatile emission pattern pointed out the potentially important jasmonic acid independent regulation of plant defense against herbivorous insects.

Regulation of Induced Volatile Emission

The regulation of diurnal cycle dependent volatile emission was studied previously from the perspective of expression of *TPS* genes. Some *TPS* genes have been shown to be expressed in a diurnal cycle dependent manner (Martin et al., 2003; Cheng et al., 2007). However, our results revealed that the gene expression level of regulation is important, yet *TPS* gene may not be the key regulatory point. The expression of *TPS* genes did fluctuate during the diurnal

cycle. Nevertheless, the insect induced gene expression of *TPS* genes, especially the *OsLIS* gene, exhibited a high baseline level. The baseline level of gene expression induction is strong enough to lead to strong terpenoid volatile emission. Therefore, other mechanisms may be involved in the regulation of terpenoid volatile emission.

Substrate availability was proposed to be another level of regulation. As aforementioned, the level of calvin cycle may be important for monoterpene biosynthesis, which can partly explain the light dependent terpenoid volatile emission. On the other hand, the diurnal cycle cannot be readily explained because the volatile emission in consistent light still shows the diurnal cycle dependent pattern. The examination of up-stream terpene biosynthesis gene led to find the 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR or IspC) gene to be differentially regulated during the day time and night time. Most of the other terpenoid biosynthesis genes are up-regulated in both daytime and night time. The 1-deoxy-d-xylulose-5-phosphate reductoisomerase is the second enzyme in the MEP pathway and it is the only copy of DXR in rice genome. The enzyme has been indicated to be one of the speed limiting enzymes since it is the first committed step for GPP biosynthesis (Tholl, 2006). DXR is the second step enzyme in the pathway as shown in Figure 4.5. Previous research has shown the tissue specific expression of both DXPS and DXR, but has not for some down-stream enzymes such as HMGR. DXPS was previously shown to express differentially following the diurnal cycle in snapdragon flower.

However, our microarray experiments showed no differential expression of DXPS during the diurnal cycle (Dudareva et al., 2005). The on and off expression pattern of DXR implied that the gene may be a key enzyme controlling the substrate availability and potentially important in the control of diurnal cycle dependent volatile emission.

Besides the DXR gene, other mechanisms may be involved in the diurnal cycle dependent terpenoid volatile emission. These include the enzyme modification and emission control. Considering that plant defense depends on a synergetic up-regulation of multiple genes, it might be more efficient to down-regulate one or two key enzymes in the pathway instead of down-regulating the entire pathway to regulate the diurnal cycle dependent volatile emission in the induced responses.

The Evolutionary and Ecological basis for Diurnal cycle Dependent Volatile Emission

The diurnal cycle dependent terpenoid volatile emission has clear evolutionary advantages for plants. Most of the parasitoids and predators forage or parasitize during the day time with both olfactory and visual cues to increase their efficiency to locate the herbivorous insects. The volatile emission during the day time is therefore particularly important to increase the fitness of natural enemies and decrease the fitness of herbivorous insects in the tritrophic interaction. Additionally, from a resource availability perspective, the carbon resource is

more abundant during the daytime, and thus carbon based indirect defense may be more readily evolved as compared to the often nitrogen based direct defense from a micro-system scale. The lower emission of terpenoid volatile compounds at night may help to reserve resources for plants, since there is always a balance of plant defense and growth. On one side, plants try to release different compounds to kill or repel the herbivorous insects directly or indirectly, on the other side, plants develop mechanisms to out-grow the herbivorous insect damage. The diurnal cycle dependent emission of volatile terpenoid will allow the preservation of energy which is necessary for plant growth to adapt to insect damages. Furthermore, some herbivorous insects have been shown to be able to use plant emitted volatile compounds including terpenoids as cues to locate their food or avoid competition to increase their fitness (De Moraes et al., 2001; Kessler and Baldwin, 2001). Since fall armyworms are nocturnal, it is therefore important for the plants to emit volatile terpenoid when necessary, and not to emit when it may cause danger. The diurnal cycle dependent volatile terpenoid emission therefore may help to increase the fitness of plants.

The Global Gene Expression Pattern

The comparison of global gene expression profiling between day and night time shows a similarity between the day time and night time insect induced gene expression pattern. In particular, the cluster of similarly highly expressed genes was mostly insect defense specific genes. The similarity indicated that the transcriptional level regulation is mostly consistent during the diurnal cycle and

the induced defense response is not diurnal cycle dependent. Overall, the differences in volatile profiling may just be due to the differential expression of the key genes in the pathway instead of the entire pathway, which will best relocate the resource in plant to balance defense and growth.

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Appendix

Table 4.1. Rice genes up-regulated in rice plants when damaged by FAW during the night.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
Terpene synthase family, metal binding domain, putative	LOC_Os04g27190	TR009868	5.77	0.50	0.00	54.39
Terpene synthase family, metal binding domain, putative	LOC_Os08g07100	TR015140	5.43	0.60	0.00	43.24
O-methyltransferase, putative	LOC_Os10g02880	TR016885	5.41	0.37	0.00	42.44
Terpene synthase family, metal binding domain, putative	LOC_Os08g07080	TR015138	5.33	0.43	0.00	40.34
Putative antifungal zeamatin-like protein	LOC_Os03g46070	TR008151	5.20	0.45	0.00	36.77
expressed protein	LOC_Os01g15340	TR000846	5.03	0.72	0.00	32.60
Bowman-Birk serine protease inhibitor family, putative	LOC_Os01g03680	TR000159	4.94	0.61	0.00	30.70
transposon protein, putative, unclassified	LOC_Os10g37160	TR018042	4.94	1.18	0.00	30.70
Similar to plant metallothionein-like protein	LOC_Os12g38010	TR020039	4.80	0.57	0.00	27.94
hypothetical protein	AK059202	TR018561	4.79	1.68	0.00	27.71
SAM dependent carboxyl methyltransferase	LOC_Os02g48770	TR005327	4.77	1.45	0.00	27.22
Potato inhibitor I family	LOC_Os01g42860	TR001909	4.76	0.90	0.00	27.17
contains similarity to hedgehog-interacting protein~gene_id:MYH19.17	LOC_Os12g37200	TR019985	4.73	0.64	0.00	26.45
Helix-loop-helix DNA-binding domain, putative	LOC_Os04g23550	TR009780	4.69	1.16	0.00	25.86
LEA protein - rice	LOC_Os05g46480	TR012195	4.53	0.80	0.00	23.03
expressed protein	LOC_Os07g34280	TR013974	4.49	0.68	0.00	22.49
contains ESTs	LOC_Os01g09220	TR000489	4.47	0.16	0.00	22.21
oxidoreductase, zinc-binding dehydrogenase family	LOC_Os04g15920	TR009650	4.45	0.45	0.00	21.93
Protein kinase domain, putative	LOC_Os08g28710	TR015823	4.43	0.74	0.00	21.53
C2H2-type zinc finger protein ZFP36	LOC_Os03g32230	TR007746	4.38	1.69	0.00	20.79
leucoanthocyanidin dioxygenase-like protein	LOC_Os03g18030	TR007127	4.36	1.24	0.00	20.50
monosaccharide transporter 4 22	LOC_Os03g11900	TR006722	4.27	0.49	0.00	19.29
expressed protein	LOC_Os05g44060	TR012111	4.17	0.62	0.00	18.01
expressed protein	LOC_Os03g52410	TR008494	4.16	0.51	0.00	17.89
Putative hydrolase Oryza sativa (japonica cultivar-group)	LOC_Os03g61360	TR009029	4.15	0.36	0.00	17.81
Glycosyl hydrolases family 17	LOC_Os01g71340	TR003576	4.11	0.86	0.00	17.26
expressed protein	LOC_Os03g22820	TR007441	4.10	0.29	0.00	17.09
hypothetical protein	LOC_Os03g48400	TR008264	4.03	0.26	0.00	16.32
Similar to lipoxygenase	LOC_Os12g37320	TR019991	4.02	0.64	0.00	16.23
expressed protein	LOC_Os03g15270	TR006937	4.01	0.34	0.00	16.13
Jacalin homolog - barley	LOC_Os12g14440	TR019445	4.01	0.63	0.00	16.08
Chalcone and stilbene synthases, C-terminal domain, putative	TA65650_4530	TR017039	3.98	1.74	0.00	15.82
Putative esterase	LOC_Os03g57640	TR008795	3.94	1.20	0.00	15.36
AMP-binding enzyme, putative	LOC_Os04g58710	TR011439	3.92	0.27	0.00	15.11
probable wrky transcription factor 62 (wrky dna-binding protein 62).	LOC_Os11g02520	TR018424	3.90	1.39	0.00	14.93
Peroxidase	LOC_Os11g02100	TR018406	3.89	0.56	0.00	14.87
Lipoxygenase L-2; lipoxygenase	LOC_Os03g52860	TR008522	3.88	1.15	0.00	14.69
proteinase inhibitor - rice	LOC_Os03g03810	TR006200	3.88	0.55	0.00	14.69
AP2 domain, putative	LOC_Os04g32620	TR010065	3.87	0.10	0.00	14.60
contains EST AU031368(E61432)						
unknown protein	LOC_Os01g50940	TR002330	3.86	0.66	0.00	14.48
expressed protein	LOC_Os03g47280	TR008206	3.84	0.85	0.00	14.33
cold regulated protein	LOC_Os05g39250	TR012002	3.82	1.37	0.00	14.09
expressed protein	LOC_Os10g35770	TR017969	3.81	0.51	0.00	14.06

Table 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
OsNAC5 protein [imported] – rice	LOC_Os05g34830	TR011901	3.72	1.41	0.00	13.17
Unknown protein	LOC_Os03g08310	TR006501	3.71	0.53	0.00	13.04
Ribosome inactivating protein	LOC_Os01g06740	TR000345	3.68	0.31	0.00	12.81
Similar to sesquiterpene synthase 1	LOC_Os04g01810	TR009269	3.65	1.26	0.00	12.51
expressed protein	LOC_Os10g36180	TR017992	3.59	0.76	0.00	12.07
Unknown protein	LOC_Os02g32580	TR004757	3.56	0.75	0.00	11.83
Bowman-Birk serine protease inhibitor family, putative	LOC_Os01g04050	TR000179	3.56	0.96	0.00	11.76
Hypothetical protein	LOC_Os12g37150	TR019983	3.54	0.82	0.00	11.62
Zinc finger, C2H2 type, putative	LOC_Os05g37190	TR011962	3.53	1.59	0.00	11.57
expressed protein	LOC_Os01g58130	TR002802	3.53	0.47	0.00	11.54
Terpene synthase family, metal binding domain, putative	TA63906_4530	TR009877	3.53	0.96	0.00	11.53
Similar to probable WRKY-type DNA binding protein [imported]	LOC_Os11g02540	TR018425	3.52	0.78	0.00	11.46
expressed protein	LOC_Os01g50350	TR002298	3.52	1.16	0.00	11.45
expressed protein	LOC_Os03g03200	TR006160	3.51	0.39	0.00	11.36
UDP-glucuronosyl and UDP-glucosyl transferase	LOC_Os02g51930	TR005546	3.50	0.63	0.00	11.31
expressed protein	LOC_Os03g32420	TR007758	3.48	1.20	0.00	11.18
expressed protein	LOC_Os11g10800	TR018575	3.42	0.15	0.00	10.68
Eukaryotic-type carbonic anhydrase	LOC_Os08g36630	TR016183	3.41	0.63	0.00	10.65
Histone deacetylase family, putative	LOC_Os02g12380	TR004163	3.37	0.16	0.00	10.35
expressed protein	LOC_Os01g51670	TR002374	3.33	0.33	0.00	10.08
1-deoxy-D-xylulose-5-phosphate synthase	LOC_Os07g09190	TR013099	3.33	2.64	0.01	10.07
Zinc finger, C3HC4 type (RING finger), putative	LOC_Os02g52210	TR005560	3.31	0.96	0.00	9.93
putative sugar-starvation induced protein	LOC_Os03g49440	TR008314	3.28	0.46	0.00	9.72
putative roteinase inhibitor	LOC_Os03g52390	TR008492	3.26	0.37	0.00	9.56
expressed protein	LOC_Os04g32480	TR010053	3.26	0.34	0.00	9.56
2-oxo acid dehydrogenases acyltransferase (catalytic domain), putative	LOC_Os01g21160	TR001076	3.24	0.30	0.00	9.44
heavy metal-associated domain, putative	LOC_Os03g05750	TR006337	3.21	0.23	0.00	9.22
Hypothetical protein	AK121553;AK058841	TR009327	3.20	0.37	0.00	9.16
ATPase, AAA family, putative	LOC_Os05g51130	TR012244	3.18	1.00	0.00	9.04
diaminopimelate epimerase	LOC_Os12g37960	TR020035	3.16	0.82	0.00	8.97
aminotransferase, class III	LOC_Os04g52440	TR011087	3.15	0.94	0.00	8.89
WRKY DNA –binding domain, putative	LOC_Os01g43650	TR001960	3.15	0.35	0.00	8.87
myb protein homolog – rice	LOC_Os04g43680	TR010597	3.15	0.37	0.00	8.86
Probable submergence induced protein 2 – rice	LOC_Os10g28350	TR017602	3.11	0.23	0.00	8.66
Universal stress protein family, putative	LOC_Os02g47650	TR005257	3.11	0.37	0.00	8.60
Glutathione S-transferase, N-terminal domain, putative	LOC_Os01g72120	TR003601	3.07	0.46	0.00	8.39
Pyridine nucleotide-disulphide oxidoreductase, putative	LOC_Os08g04630	TR015031	3.05	1.10	0.00	8.27
Cytochrome P450	LOC_Os08g39730	TR016354	3.04	0.57	0.00	8.21
F13B4.1 protein – Arabidopsis thaliana, putative	LOC_Os08g31860	TR015969	3.03	0.53	0.00	8.19
GH3 auxin-responsive promoter	LOC_Os01g55940	TR002662	3.03	0.29	0.00	8.17
contains ESTs	LOC_Os01g45640	TR002025	2.98	0.39	0.00	7.87
Hypothetical protein	LOC_Os01g05540	TR000275	2.97	0.63	0.00	7.84
No apical meristem (NAM) protein, putative	LOC_Os01g60020	TR002912	2.95	0.38	0.00	7.74
root specific pathogenesis-related protein 10	LOC_Os12g36830	TR019969	2.94	0.58	0.00	7.69
aminotransferase, putative	LOC_Os02g02210	TR003841	2.92	0.95	0.00	7.56
Clp amino terminal domain, putative	LOC_Os02g32520	TR004753	2.92	0.76	0.00	7.55
Peroxidase, putative	LOC_Os07g48020	TR014741	2.91	0.33	0.00	7.53
putative protein kinase	LOC_Os10g33040	TR017812	2.88	1.30	0.00	7.36
Hypothetical protein	chr12:23925573	TR020085	2.88	0.30	0.00	7.35
Core-2/1-Branching enzyme, putative	LOC_Os04g23580	TR009781	2.88	0.25	0.00	7.34

Tab 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
expressed protein	LOC_Os01g45250	TR002002	2.87	0.19	0.00	7.30
expressed protein	LOC_Os05g08620	TR011721	2.86	0.55	0.00	7.27
No apical meristem (NAM) protein, putative	LOC_Os07g12340	TR013252	2.86	0.65	0.00	7.24
putative thiolase	LOC_Os10g31950	TR017765	2.84	0.38	0.00	7.17
AMP-binding enzyme, putative	LOC_Os03g03790	TR006199	2.84	1.04	0.00	7.15
Lipoxygenase	LOC_Os12g37260	TR019987	2.83	2.03	0.00	7.11
1-aminocyclopropane-1-carboxylate synthase (EC 4.4.1.14) 2 - wheat	LOC_Os04g48850	TR010908	2.83	0.25	0.00	7.11
Hydrolase, alpha/beta fold family, putative	LOC_Os05g46460	TR012193	2.81	0.67	0.00	7.01
expressed protein	LOC_Os03g58850	TR008874	2.80	0.61	0.00	6.95
Late embryogenesis abundant protein	AF323612	TR001082	2.78	0.61	0.00	6.89
Bowman-Birk serine protease inhibitor family, putative	LOC_Os01g03320	TR000144	2.77	0.47	0.00	6.84
Pathogen-related protein	LOC_Os03g18850	TR007189	2.76	0.23	0.00	6.79
zinc finger domain, LSD1 subclass, putative	N/A	TR015092	2.76	0.59	0.00	6.78
Sialyltransferase family, putative	LOC_Os01g63970	TR003149	2.75	0.26	0.00	6.72
expressed protein	LOC_Os02g40700	TR005082	2.75	0.40	0.00	6.72
Subtilase family, putative	LOC_Os01g58290	TR002812	2.73	0.41	0.00	6.64
putative ammonium transporter	LOC_Os03g62200	TR009079	2.73	1.25	0.00	6.64
Similar to lob domain protein 1	LOC_Os03g17810	TR007112	2.72	0.85	0.00	6.58
Hypothetical protein	AK067879	TR002814	2.70	0.54	0.00	6.50
aromatic-L-amino-acid decarboxylase	LOC_Os08g04560	TR015026	2.70	0.98	0.00	6.49
expressed protein	LOC_Os11g10470	TR018554	2.69	1.18	0.00	6.45
putative Cys2/His2 zinc-finger protein	LOC_Os03g60560	TR008975	2.69	1.14	0.00	6.44
putative Serine/threonine phosphatases	LOC_Os03g16170	TR007005	2.66	0.77	0.00	6.33
ATPase, AAA family, putative	LOC_Os01g19260	TR001011	2.66	1.35	0.00	6.31
No apical meristem (NAM) protein, putative	LOC_Os07g48450	TR014775	2.65	0.45	0.00	6.30
Terpene synthase family, metal binding domain, putative	LOC_Os03g22634	TR007429	2.65	0.37	0.00	6.28
Glutaredoxin, putative	LOC_Os01g47760	TR002134	2.65	0.53	0.00	6.26
3-oxoacyl-(acyl-carrier-protein) reductase, putative	LOC_Os02g30060	TR004669	2.65	0.79	0.00	6.26
Myb-like DNA-binding domain, putative	LOC_Os12g37690	TR020013	2.64	0.42	0.00	6.23
Homeobox domain, putative	LOC_Os04g45810	TR010713	2.62	0.23	0.00	6.14
GH3 auxin-responsive promoter	LOC_Os07g40290	TR014299	2.62	1.59	0.00	6.13
Hypothetical protein	LOC_Os08g07160	TR015142	2.59	1.11	0.00	6.01
Choline kinase N terminus, putative	LOC_Os01g51920	TR002393	2.59	0.37	0.00	6.00
ABC transporter, putative	LOC_Os04g13210	TR009582	2.58	0.92	0.00	5.99
Hypothetical protein	LOC_Os08g37620	TR016221	2.57	0.25	0.00	5.95
Hypothetical protein	LOC_Os04g43650	TR010594	2.57	0.36	0.00	5.93
Hypothetical protein	LOC_Os03g04420	TR006244	2.56	0.26	0.00	5.91
Similar to saccharopin dehydrogenase-like protein	LOC_Os02g54254	TR005699	2.56	0.91	0.00	5.89
putative helix-loop-helix DNA-binding protein	LOC_Os03g53020	TR008534	2.52	1.51	0.00	5.75
expressed protein	LOC_Os04g05650	TR009388	2.52	0.87	0.00	5.72
putative indole-3-acetic acid-regulated protein	AK109491;AK068270	TR018022	2.50	0.95	0.00	5.67
Hypothetical protein	TA56857_4530	TR008594	2.49	0.78	0.00	5.61
UDP-glucuronosyl and UDP-glucosyl transferase	LOC_Os04g47720	TR010840	2.48	0.92	0.00	5.59
Terpene synthase family, metal binding domain, putative	LOC_Os04g27720	TR009879	2.48	1.34	0.00	5.59
AMP-binding enzyme, putative	LOC_Os08g34790	TR016102	2.48	0.26	0.00	5.58
Plastocyanin-like domain, putative	LOC_Os08g37670	TR016223	2.48	0.95	0.00	5.57
No apical meristem (NAM) protein, putative	LOC_Os01g64310	TR003165	2.44	0.43	0.00	5.44
Hypothetical protein	LOC_Os03g12820	TR006790	2.43	0.51	0.00	5.40
hypothetical protein	LOC_Os01g60640	TR002950	2.42	0.91	0.00	5.35
Auxin responsive protein, putative	LOC_Os01g56240	TR002684	2.42	0.43	0.00	5.34
signal recognition particle protein SRP54	LOC_Os05g43390	TR012089	2.41	1.26	0.00	5.30

Table 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
N/A	LOC_Os02g21040	TR004457	2.40	0.41	0.00	5.29
Protein kinase domain, putative	LOC_Os02g02780	TR003887	2.37	0.75	0.00	5.17
Expressed protein	chr01:6255266	TR000621	2.36	0.46	0.00	5.14
Ribonuclease T2 family	LOC_Os08g33710	TR016055	2.36	0.77	0.00	5.14
ribose-phosphate pyrophosphokinase, putative	LOC_Os01g52530	TR002438	2.36	0.57	0.00	5.13
Exostosin family	LOC_Os03g05070	TR006290	2.35	0.41	0.00	5.10
Expressed protein	LOC_Os04g39320	TR010355	2.35	1.03	0.00	5.10
putative trypanothione-dependent peroxidase	LOC_Os03g29190	TR007641	2.35	0.22	0.00	5.09
putative quercetin 3-O-glucoside-6"-O-malonyltransferase	LOC_Os02g28170	TR004600	2.34	0.50	0.00	5.06
Expressed protein	LOC_Os08g42960	TR016535	2.33	0.10	0.00	5.02
hypothetical protein	AK105524	TR007623	2.33	0.32	0.00	5.02
Expressed protein	LOC_Os03g59320	TR008913	2.31	0.71	0.00	4.97
Putative stress-related protein	LOC_Os03g53900	TR008591	2.31	1.36	0.00	4.97
Expressed protein	LOC_Os02g26790	TR004553	2.31	0.60	0.00	4.96
Putative transcription factor	LOC_Os03g48450	TR008266	2.31	0.63	0.00	4.96
hypothetical protein	LOC_Os12g38990	TR020087	2.31	0.37	0.00	4.95
Expressed protein	LOC_Os01g62970	TR003098	2.30	0.27	0.00	4.93
Terpene synthase family, metal binding domain, putative	LOC_Os08g04500	TR015023	2.30	0.89	0.00	4.92
Protein kinase domain, putative	LOC_Os01g50370	TR002300	2.29	0.82	0.00	4.91
Protein kinase domain, putative	LOC_Os07g44290	TR014533	2.29	0.62	0.00	4.88
hypothetical protein	LOC_Os01g27500	TR001299	2.29	0.38	0.00	4.88
putative osmotic stress-activated protein kinase -TRUNCATED-	LOC_Os02g34600	TR004851	2.28	0.64	0.00	4.87
Myb-like DNA-binding domain, putative	LOC_Os03g13790	TR006845	2.28	0.40	0.00	4.86
Zinc finger, C2H2 type, putative	LOC_Os01g62130	TR003040	2.28	1.05	0.00	4.86
hypothetical protein	LOC_Os05g46790	TR012198	2.28	0.92	0.00	4.85
indole-3-glycerol phosphate synthase, putative	LOC_Os08g23150	TR015647	2.27	0.95	0.00	4.83
AP2 domain, putative	LOC_Os03g09170	TR006562	2.27	0.20	0.00	4.81
Protein kinase domain, putative	LOC_Os02g06930	TR004082	2.27	0.62	0.00	4.81
AP2 domain, putative	LOC_Os04g52090	TR011061	2.26	0.55	0.00	4.80
Zinc finger, C2H2 type, putative	LOC_Os12g39400	TR020106	2.26	0.45	0.00	4.79
Eukaryotic aspartyl protease, putative	LOC_Os02g48870	TR005334	2.25	0.36	0.00	4.76
Expressed protein	LOC_Os01g64470	TR003174	2.24	0.45	0.00	4.74
C2 domain, putative	LOC_Os08g38440	TR016269	2.24	0.62	0.00	4.73
AP2 domain, putative	LOC_Os03g08470	TR006514	2.24	0.31	0.00	4.72
phage head-tail adaptor, putative	LOC_Os12g08850	TR019286	2.24	0.99	0.00	4.71
Peroxidase, putative	LOC_Os01g73200	TR003680	2.22	0.40	0.00	4.65
Expressed protein	LOC_Os10g43060	TR018397	2.22	1.13	0.00	4.65
Similar to probable wrky transcription factor 24 (wrky dna-binding protein 24)	LOC_Os03g20550	TR007305	2.22	0.21	0.00	4.65
putative lipase	LOC_Os10g25400	TR017504	2.21	0.60	0.00	4.64
Transposable element protein, putative	LOC_Os01g09640	TR000514	2.21	0.20	0.00	4.62
hypothetical protein	LOC_Os01g24960	TR001216	2.20	0.71	0.00	4.60
Peroxidase, putative	LOC_Os01g73170	TR003677	2.19	0.07	0.00	4.58
Early nodulin 93 ENOD93 protein, putative	LOC_Os06g04990	TR012464	2.19	0.76	0.00	4.56
anthranilate phosphoribosyltransferase	LOC_Os03g03450	TR006173	2.19	0.32	0.00	4.55
Expressed protein	LOC_Os08g30510	TR015896	2.18	0.66	0.00	4.55
plant integral membrane protein TIGR01569, putative	LOC_Os07g26110	TR013648	2.18	0.77	0.00	4.54
putative indole-3-glycerol phosphate lyase	LOC_Os03g58300	TR008831	2.18	0.57	0.00	4.54
ZIP zinc/iron transport family	LOC_Os04g52310	TR011077	2.18	0.72	0.00	4.53
transposon protein, putative, unclassified	LOC_Os04g51150	TR010999	2.17	0.77	0.00	4.51
hypothetical protein	LOC_Os01g70820	TR003536	2.16	0.40	0.00	4.47
Expressed protein	LOC_Os10g39100	TR018139	2.15	0.51	0.00	4.45
Protein kinase domain, putative	LOC_Os07g35290	TR014012	2.15	0.34	0.00	4.45
Galactosyltransferase, putative	LOC_Os09g27950	TR016793	2.15	1.03	0.00	4.42
Chitinase	LOC_Os10g28050	TR017592	2.13	0.84	0.00	4.37
Putative cysteine proteinase inhibitor	LOC_Os03g31510	TR007724	2.13	0.25	0.00	4.37

Table 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
peroxidase	LOC_Os12g02080	TR018984	2.13	0.77	0.00	4.36
Protein kinase domain, putative	LOC_Os08g39170	TR016319	2.12	1.09	0.00	4.36
calmodulin-like protein	LOC_Os03g21380	TR007352	2.11	0.48	0.00	4.31
Plant neutral invertase, putative	LOC_Os01g22900	TR001141	2.11	0.28	0.00	4.31
Expressed protein	LOC_Os05g03130	TR011582	2.10	1.22	0.00	4.28
Expressed protein	LOC_Os03g28940	TR007632	2.10	0.06	0.00	4.27
hypothetical protein	LOC_Os04g47450	TR010825	2.09	0.96	0.00	4.27
Expressed protein	LOC_Os08g29600	TR015862	2.08	0.39	0.00	4.22
Barwin family	LOC_Os11g37970	TR018764	2.07	0.21	0.00	4.21
Similar to mutT domain protein	LOC_Os04g46280	TR010742	2.07	0.55	0.00	4.19
Protein kinase domain, putative	LOC_Os10g05250	TR016991	2.06	0.47	0.00	4.18
Tyrosine aminotransferase	LOC_Os11g42510	TR018889	2.06	0.55	0.00	4.18
Thaumatin/PR5-like protein	LOC_Os12g38150	TR020046	2.05	0.46	0.00	4.15
nod factor binding lectin-nucleotide phosphohydrolase	LOC_Os12g02980	TR019019	2.04	0.57	0.00	4.11
Common central domain of tyrosinase, putative	LOC_Os01g58100	TR002800	2.04	0.43	0.00	4.11
UDP-glucuronosyl and UDP-glucosyl transferase	LOC_Os07g10190	TR013161	2.04	0.24	0.00	4.11
MIP family channel proteins	LOC_Os01g74450	TR003752	2.04	0.20	0.00	4.10
WS118 protein	LOC_Os01g50910	TR002328	2.04	1.19	0.00	4.10
Expressed protein	LOC_Os01g62310	TR003053	2.03	0.61	0.00	4.10
Expressed protein	LOC_Os04g58890	TR011454	2.03	0.22	0.00	4.09
Expressed protein	LOC_Os01g32460	TR001412	2.02	0.46	0.00	4.06
peroxidase (EC 1.11.1.7) poxN [similarity] - rice	LOC_Os03g13210	TR006814	2.02	0.97	0.00	4.05
Protein kinase domain, putative	LOC_Os02g04230	TR003976	2.02	0.53	0.00	4.05
Transmembrane amino acid transporter protein	LOC_Os08g03350	TR014962	2.01	0.37	0.00	4.02
Putative nodule-specific protein	LOC_Os03g58580	TR008851	2.01	1.75	0.01	4.02
probable adenylate kinase 1, chloroplast precursor (ec 2.7.4.3)	LOC_Os04g57540	TR011360	1.99	0.54	0.00	3.98
Helix-loop-helix DNA-binding domain, putative	LOC_Os05g46370	TR012188	1.99	0.14	0.00	3.98
hypothetical protein	LOC_Os03g51650	TR008447	1.97	0.79	0.00	3.93
3-hydroxyacyl-CoA dehydrogenase, C-terminal domain, putative	LOC_Os02g17390	TR004305	1.97	0.83	0.00	3.93
Putative flavanone 3-hydroxylase	LOC_Os10g39140	TR018142	1.96	0.46	0.00	3.90
retrotransposon protein, putative, unclassified	LOC_Os01g37350	TR001616	1.96	0.13	0.00	3.90
No apical meristem (NAM) protein, putative	LOC_Os01g48460	TR002170	1.96	0.22	0.00	3.90
L-ascorbate peroxidase (EC 1.11.1.11) [validated] - rice	LOC_Os03g17690	TR007103	1.96	0.41	0.00	3.89
glutathione peroxidase 1	LOC_Os04g46960	TR010790	1.96	0.18	0.00	3.89
Possible lysine decarboxylase, putative	LOC_Os03g01880	TR006071	1.94	0.21	0.00	3.85
Cytidine deaminase, putative	LOC_Os07g14150	TR013329	1.94	0.51	0.00	3.83
acetyl-CoA acyltransferases	LOC_Os02g57260	TR005897	1.94	0.36	0.00	3.83
hypothetical protein	LOC_Os12g14320	TR019443	1.94	0.58	0.00	3.83
hypothetical protein	N/A	TR007722	1.92	0.52	0.00	3.80
Putative chitinase	LOC_Os01g64100	TR003157	1.91	0.65	0.00	3.77
Putative receptor-like kinase	LOC_Os03g56160	TR008710	1.90	0.10	0.00	3.73
Myb-like DNA-binding domain, putative	LOC_Os01g64360	TR003168	1.89	0.92	0.00	3.71
Expressed protein	LOC_Os07g44910	TR014568	1.88	0.86	0.00	3.69
Zinc finger, C3HC4 type (RING finger), putative	LOC_Os01g55110	TR002605	1.88	0.15	0.00	3.69
Transmembrane amino acid transporter protein	LOC_Os12g08130	TR019251	1.88	0.33	0.00	3.68
Expressed protein	LOC_Os12g02720	TR019008	1.88	0.62	0.00	3.67
Aldehyde oxidase and xanthine dehydrogenase,	LOC_Os07g18120	TR013440	1.87	0.95	0.00	3.66
Acyltransferase, putative	LOC_Os02g02340	TR003849	1.87	0.42	0.00	3.65
Expressed protein	LOC_Os02g30320	TR004687	1.86	0.28	0.00	3.63
Potassium uptake protein	LOC_Os04g32920	TR010077	1.86	0.10	0.00	3.62

Table 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
branched-chain amino acid aminotransferase, putative	LOC_Os03g12890	TR006794	1.85	0.67	0.00	3.62
oxidoreductase, short chain dehydrogenase/reductase family	LOC_Os07g46930	TR014677	1.85	1.27	0.00	3.61
Expressed protein	LOC_Os04g37790	TR010265	1.85	0.74	0.00	3.61
Putative beta-1,3-glucanase	LOC_Os03g45390	TR008124	1.85	0.78	0.00	3.61
hypothetical protein	N/A	TR014346	1.85	0.88	0.00	3.60
probenazole-induced protein - rice	LOC_Os12g36880	TR019971	1.84	0.36	0.00	3.59
Sulfotransferase domain	LOC_Os08g17510	TR015518	1.84	0.61	0.00	3.57
Expressed protein	LOC_Os03g07190	TR006423	1.83	0.31	0.00	3.57
Expressed protein	LOC_Os07g46030	TR014611	1.83	1.75	0.02	3.55
EF hand, putative	LOC_Os01g72080	TR003599	1.83	0.26	0.00	3.55
Sucrose synthase	LOC_Os03g22120	TR007405	1.83	0.19	0.00	3.55
No apical meristem (NAM) protein, putative	LOC_Os01g01430	TR000025	1.83	0.66	0.00	3.54
Inositol-3-phosphate synthase (ec 5.5.1.4)	LOC_Os03g09250	TR006569	1.82	0.11	0.00	3.54
WRKY DNA-binding domain, putative	LOC_Os08g29660	TR015866	1.82	0.47	0.00	3.54
Dynein light chain type 1	LOC_Os01g55510	TR002631	1.82	0.62	0.00	3.53
malate dehydrogenase, NAD-dependent	LOC_Os08g33720	TR016056	1.82	0.81	0.00	3.53
C2 domain, putative	LOC_Os07g31720	TR013876	1.81	0.24	0.00	3.51
Expressed protein	LOC_Os04g39360	TR010357	1.81	0.68	0.00	3.50
senescence-associated protein-like	LOC_Os12g24020	TR019624	1.80	0.49	0.00	3.49
Expressed protein	LOC_Os07g02850	TR012735	1.80	1.28	0.00	3.49
phospholipid-translocating P-type ATPase, flippase	chr03:11864277	TR007330	1.80	0.34	0.00	3.48
Expressed protein	LOC_Os01g53090	TR002476	1.79	0.19	0.00	3.47
Expressed protein	LOC_Os01g56560	TR002708	1.79	0.32	0.00	3.47
Putative AMP-binding protein	LOC_Os03g04120	TR006224	1.79	0.34	0.00	3.46
Similar to beta-glucosidase-like protein	LOC_Os04g43390	TR010579	1.79	1.80	0.02	3.45
putative multiple inositol polyphosphate phosphatase	LOC_Os03g60370	TR008964	1.78	0.59	0.00	3.45
heat shock protein-like	LOC_Os01g42190	TR001866	1.77	0.38	0.00	3.40
chlorophyll a/b binding protein 1.	LOC_Os09g17740	TR016725	1.76	0.32	0.00	3.40
WRKY DNA-binding domain, putative	LOC_Os01g53040	TR002473	1.76	0.47	0.00	3.39
hypothetical protein	N/A	TR007772	1.76	0.44	0.00	3.39
Terpene synthase family, metal binding domain, putative	LOC_Os04g27430	TR009873	1.76	0.26	0.00	3.38
Expressed protein	LOC_Os01g63060	TR003102	1.76	0.17	0.00	3.38
Zinc finger C-x8-C-x5-C-x3-H type (and similar), putative	LOC_Os08g06330	TR015094	1.76	0.24	0.00	3.38
Expressed protein	LOC_Os01g13930	TR000776	1.75	0.40	0.00	3.38
beta-galactosidase	LOC_Os03g15020	TR006922	1.75	0.14	0.00	3.38
AP2 domain, putative	LOC_Os05g41780	TR012054	1.75	0.46	0.00	3.36
Expressed protein	LOC_Os10g20470	TR017333	1.75	0.55	0.00	3.36
hypothetical protein	AK111371	TR015356	1.74	0.63	0.00	3.34
Expressed protein	LOC_Os12g39840	TR020131	1.74	0.71	0.00	3.33
hypothetical protein	chr03:18685339	TR007770	1.73	0.92	0.00	3.33
Protein kinase domain, putative	LOC_Os07g35390	TR014020	1.72	0.33	0.00	3.30
Similar to 1-aminocyclopropane-1-carboxylate oxidase (EC 1.4.3.-)	N/A	TR011639	1.72	0.50	0.00	3.30
Mitochondrial carrier protein, putative	LOC_Os08g40850	TR016408	1.72	0.35	0.00	3.29
DD1A protein, putative	LOC_Os07g08240	TR013041	1.71	0.84	0.00	3.28
Expressed protein	LOC_Os03g44810	TR008098	1.70	0.78	0.00	3.25
Metallothionein	LOC_Os12g38064	TR020041	1.70	0.25	0.00	3.25
Unknown protein	LOC_Os03g08320	TR006502	1.70	0.46	0.00	3.25
Expressed protein	LOC_Os05g02770	TR011557	1.70	0.93	0.00	3.24
Expressed protein	LOC_Os11g25454	TR018669	1.70	0.22	0.00	3.24
putative ORFX	LOC_Os04g38790	TR010317	1.69	0.25	0.00	3.24
acetyl-CoA acyltransferases	LOC_Os01g02020	TR000060	1.68	0.28	0.00	3.21
hypothetical protein	LOC_Os01g04100	TR000183	1.68	0.42	0.00	3.21
oxidoreductase, 2OG-Fe(II) oxygenase family, putative	LOC_Os04g10350	TR009496	1.68	0.28	0.00	3.20
Putative lipid transfer protein	LOC_Os10g36110	TR017990	1.68	0.86	0.00	3.20
Expressed protein	LOC_Os12g32190	TR019820	1.67	0.28	0.00	3.18

Table 4.1. continued.

Gene Function	Gene ID	Probe ID	Log2R	STD	P	Ratio
Protein kinase domain, putative	LOC_Os01g18800	TR000992	1.67	0.36	0.00	3.18
ribosomal protein S17, putative	LOC_Os08g10604	TR015308	1.66	0.22	0.00	3.17
Expressed protein	LOC_Os01g54670	TR002576	1.66	0.29	0.00	3.17
Expressed protein	LOC_Os01g61230	TR002983	1.66	0.97	0.00	3.16
Expressed protein	LOC_Os12g26960	TR019685	1.66	0.80	0.00	3.16
No apical meristem (NAM) protein, putative	LOC_Os08g02300	TR014925	1.66	0.77	0.00	3.16
hypothetical protein	LOC_Os08g13380	TR015354	1.66	0.44	0.00	3.16
Similar to At4g16146	LOC_Os01g19940	TR001042	1.66	0.24	0.00	3.15
aconitate hydratase 1	LOC_Os03g04410	TR006243	1.65	0.21	0.00	3.14
unknown protein	AK070315	TR013074	1.65	0.36	0.00	3.13
NAD-dependent glycerol-3-phosphate dehydrogenase	LOC_Os01g58740	TR002845	1.65	0.22	0.00	3.13
syntxin 132 (atsyp132)	LOC_Os06g07200	TR012553	1.65	0.25	0.00	3.13
CHCH domain, putative	LOC_Os04g44550	TR010631	1.64	0.22	0.00	3.12
Dihydroorotate dehydrogenase, putative	LOC_Os02g50350	TR005430	1.64	0.27	0.00	3.12
Expressed protein	LOC_Os03g08840	TR006540	1.64	1.04	0.00	3.12
vacuolar targeting receptor bp-80	LOC_Os03g21720	TR007376	1.64	0.63	0.00	3.11
B3 DNA binding domain, putative	LOC_Os01g49830	TR002263	1.64	0.17	0.00	3.11
Ethylene insensitive 3, putative	LOC_Os07g48630	TR014789	1.63	0.50	0.00	3.09
AP2 domain, putative	LOC_Os07g42510	TR014426	1.63	0.37	0.00	3.09
Expressed protein	LOC_Os03g56860	TR008762	1.63	0.61	0.00	3.09
hypothetical protein	LOC_Os08g03460	TR014971	1.62	0.48	0.00	3.08
Expressed protein	LOC_Os12g36910	TR019973	1.62	0.52	0.00	3.08
Expressed protein	LOC_Os03g37090	TR007831	1.62	0.31	0.00	3.08
Transketolase, pyridine binding domain, putative	LOC_Os07g07470	TR012995	1.62	0.62	0.00	3.06
Putative cytokinin oxidase	LOC_Os01g10110	TR000539	1.61	0.59	0.00	3.06
Expressed protein	LOC_Os07g44410	TR014542	1.61	0.36	0.00	3.05
Expressed protein	LOC_Os07g02460	TR012716	1.61	0.35	0.00	3.05
pyruvate kinase	LOC_Os01g16960	TR000929	1.61	0.51	0.00	3.04
hypothetical protein	LOC_Os04g42680	TR010551	1.61	0.90	0.00	3.04

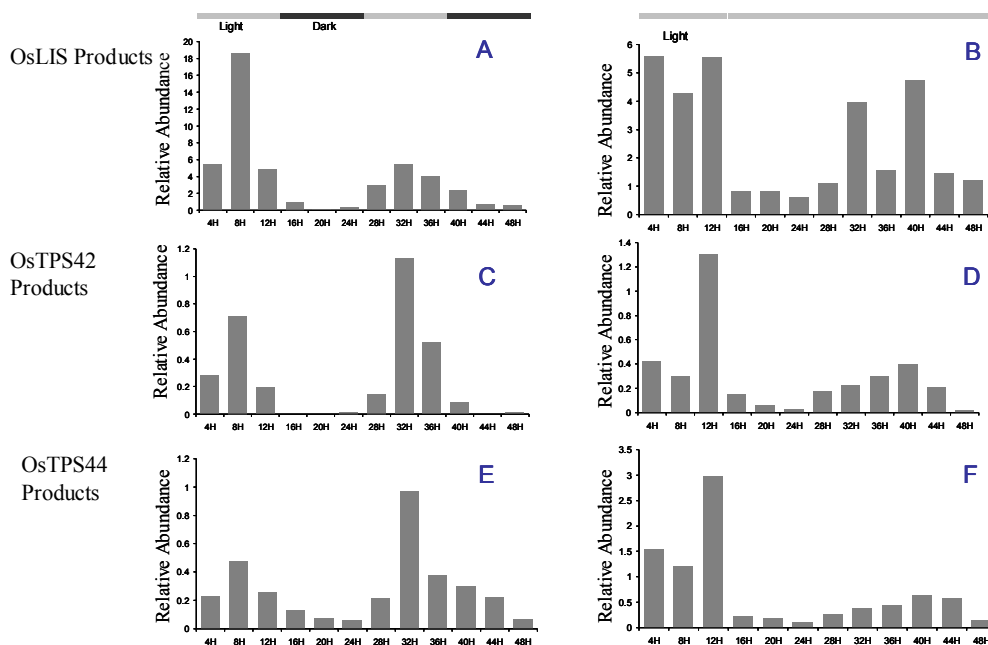


Figure 4.1. The insect induced volatile emission during the diurnal cycle. The Y axes are normalized relative abundance of volatile emission, and the X axes are the time point after the 12 hour fall armyworm treatment. A, C, and E are the emission patterns under diurnal light-dark cycle for the terpenoid products of OsLIS, OsTPS42 and OsTPS44, respectively. B, D, and F are the emission patterns under constant light for the terpenoid products of OsLIS, OsTPS42 and OsTPS44, respectively. A clear diurnal cycle dependent volatile emission pattern can be found for products of all genes, even though the baseline levels are different for each gene.

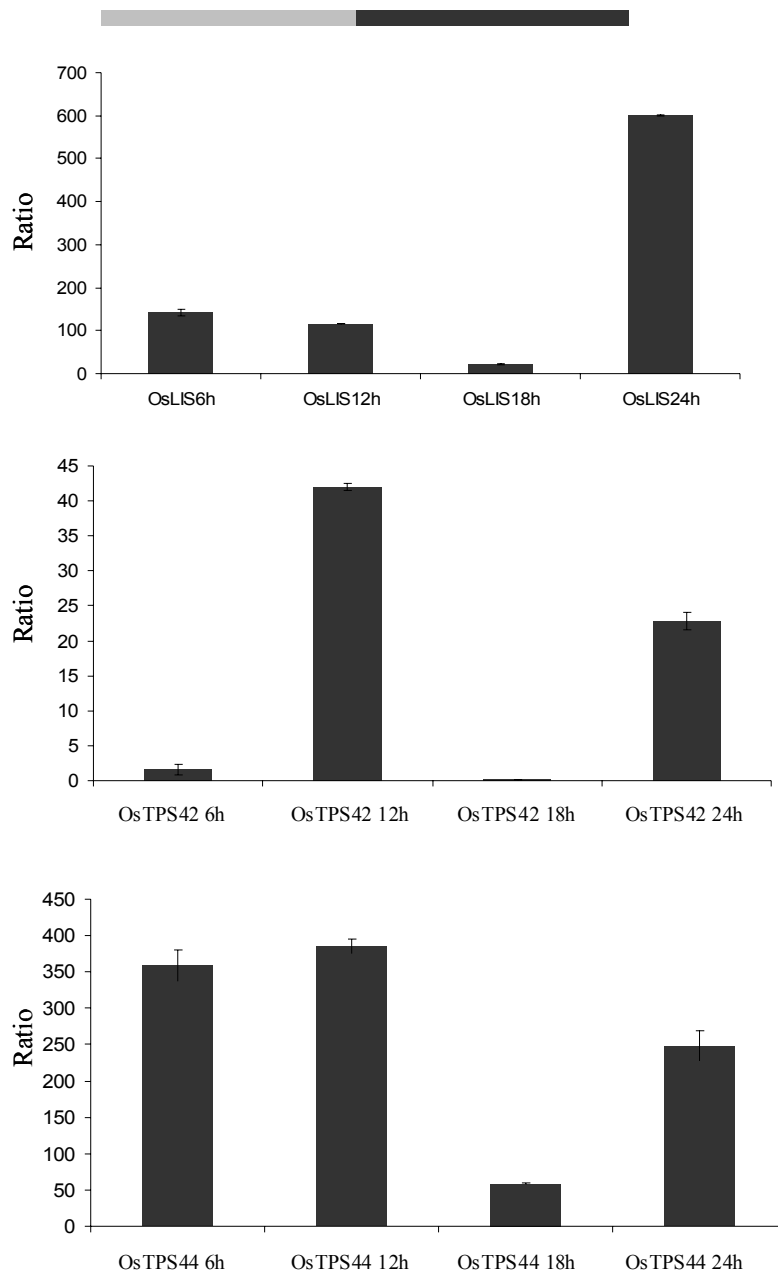
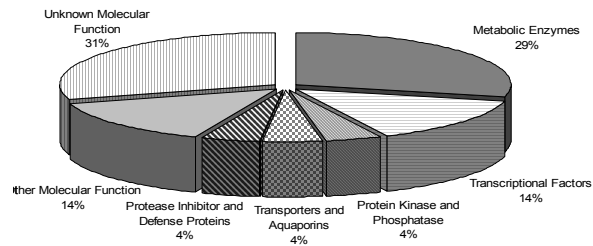
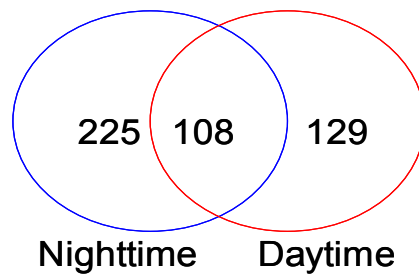


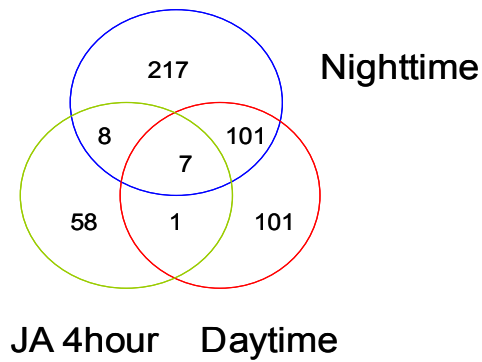
Figure 4.2. Real-time PCR results of three *TPS* genes under different light condition. The light starts at 0 hour and ends at 12 hour. Therefore 18 hour represents the dark point and 6 hour represents the light point. The Y axis represents the fold changes, and X represents different time point.



A



B



C

Figure 4.3. Summary of microarray data. A. The gene classification for the up-regulated genes. B. The number of shared up-regulated genes by daytime and nighttime. C. The number of shared up-regulated gene by JA treatment and insect treatment.

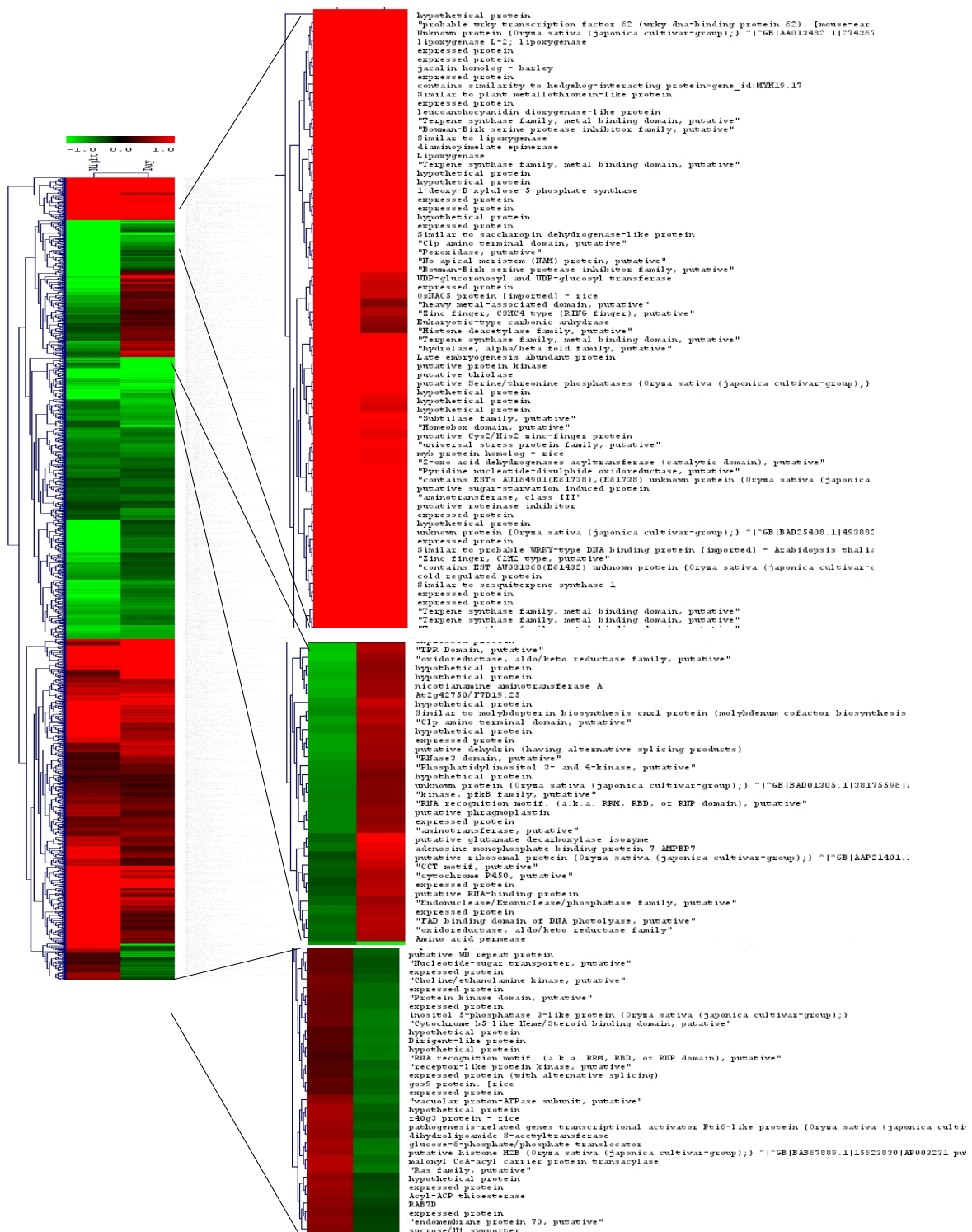


Figure 4.4. Cluster analysis of daytime and night time gene expression. Red color indicated up-regulation and green color indicated down-regulation.

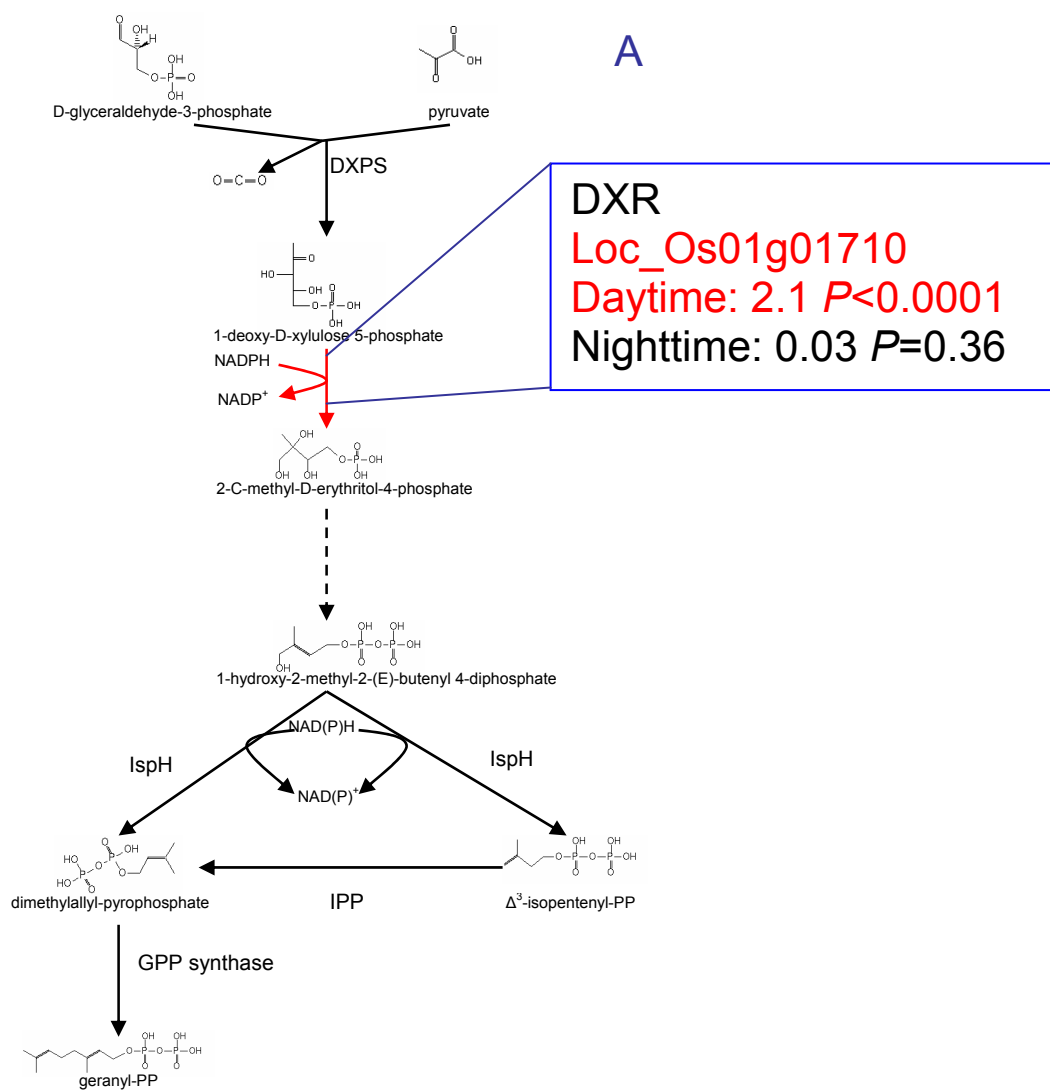


Table 4.5. continued.

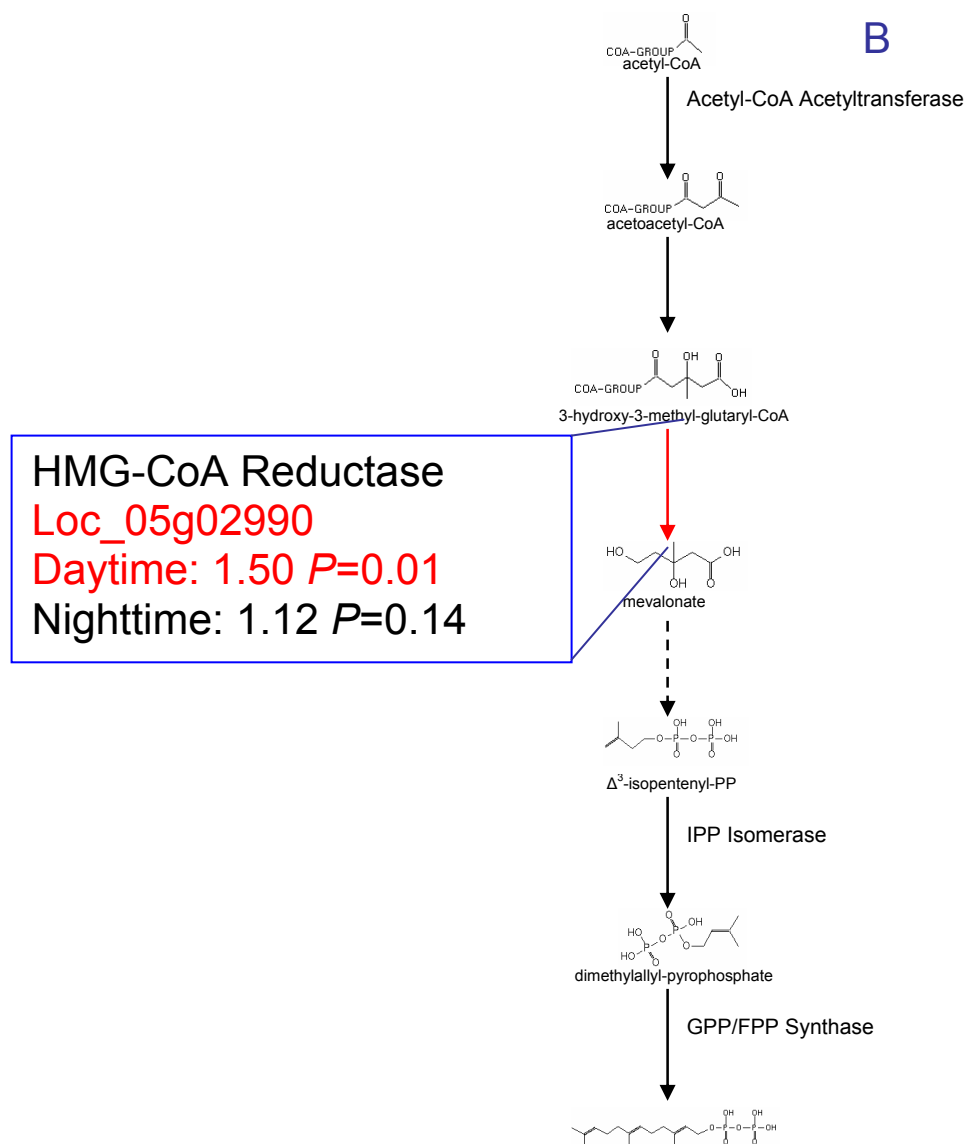


Figure 4.5. The differential regulation of key genes during daytime and night time in both mevalonate and non-mevalonate pathways. DXR stood for 1-deoxy-D-xylulose-5-phosphate reductoisomerase.

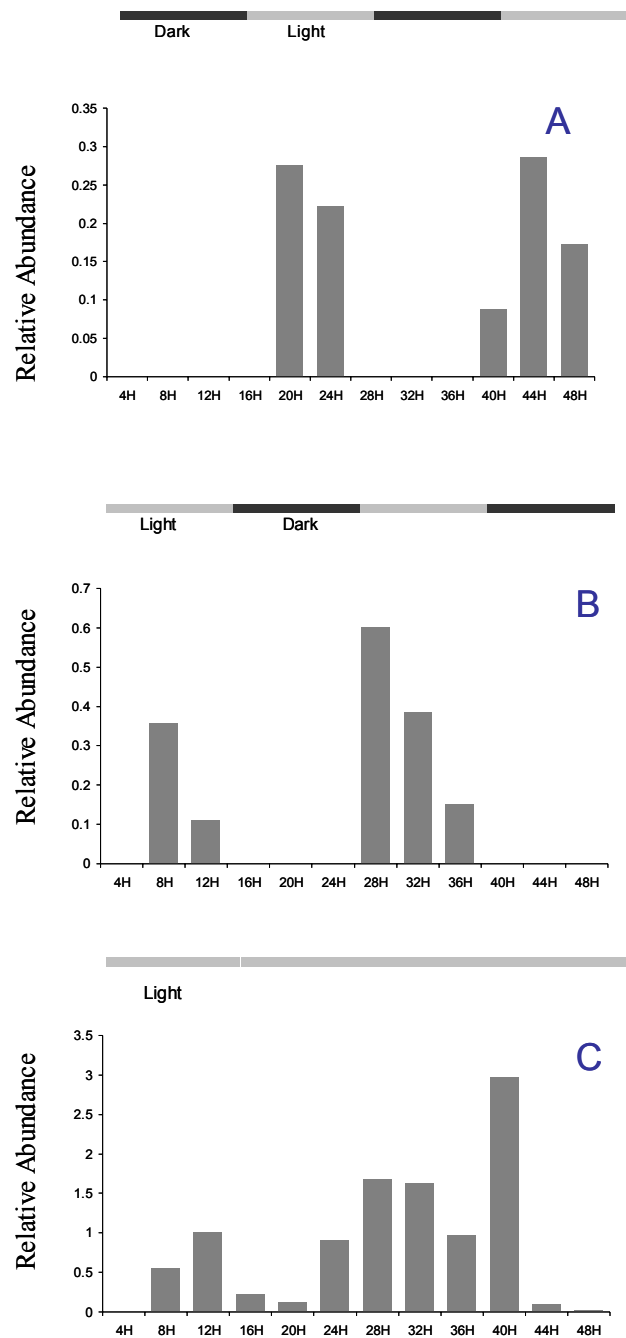


Figure 4.6. Jasmonic induced linalool emission during the diurnal cycle. The Y axes showed the relative amount of linalool and the X axes showed the time point. A was the dark and light cycle with treatment starting at dark. B was the

Figure 4.6. continued.

light and dark cycle with treatment starting at light. C was the consistent light condition.

Chapter V. Characterization of a Poplar Terpene Synthase Guided by Comparative Genome Analysis

Adapted from:

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Abstract

Volatile terpenoids are an important class of volatile organic compounds (VOCs) involved in indirect plant defense. All volatile terpenoids are synthesized using terpene synthase (TPS). Studying the evolution of *TPS* genes across species have allowed us to further understand the molecular mechanisms and evolution of indirect plant defense against herbivorous insects. Such study is enabled by the recent completion of rice and poplar genome. In this research, we first identified 52 candidate rice *TPS* genes and 54 candidate poplar *TPS* genes through reiterative sequence similarity search. Relatedness of *TPS* genes within and across species was studied. Comparative genome analysis revealed rapid evolution of *TPS* genes, and the expansion of *TPS* gene family mostly occurred after the species divergence between monocot and dicot as well as between woody perennial species and herbaceous annual species. Moreover, a comprehensive phylogenic analysis of most previously identified *TPS* genes confirmed the rapid evolution of the gene family, yet also led to identification of a group of conserved monoterpene synthase genes. Biochemical analysis revealed one of the poplar *TPS* genes (*PtLIS*) to be a linalool synthase. Gene expression analysis suggested the defense function of the *PtLIS* gene. Overall, comparative genome analysis indicated the rapid evolution of *TPS* genes as a mechanism for plants to adapt to the changing environment, and the features of gene family may be utilized for gene discovery.

Introduction

Terpenoids are the largest group of secondary metabolites and have been shown to be involved in a variety of biological functions (Dudareva *et al.*, 2006). The biological functions of terpenoids are relevant to their chemical structures. All terpenoids are composed of a five carbon based isoprene structure. Depending on the number of five carbon base units, terpenoids can be classified into monoterpene with ten carbons, sesquiterpene with fifteen carbons, diterpene with twenty carbons, triterpene with thirty carbons, tetraterpene with forty carbons, and polyterpene with longer chain of five carbon units (Bohlmann *et al.*, 1998; Keeling and Bohlmann, 2006). Among these different types, monoterpenes and sesquiterpenes are often part of the volatile organic compounds emitted in the induced defense response when plants are challenged with herbivorous insects (Keeling and Bohlmann, 2006). Volatile terpenoids are believed to play an important role in indirect plant defense serving as info-chemicals for natural enemies to best locate herbivorous insects (Takabayashi and Dicke, 1996; Kessler and Baldwin, 2001). Volatile terpenoids like linalool was shown to preferentially attract natural enemies of herbivorous insects in both laboratory and field experiments (Dicke *et al.*, 1990; Kessler and Baldwin, 2001). Moreover, preferential attraction of nature enemies as a result of increased production of volatile terpenoids was found with transgenic plants over-expressing terpene synthase genes (Schnee *et al.*, 2006). One of the key aspects of studying ecological function of terpenoids is to identify the genes

involved in their biosynthesis, which will help to understand the molecular basis for the volatile production and regulation.

Regardless of the diverse structure, terpenoids are mostly synthesized from terpene synthase. Based on the substrate and product specificity, terpene synthases can be classified into monoterpene synthases, sesquiterpene synthases, diterpene synthases and such. Monoterpene synthases catalyze the conversion from GPP (geranyl pyrophosphate) to a variety of monoterpenes containing 10 carbons; sesquiterpene synthases catalyze the conversion from FPP (farnesyl pyrophosphate) to sesquiterpenes containing 15 carbons; and diterpene catalyze the conversion from GGPP (geranylgeranyl pyrophosphate) to diterpenes containing 20 carbons (Bohlmann *et al.*, 1998; Tholl, 2006). GPP and GGPP are synthesized through the MEP (2-C-methyl-D-erythritol 4-phosphate, non-mevalonate) pathway in the plastid, whereas FPP is synthesized from mevalonate pathway in the cytosol (Tholl, 2006). In higher plants, terpene synthase genes exist as gene families. *Arabidopsis* terpene synthase gene family is the only adequately analyzed gene family in higher plants with 32 *TPS* genes and 8 pseudogenes (Aubourg *et al.*, 2002). A total of more than one hundred *TPS* genes responsible for low molecular weight terpenoid (monoterpene, sesquiterpene, and diterpene) biosynthesis have been identified over the last three decades in more than thirty species. In *Arabidopsis*, several terpene synthase genes have been characterized for low molecular weight terpenoid biosynthesis in flowers, roots and leaves (Chen *et al.*, 2003; Chen *et al.*, 2004;

Tholl *et al.*, 2005; Kishimoto *et al.*, 2006; Ro *et al.*, 2006). A single terpene synthase can be responsible for multiple products, especially for sesquiterpene synthase (Chen *et al.*, 2003; Kollner *et al.*, 2004; Tholl *et al.*, 2005). In fact, three terpene synthase genes are responsible for most of the volatile terpenoids produced in *Arabidopsis* flowers (Chen *et al.*, 2003; Tholl *et al.*, 2005). Broad biological and ecological functions are speculated for these genes. The diverse members of *TPS* gene family imposed several important questions: which gene is for which product(s)? How are these genes regulated? What are the exact ecological functions of these genes? The genome-wide in silico analysis of terpene synthase gene family has helped the process of gene discovery and gene evolution studies.

The evolution of the *TPS* gene family has been well-studied. The *TPS* gene family provides a perfect model for the study of the expansion of gene families with dynamic evolution. In fact, the *TPS* gene family along with the P450 gene family, the N- and O- methyltransferase gene family, and others, consists of a group of gene families important in plant defense, and has grown due to the plant environment interactions. The study of the evolution of these gene families will elucidate the molecular basis for the evolution of insect defense in plants. According to previous gene family studies, the *TPS* genes for low molecular weight terpenoid biosynthesis can be classified into six groups based on phylogenetic analysis (Bohlmann *et al.*, 1998; Aubourg *et al.*, 2002). Based on structure, the angiosperm terpene synthases can be classified into two groups,

TPSI and TPSIII (Bohlmann *et al.*, 1998; Trapp and Croteau, 2001; Aubourg *et al.*, 2002; Tholl, 2006). Class I *TPS* genes have a 200 amino acid feature sequence that is lacking in the other class of *TPS* genes (Cseke *et al.*, 1998; Aubourg *et al.*, 2002). Type I class *TPS* genes in agiosperms have a common ancestor gene while class III type *TPS* genes can be classified into sub-classes (Trapp and Croteau, 2001; Aubourg *et al.*, 2002; Tholl, 2006).

As aforementioned, the monoterpene synthases and sesquiterpene synthases could be distinguished from one another by the presence of transient peptides (Aubourg *et al.*, 2002). However, the subclass (a to f) classification could not be correlated with enzyme activity. The classification was more of a reflection of evolutionary process of *TPS* genes rather than the substrate and product specificity (Aubourg *et al.*, 2002). In fact, the product profiles for a certain *TPS* gene can hardly be predicted through sequence only since minor changes in gene sequence can result in quite different product profile (Yoshikuni *et al.*, 2006; Xu *et al.*, 2007). Phenomena have been found among the genes evolved during tandem duplication, and also in the same gene from different cultivar (Kollner *et al.*, 2004; Tholl, 2006; Xu *et al.*, 2007). For example, two maize sesquiterpene synthases from different cultivars were found to produce different terpenoid volatile profiles (Kollner *et al.*, 2004). The plasticity in enzyme activity is a common rule rather than an exception, since many plant species displayed a diverse profile of volatile terpenoids among different cultivars (Degen *et al.*, 2004; Kollner *et al.*, 2004; Lou *et al.*, 2006).

One important feature of *TPS* gene function is that the exact biochemical function of *TPS* genes cannot be readily derived from gene structure analysis, which is due to the rapid evolution of the *TPS* gene family (Xu *et al.*, 2007). Tandem duplication is prevalent in the new gene birth in the *TPS* gene family, which has resulted in multi-member *TPS* gene family in many, if not all, angiosperm species. It is commonly believed that new genes either evolve new function or lose activity during the evolution due to dose effects. This may not always be true in plants, especially in the case of terpene synthase genes, where two recently duplicated *TPS* genes can be responsible for similar product profiles (Chapter III). However, an evolutionary force seems to exist to drive the development of new function for duplicated genes since many tandem duplicated genes not only have different biochemical function, but also have different regulation of expression in different physiological and pathological processes (Chapter I). Therefore, it is common that the same gene in a different cultivar or highly similar genes in the same plant are responsible for quite different product profiles (Xu *et al.*, 2007). Another feature of *TPS* gene evolution is the widespread convergent evolution. Many *TPS* genes responsible for the same products are from quite different sequences (Chapter III). The phylogenic analysis of all limonene synthase genes among different species indicated that convergent evolution might be one of major mechanisms of evolution of *TPS* genes with same or similar product profile. Overall, the *TPS* gene family evolved rapidly leading to genes with diverse functions and product

profiles. Despite the progresses with gene evolution study in the *TPS* gene family, questions still remain about how gene evolution is relevant to species evolution. Considering the diverse structure of the *TPS* genes, we are unsure if any existing *TPS* gene group is from a common ancestor retaining original function. In order to further understand the evolution of the *TPS* gene family, we decided to perform comparative gene family analysis across the species based on recent available rice and poplar genome sequence.

In this research, we aimed to both investigate the evolution of the terpene synthase gene family and characterize a poplar terpene synthase gene from a conserved group of terpene synthase genes with a deep evolutionary origin. Our research indicated that rice has 52 candidate *TPS* genes and poplar has 54 candidate *TPS* genes. Within species phylogenetic analysis revealed that the relatedness of genes correlated with the structure domain evolution. The cross-species phylogenetic analysis indicated that the *TPS* gene family expansion happened mainly after the divergence of monocot species and dicot species as well as the divergence of annual herbaceous species and perennial woody species. Moreover, the rapid evolution of the gene family also supported the importance of convergence evolution for biochemical function of *TPS* genes. Nevertheless, a conserved group of *TPS* genes with several characterized linalool synthases and other monoterpenes synthases were identified through comparative genome analysis of most identified *TPS* genes. Three poplar genes were in this group, and we cloned and characterized the cDNA of one of them to

be a linalool synthase gene. The expression pattern of the gene was surveyed and the biological function was discussed.

Methods

Sequence retrieval and analysis

Arabidopsis TPS genes were selected according to previous publications (Aubourg *et al.*, 2002). The protein sequence of several rice and *Arabidopsis* TPS genes were used initially as a query sequence to search against the rice (<http://www.tigr.org/tdb/e2k1/osa1>), and the poplar genome database (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>), respectively, using the BLASTP algorithm (Altschul *et al.* [1990](#)). The cutoff *e* value was set to be e-6.

Multiple sequence alignment and phylogenetic analysis

Multiple protein sequence alignments were constructed using Vector NTI AlignX (Invitrogen Inc., Calsad, CA).

Motif Search

To discover conserved motifs in TPS genes, the sequences of all predicted were analyzed with MEME program (<http://www.meme.nbcr.net/meme/meme-intro.html>).

Quantitative Real-time PCR

Quantitative real-time PCR experiments were carried out as previously described (Yuan *et al.*, 2005). Complementary DNA was synthesized from 1 µg

of total RNA for different treatment and control samples with Iscript cDNA Synthesis Kit (Bio-rad, Inc) according to manufacturer's instructions. Samples were diluted into 20 ng/μl, 4 ng/μl and 0.08 ng/μl concentration series. Three replicates of real-time PCR experiments were performed for each concentration using an ABI 7000 Sequence Detection System from Applied Biosystems (Foster City, CA) with the PowerSYBR mix (Applied Biosystems). The primers for target genes were designed by Primer Express software (Applied Biosystems) and the primer sequences were as shown in Supplementary Table 5.1. Ct numbers were extracted for both reference genes and target genes with auto baseline and manual threshold. Amplification efficiency for the reactions was estimated as described previously, and multiple regression models were used to derive point estimation of $\Delta\Delta C_t$, p value, standard error and 95% confidence intervals with the SAS 9.1 programs provided (SAS institute, Cary, NC) (Yuan et al., 2006).

Full length cDNA cloning and protein expression in E. coli

Full length cDNAs of poplar *TPS* gene were cloned from jasmonic acid treated poplar leaves using RT-PCR. cDNA synthesis was performed in the same way as described for real-time PCR. The primers used were 5'-ATGAAGCCCATCCTCAAAGAATTTAAG-3' (forward) and 5'-GAGGAGAGATTTCATATGCTCCTCA-3' (reverse). PCR was carried out with the BD Advantage 2 Enzyme (BD Biosciences, Rockville, MD) with the following conditions: 95°C for 2 min, followed by 35 cycles at 95°C for 30 sec;

56°C for 30 sec and 68°C for 2 min, and a final elongation step at 70°C for 10 min. The resulting fragments were cloned into the vector pCRT7/CT-TOPO (Invitrogen, Carlsbad, CA). An *E. coli* BL21 Codon Plus strain, transformed with the appropriate expression construct, was used for protein expression. Induction was performed at 25°C overnight with 1 mM isopropyl-1-thio--D-galactopyranoside.

Enzyme Assay

Fifty-mL cells from induced culture were harvested at 4°C and washed with 10-mL enzyme extraction buffer (50 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, 5 mM MgCl₂, 5 mM DTT, 5 mM sodium ascorbate, and 0.5 mM phenylmethylsulfonyl fluoride). Cells were then disrupted by sonication in 3-mL enzyme extraction buffer, and then centrifuged at 13,000g at 4°C for 5 min. Buffer exchange was achieved by passing through a size exclusion Sephadex column (GE Healthcare, Piscataway, NJ), and the elution was collected in 4-mL of assay buffer containing 10 mM 3-(N-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, and 1 mM DTT. The enzyme assay was carried out in 1-mL containing 300-μL of enzyme extract, and 700-μL assay buffer containing 20 mM MgCl₂, 0.2 mM MnCl₂, 0.2 mM NaWO₄, 0.1 mM NaF, and 40 μM geranyl diphosphate or farnesyl diphosphate (Echelon Research Laboratories, Salt Lake City, UT). The assay was overlaid with 200-μL n-pentene and was performed in a glass tube for 2 hours at 30°C. The volatiles were collected by vortex of the assay at maximum

speed for 15 seconds. The assay was then centrifuged for one minute at 8000g at 4°C for phase separation. About 100 µL of the organic phase was collected for the gas chromatography–mass spectrometry analysis.

GC-MS Analysis

Samples from volatile collections were eluted into methylchloride and analyzed with Shimadzu QP5050A GC-MS instrument. The experiments were carried out at the following conditions, flow rate of 5mL/min of carrier gas helium, a splitless injection of 3 µL, and a temperature gradient of 5°C/min from 40°C (3-min hold) to 240°C.

Results

TPS Genes in Arabidopsis, Rice and Poplar

Through the reiterative blast search, we identified 52 rice gene sequences similar to known *TPS* genes. In the same way, 54 poplar *TPS* similar sequences were identified. Table 5.1 showed all predicted rice and poplar *TPS* genes, their ID, and chromosome. For rice *TPS* genes, the TIGR (The Institute for Genomic Research) rice locus ID was used, and DOE-JGI (Department of Energy Joint Genomic Institute) poplar gene model ID was used for poplar *TPS* genes.

Previous genome analysis showed 32 *AtTPS* genes and 8 *pseudo-TPS* genes (Aubourg *et al.*, 2002). Despite the comprehensive work from Aubourg *et al.* (2002), the gene names such as *AtTPS1* were not recorded according to the chromosome location, which is the convention for naming genes in the family. In order to avoid this confusion, we used *Arabidopsis* gene ID from TAIR (The *Arabidopsis* Information Resource, <http://www.arabidopsis.org/>) to represent the *Arabidopsis* *TPS* genes.

The in silico sequence from the database sometimes mis-annotated the gene structures, especially at 5' end. Detailed analysis for pseudo gene predictions was not carried out for either rice or poplar *TPS* genes since it is beyond the purpose of this research as comparative genome analysis based gene discovery, and the cDNA and EST resources are limited for both species as compared to that of *Arabidopsis*. For the same reason, intron/exon analysis and chromosome distribution were not described.

Relatedness and Gene Structure of AtTPS, OsTPS and PtTPS

Phylogenetic analysis was carried out to study the relatedness of *TPS* genes in all three species as shown in Figure 5.1. In all three species, the majority of the genes belonged to Class I *TPS* genes. In *Arabidopsis*, there are three Class III *TPS* genes, and there are four Class III *TPS* genes in rice and poplar, respectively. Most of these genes turned out to be diterpene synthase genes, and several of them were characterized to be involved in GA biosynthesis.

In both *Arabidopsis* and rice, several terpene synthase genes have already been characterized for their biochemical function. As shown in Figure 5.1 A and B, monoterpene synthases tended to share clades with monoterpene synthases and sesquiterpene synthases tended to share clades with sesquiterpene synthases.

However, in *Arabidopsis*, there is a group of *TPS* genes that consist of recently expanded genes mainly in chromosome 3 and 4. These genes have not been characterized to date. The rice *TPS* gene family also had a similar recently expanded group mainly through tandem duplication on chromosome 4. Two of these genes have been characterized to be OsLMSes. Moreover, for both rice and *Arabidopsis*, the linalool synthase genes were close to diterpene synthase genes in the phylogenetic analysis.

Comparative Genome Analysis of Rice, Poplar and Arabidopsis TPS Genes

The phylogenetic analysis of rice, *Arabidopsis*, and poplar *TPS* genes together reveals several features. First, most of the major clades are species specific. In

other words, most *AtTPS* genes shared clades together, so did *PtTPS* and *OsTPS* genes. Second, most of the *Arabidopsis* and rice TPS genes had similar distance with one another, which indicated their evolutionary speed to be similar. However, the *PtTPS* genes in two of the poplar clades showed higher similarity among the genes, which indicates a rapid evolution of that particular group of *PtTPS* genes. Thirdly, there were two small clades shared by genes from all three species. Shared clade I contained several diterpene synthases involved in GA biosynthesis. The existence of this group is not out of expectation since GA biosynthesis should be conserved during the evolution considering the importance of the plant hormone in plant growth and development. The shared clade II contained rice and *Arabidopsis* linalool synthase as well as several poplar *TPS* genes. The existence of shared clade II is out of expectation since linalool is a secondary metabolite and the evolution of *TPS* genes involved in secondary metabolite biosynthesis is highly dynamic in the evolution.

Comparative Genome Analysis of Most Identified TPS Genes

Comparative genome analysis was also carried out for most of the *TPS* genes characterized as indicated by previous publications and NCBI annotations. The phylogenic analysis of all of these *TPS* genes revealed several features. First, the monoterpene synthase genes tend to share clade with monoterpene synthase genes, whilst the sesquiterpene genes tend to share clades with sesquiterpene synthase genes, which is similar to the three species phylogenic analysis. Second, there are very few large clades composed of genes with only one type of product

profile. For example, limonene synthase genes can share clades with other monoterpene synthase genes, yet very few large clades included terpene synthase genes for only one product. Third, the terpene synthase genes for the same product(s) can be in different clades. For example, there were several clades with linalool synthase genes. Forth, the genes within the same or closely relevant species tended to share the same clade. This observation is a predominant phenomenon. Monoterpene synthase genes or sesquiterpene synthase genes from the same or closely related species were found to share a separate clade. These genes could be for similar or different activities. Fifth, a similar conserved clade as found in Figure 5.2 was also found in Figure 5.3, which indicated the conserved group of gene included a wider range of species including rice, *Arabidopsis*, poplar, Medicago and such. This clade was shared by three *Antirrhium majus* monoterpene synthase, three medicargo monoterpene synthase, one *Arabidopsis* monoterpene synthase, three poplar monoterpene synthase, two rice monoterpene synthase and two maize monoterpene synthase genes. Both *Arabidopsis* and rice terpene synthase genes in this clade encoded for linalool synthase genes. The dicot *TPS* genes in the clade are closer relevant to one another as compared to the monocot genes. We therefore inquired about the biochemical function of the *PtTPS* genes in this clade, which may help us to understand the evolution of *TPS* genes across the species.

Characterization of PtLIS in the conserved clade

We cloned both full length and truncated *PtTPS17* gene and expressed them in *E. coli*. Enzyme assays were carried out for both, and only truncated *PtTPS* genes exhibited activity toward GPP. Monoterpene synthase genes were normally located in the plastid, and transient peptides often interfered with the enzyme assay, which explained why truncation is often necessary for enzymatic assays of monoterpene synthase (Bohlmann and Croteau, 1999; Aubourg et al., 2002; Chen et al., 2003). As shown in Figure 5.4, the enzyme assay for the truncated gene shows activity toward GPP producing linalool as the single product. Considering the plastid intracellular location, we considered this gene to be a *PtLIS* gene.

Gene Expression analysis of PtLIS Gene

Real-time PCR experiments were carried out to examine the gene expression pattern of the *PtLIS* gene identified. As shown in Figure 5.5, the *PtLIS* gene is up-regulated by essentially all plant hormone treatments including JA, SA, ABA, BA and such. Expression was increased more than 2 fold in most cases. Under normal conditions, the *PtLIS* gene expresses more in shoot tip tissue as compared to the whole plant. No gene expression was detected for old leaves, root or bark. Regardless the gene expression pattern, we have performed volatile analysis with SPME, and no significant amount of linalool was found in any of the conditions where the gene was over-expressed.

Multiple Sequence Alignment

Multiple sequence alignment and phylogenetic analysis were carried out for all previously reported LIS genes (Dudareva *et al.*, 1996; Cseke *et al.*, 1998; Jia *et al.*, 1999; van Schie *et al.*, 2007). As shown in Figure 5.6A, the multiple sequence alignment revealed that the sequences from LIS genes out of the different species tested were conserved. However, both *Clarkia breweri* and pink ribbon LIS had longer 5' sequences, and they were among the earliest identified LISes. In fact, both genes were more similar to diterpene synthases as compared to other genes. The phylogenetic analysis revealed that *Arabidopsis*, poplar and rice LIS genes shared higher similarity, whereas *Clarkia breweri* and pink ribbon LISes shared higher similarity. From the analysis, we expected the LISes were evolved from convergent evolution, which is also confirmed by Figure 5.4.

Discussion

We presented the comparative analysis of *TPS* genes within and across species, as well as cloning and characterization of a *PtLIS* gene based on the comparative genome analysis. The results revealed several important features of the *TPS* gene and its gene family evolution and the potential of using gene family analysis for gene discovery.

The Evolution of TPS Genes within the Species

From the gene family analysis, we observed that tandem duplication is prevalent in the *TPS* gene family evolution, where it becomes the major source for new gene generation in each species. In all three species, the recent expansion of the gene family seemed to involve tandem duplication at several different chromosome locations. The new genes in the family can either obtain new functions, to retain original functions, or to become pseudogenes. All three gene fates exist in the terpene synthase gene family evolution and we could not draw any conclusion regarding which is more common than the other.

For example, in rice, three sesquiterpene synthase genes (*OsTPS37*, *OsTPS38*, *OsTPS39*, *TPS_Pseudogene_E*) seemed to originate from a common ancestor from recent tandem duplication events. *OsTPS37* and *OsTPS38* evolved very different biochemical function according the product profile (Chapter II). In fact, the two genes are responsible for most of the sesquiterpenes emitted during insect damage of japonica rice, even though the regulation of these genes during

the insect defense was different. However, *TPS_Pseudogene_E* turned out to be a pseudogene, and this pseudogene has more than 90% sequence similarity to *OsTPS38*. The third gene *OsTPS39* was not detected by RT-PCR in the insect treated japonica rice, which indicated that it doesn't have a insect defense function.

The case for *OsTPS22* and *OsTPS23* is quite different, where both genes produced limonene as major products. The biochemical function for the two genes is very similar. The gene regulation is slightly different, where *OsTPS25* is more responsive to jasmonic acid treatment (Chapter III). We can argue that evolution of *TPS* gene is a highly dynamic process, and what we observed in this research is just a snapshot of a time point during the dynamic changes. Therefore, the two OsLMS may end up having evolved two different biochemical and biological functions, at a minimum, different regulatory responses. However, the prevalent phenomena of co-existence of *TPS* genes with same products in the same species seems to indicate that rice as other plants does not have a strong selection against co-existence of the same gene for multiple copies. Considering many higher plant genomes including rice, poplar and *Arabidopsis*, all having evolved from one or more whole genome duplication events, plants may be more tolerant to multiple copies of the same gene in genome as compared to animal species (De Bodt *et al.*, 2005). Our finding that multiple *TPS* genes with same biochemical function in a certain species also supports these assumptions.

The evolution of TPS gene family across species

The phylogenetic analysis of *TPS* gene families across species showed a significant pattern of species specificity. In other words, the genes in the same or closely relevant species tended to share common clades, rather than the genes with the same biochemical activity across different species. The same phenomena had been found in the phylogenetic analysis of three species *TPS* genes and the phylogenetic analysis of multiple species *TPS* genes. On one side, the phenomena indicated the rapid and dynamic evolution of *TPS* gene family. In fact, the expansion of most members of *TPS* gene families in rice, *Arabidopsis* and poplar is expected to be after the speciation between monocot and dicot as well as the perennial woody species and herbaceous annual plants. The monocot and dicot diverged around 120 million years ago, and most of the *TPS* genes seemed to have evolved after the divergence (De Bodt *et al.*, 2005). *Arabidopsis* and poplar shared much closer lineage, where both of them belonged to Eudicots and the Rosids. *Arabidopsis* belongs to Brassicales out of Eurosoid II, while poplar belongs to Malpighiales out of Eurosoid I. The divergence between *Arabidopsis* and poplar is expected to be around 50 to 70 million years ago. The species specific pattern of comparative genome analysis for poplar and *Arabidopsis* *TPS* genes indicated the very recent expansion of *TPS* gene family.

On the other side, the species specific pattern indicated the importance of convergent evolution in evolving biochemical and ecological function of *TPS*

genes. It is a rare event for a common ancestor gene for certain terpenoid volatile compound(s) biosynthesis to retain its biochemical function for a long period time during the evolution considering the rapid evolution of the gene family. The *TPS* genes for the same products, therefore, often evolve through convergent evolution. The functional evolution of *TPS* genes is highly dynamic, assumingly due to the need of quick adaptation to the environment.

Biochemical activity and evolution

The evolution of new biochemical function for terpenoid biosynthesis has been intensively studied utilizing structural biology approaches. Generally speaking, these studies indicated the highly flexible *TPS* gene structure in producing new compounds. Changes in few amino acids could lead to different product profile, which has been shown to be true in the case of *OsLMS* (Hyatt *et al.*, 2007) (Chapter III). The changes of product profile could either be the proportion of different compounds or the contents of different compounds, both of which have been indicated to be important in the sending information to natural enemies of herbivorous insects (Dudareva *et al.*, 2006) (Chapter I).

The structure flexibility along with the rapid evolution has made the *TPS* gene family a unique component in plant adaptation to the ever-changing environment. The driving force for the evolution of new *TPS* gene function is expected to be due to herbivorous insect damage (Dudareva *et al.*, 2006). However, other environmental factors such as abiotic stress and plant diseases

should not be totally ignored (Dudareva *et al.*, 2006). In the environment abundant with both herbivorous insects and their natural enemies, new biochemical functions of existing *TPS* genes is expected to be evolved rapidly. The evolution could be on the enzyme itself or the promoter region. The changes in enzyme protein sequences could lead to new products that can more effectively attract natural enemies of herbivorous insects in the environment. Moreover, the changes in the promoter region could allow the enzymes to be more responsive to herbivory damage and re-define the function for the existing enzymes.

The existence a clade of *TPS* genes with deep evolution origin in the phylogenic analysis of all *TPS* genes was somehow out of expectation considering the rapid evolution of the gene family. According to the phylogenic analysis, the group may represent an ancient group of *TPS* genes, and they could also represent the ancestor genes for some other genes. The biochemical function of the group seems to be retained well, since *Arabidopsis*, poplar and rice genes were all responsible for linalool biosynthesis. Linalool was shown as one of the most effective volatile compounds attracting parasitoids and predators for herbivorous insects with both field and laboratory experiments (Kessler and Baldwin, 2001). In field studies, linalool has been shown to reduce insect infestation as effective as the plant defense hormone derivative, methyl jasmonate (Kessler and Baldwin, 2001). Linalool could be easily converted from GPP, which enables the plant to require less cost/energy in its biosynthesis. In fact, linalool has been

serving as pollination signaling compounds in flowers as well (Dudareva *et al.*, 1996). A compound like linalool would be ideal for locating forage for natural enemies of herbivorous insects. Linalool therefore may serve as an infochemical for indirect defense in a broad range of ecosystems (Jia *et al.*, 1999; Aharoni *et al.*, 2003; Iijima *et al.*, 2004; Noe *et al.*, 2006; van Schie *et al.*, 2007). The significant differences in the effectiveness of linalool as compared to other terpenoid compounds in indirect defense may be the evolutionary driving force to keep the biochemical activity of the ancient group of enzyme.

The Function of PtTPS gene identified

We have performed volatile analysis of poplar plants treated with different plant hormone treatments to expect the production of linalool (data not shown).

However, under current conditions including jasmonic acid and salicylic acid treatment, no significant amount of linalool was emitted, which is slightly contradictory to the gene expression pattern of the genes under plant hormone treatment, since the *PtLIS* gene is slightly up-regulated by both plant hormones. It should be noted that if other monoterpene synthase genes such as ocimene synthase were induced to a higher level than linalool synthase, we would expect no linalool production, due to the substrate competition. Since we found a high level of ocimene and 1-8 cineole during the treatments, we expect that linalool is not released due to substrate competition.

Even though linalool is not induced by the defense hormone jasmonic acid, forest tent caterpillar (*Malacosoma disstria*) treatment induced production of linalool in hybrid poplar, which indicates linalool has an important role as a defense compound (Arimura et al., 2004; Kendrick and Raffa, 2006). Further research needs to be carried out to characterize more monoterpene synthase genes and up-stream terpene biosynthesis genes in poplar. The function and regulation of these genes under different insect responses should be studied to further understand the molecular mechanisms of insect defense in poplar (Ralph et al., 2006). We would expect the *PtLIS* to retain its biochemical and biological function during the dynamic evolution of TPS gene family at least partially due to the pressure from herbivorous insects.

Moreover, since *PtLIS* is up-regulated by a variety of plant hormones, the gene may also be involved in biological processes other than plant defense against insects. Terpenoids have been indicated for a broad range of functions including both biotic and abiotic stresses (Dudareva et al., 2006). The fact that *PtLIS* is up-regulated by plant hormones like ABA indicates that this gene may be involved in abiotic stress response as well. Overall, more studies need to be performed to study the *TPS* gene function in poplar as a model species for woody perennial plant.

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Appendix

Table 5.1. Candidate *TPS* genes in rice and poplar.

Gene	ID	Chr
Rice TPS Genes		
OsTPS1	LOC_Os01g23530	1
OsTPS2	LOC_Os01g42610	1
OsTPS3	LOC_Os02g02930	2
OsTPS4	LOC_Os02g17780	2
OsTPS5	LOC_Os02g26014	2
OsTPS6	LOC_Os02g36210	2
OsTPS7	LOC_Os02g36140	2
OsTPS8	LOC_Os02g36220	2
OsTPS9	LOC_Os02g36264	2
OsTPS10	LOC_Os03g22620	3
OsTPS11	LOC_Os03g22634	3
OsTPS12	LOC_Os03g24650	3
OsTPS13	LOC_Os03g24680	3
OsTPS14	LOC_Os03g24690	3
OsTPS15	LOC_Os03g24760	3
OsTPS16	LOC_Os03g24710	3
OsTPS17	LOC_Os03g31430	3
OsTPS18	LOC_Os04g01810	4
OsTPS19	LOC_Os04g09900	4
OsTPS20	LOC_Os04g10060	4
OsTPS21	LOC_Os04g26960	4
OsTPS22	LOC_Os04g26980	4
OsTPS23	LOC_Os04g27070	4
OsTPS24	LOC_Os04g27190	4
OsTPS25	LOC_Os04g27340	4
OsTPS26	LOC_Os04g27400	4
OsTPS27	LOC_Os04g27430	4
OsTPS28	LOC_Os04g27540	4
OsTPS29	LOC_Os04g27670	4
OsTPS30	LOC_Os04g27720	4
OsTPS31	LOC_Os04g27760	4
OsTPS32	LOC_Os04g27470	4
OsTPS33	LOC_Os04g27790	4
OsTPS34	LOC_Os04g52210	4
OsTPS35	LOC_Os04g52230	4
OsTPS36	LOC_Os04g52240	4
OsTPS37	LOC_Os05g16980	5
OsTPS38	LOC_Os05g17010	5
OsTPS39	LOC_Os05g24500	5
OsTPS40	LOC_Os05g24520	5
OsTPS41	LOC_Os07g11790	7
OsTPS42	LOC_Os08g04500	8
OsTPS43	LOC_Os08g07080	8
OsTPS42	LOC_Os08g07100	8
OsTPS45	LOC_Os08g07120	8

Table 5.1. continued.

Gene	ID	Chr
OsTPS44	LOC_Os10g34790	10
OsTPS47	LOC_Os11g28490	11
OsTPS48	LOC_Os11g28500	11
OsTPS49	LOC_Os11g28530	11
OsTPS50	LOC_Os12g30790	12
OsTPS51	LOC_Os12g30800	12
OsTPS52	LOC_Os12g30824	12
Poplar TPS Genes		
PtTPS1	gw1.I.3687.1	1
PtTPS2	gw1.I.3688.1	1
PtTPS3	fgenes4_pm.C_LG_II000250	2
PtTPS4	gw1.IV.2101.1	4
PtTPS5	gw1.IV.2105.1	4
PtTPS6	gw1.IV.2323.1	4
PtTPS7	fgenes4_pg.C_LG_IV000547	4
PtTPS8	eugene3.00050339	5
PtTPS9	eugene3.00050699	5
PtTPS10	gw1.V.4840.1	5
PtTPS11	fgenes4_pg.C_LG_V001263	5
PtTPS12	fgenes4_pg.C_LG_VII000261	7
PtTPS13	fgenes4_pm.C_LG_VII000101	7
PtTPS14	fgenes4_pg.C_LG_VII000272	7
PtTPS15	gw1.VII.1144.1	7
PtTPS16	fgenes4_pm.C_LG_VII000258	7
PtTPS18	eugene3.00080757	8
PtTPS18	gw1.XI.2364.1	11
PtTPS19	eugene3.00110397	11
PtTPS20	fgenes4_pg.C_LG_XI001106	11
PtTPS21	estExt_Genewise1_v1.C_LG_XI0653	11
PtTPS22	eugene3.00130815	13
PtTPS23	gw1.XIII.3321.1	13
PtTPS24	eugene3.00150645	15
PtTPS25	fgenes4_pg.C_LG_XIX000175	19
PtTPS26	gw1.XIX.482.1	19
PtTPS27	gw1.XIX.1344.1	19
PtTPS28	grail3.0085006801	19
PtTPS29	gw1.XIX.1359.1	19
PtTPS30	gw1.64.12.1	S_64
PtTPS31	fgenes4_pg.C_scaffold_117000025	S_117
PtTPS32	eugene3.01170028	S_117
PtTPS33	eugene3.01170033	S_117
PtTPS34	eugene3.01170035	S_117
PtTPS35	fgenes4_pm.C_scaffold_117000002	S_117
PtTPS36	estExt_fgenes4_pg.C_1220060	S_122
PtTPS37	gw1.234.14.1	S_234
PtTPS38	eugene3.02720005	S_272
PtTPS39	gw1.283.8.1	S_283

Table 5.1. continued.

Gene	ID	Chr
PtTPS40	fgenes4_pg.C_scaffold_401000001	S_401
PtTPS41	eugene3.04010004	S_401
PtTPS42	eugene3.04010007	S_401
PtTPS43	gw1.882.3.1	S_882
PtTPS44	eugene3.09080001	S_908
PtTPS45	eugene3.09120001	S_912
PtTPS46	gw1.952.1.1	S_952
PtTPS47	eugene3.10760001	S_1076
PtTPS48	gw1.1935.4.1	S_1935
PtTPS49	gw1.3545.3.1	S_3545
PtTPS50	fgenes4_pg.C_scaffold_9875000001	S_9875
PtTPS51	eugene3.106990001	S_10699
PtTPS52	gw1.12296.3.1	S_12296
PtTPS53	fgenes4_pg.C_scaffold_14117000001	S_14117
PtTPS54	grail3.14219000101	S_14219

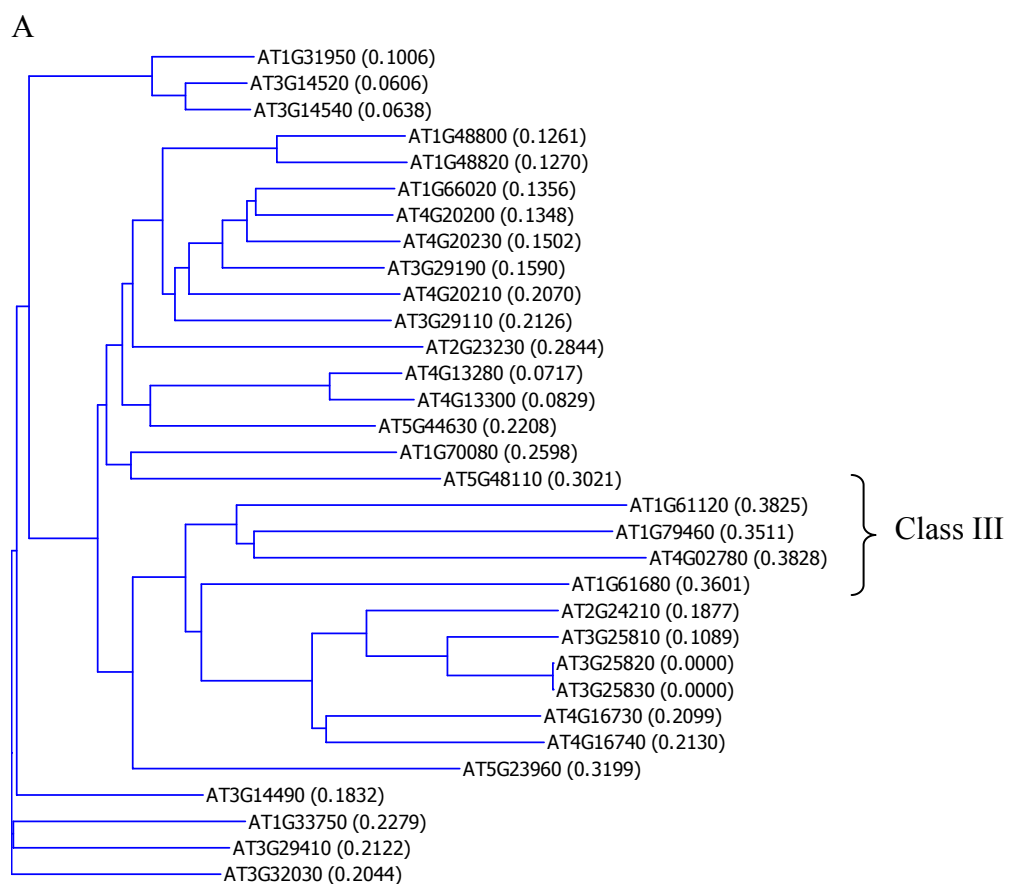


Figure 5.1. continued

B

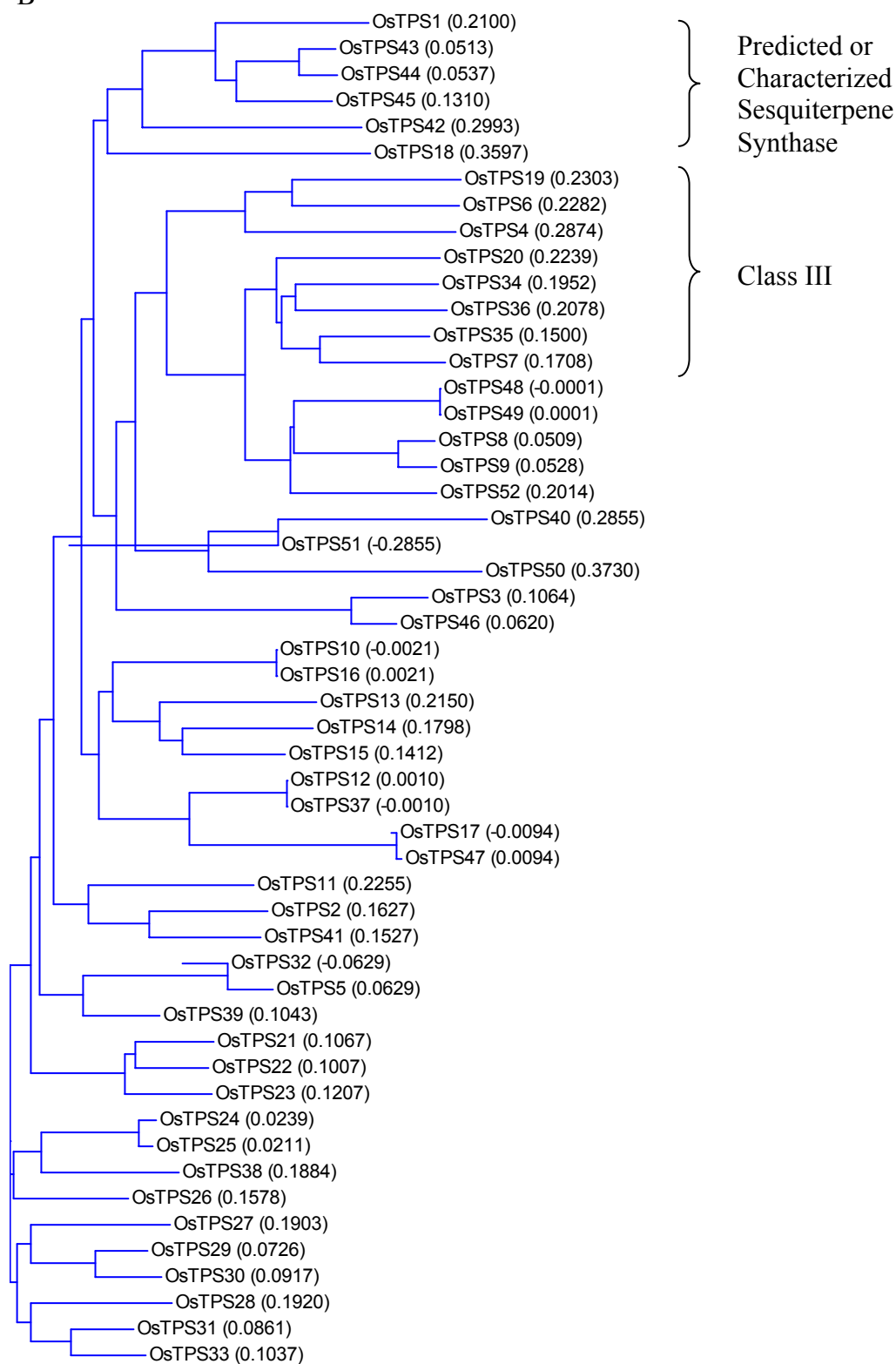


Figure 5.1.continued.

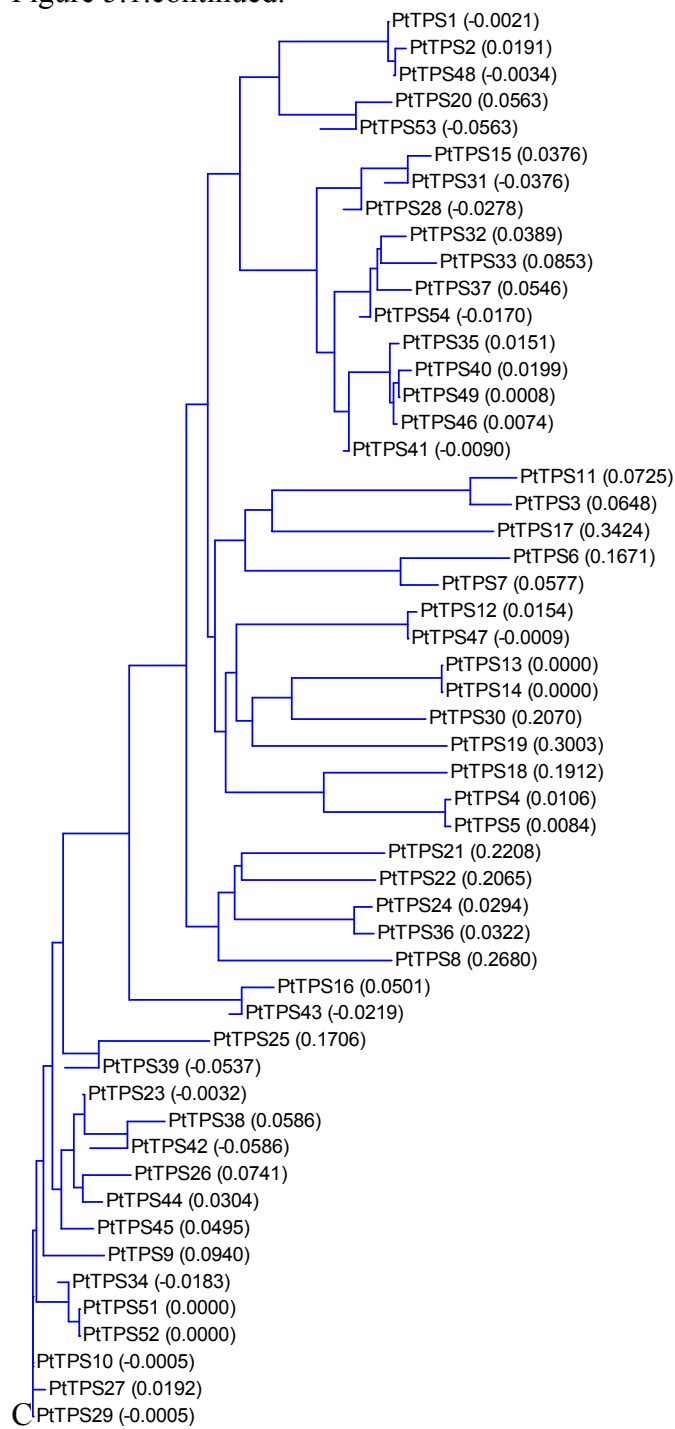


Figure 5.1. The phylogenetic analysis of *TPS* genes within the species for rice, poplar, and *Arabidopsis*, respectively. Figure 5.1A shows the phylogenetic

Figure 5.1. continued.

analysis of *AtTPS* genes; B shows the phylogenic analysis of *OsTPS* genes; C shows the phylogenic analysis of *PtTPS* genes. The Class III *TPS* genes for GA biosynthesis and other features were also shown in the figure.

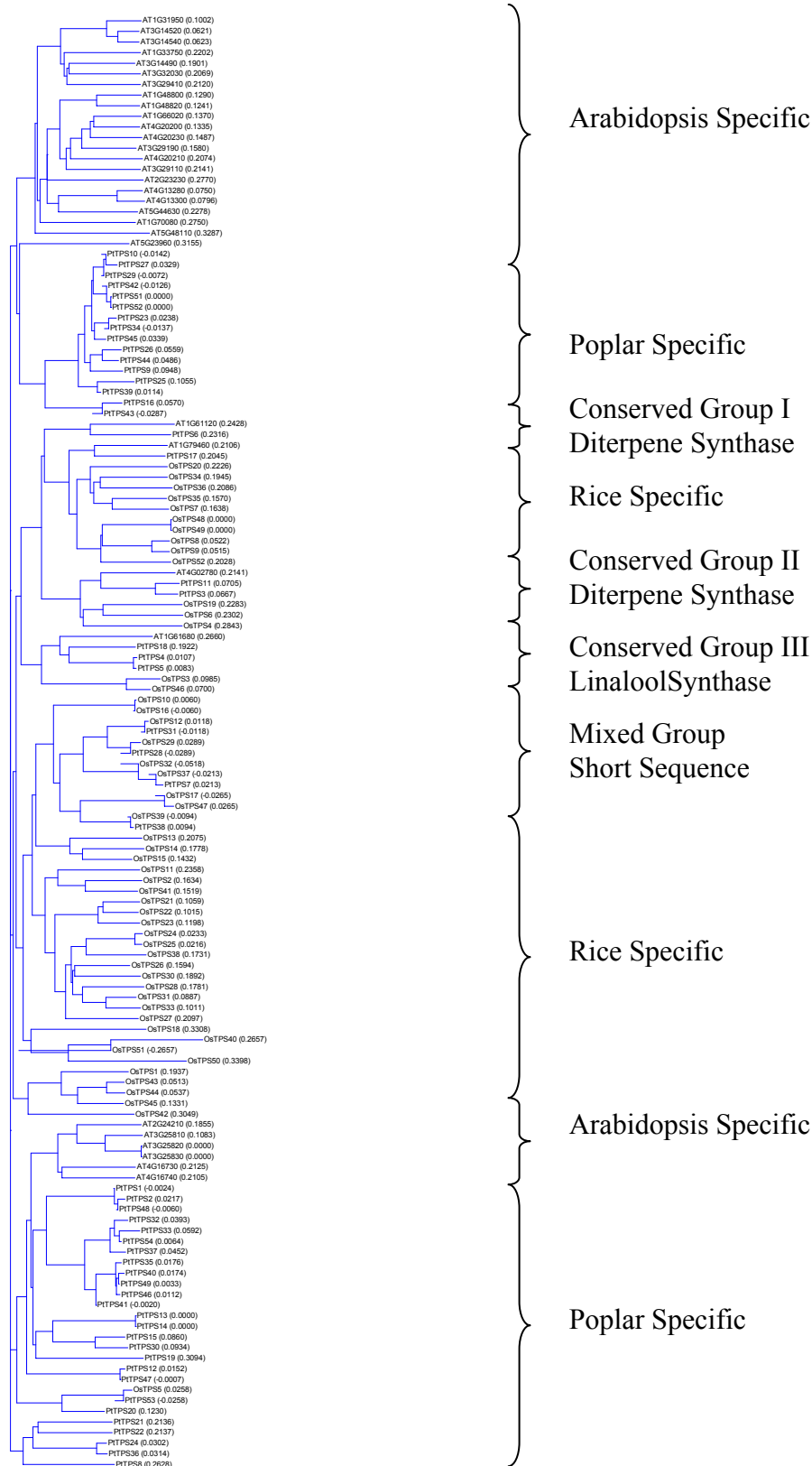


Figure 5.2. continued.

Figure 5.2. Phylogenic analysis of all *Arabidopsis*, rice and poplar putative *TPS* genes.

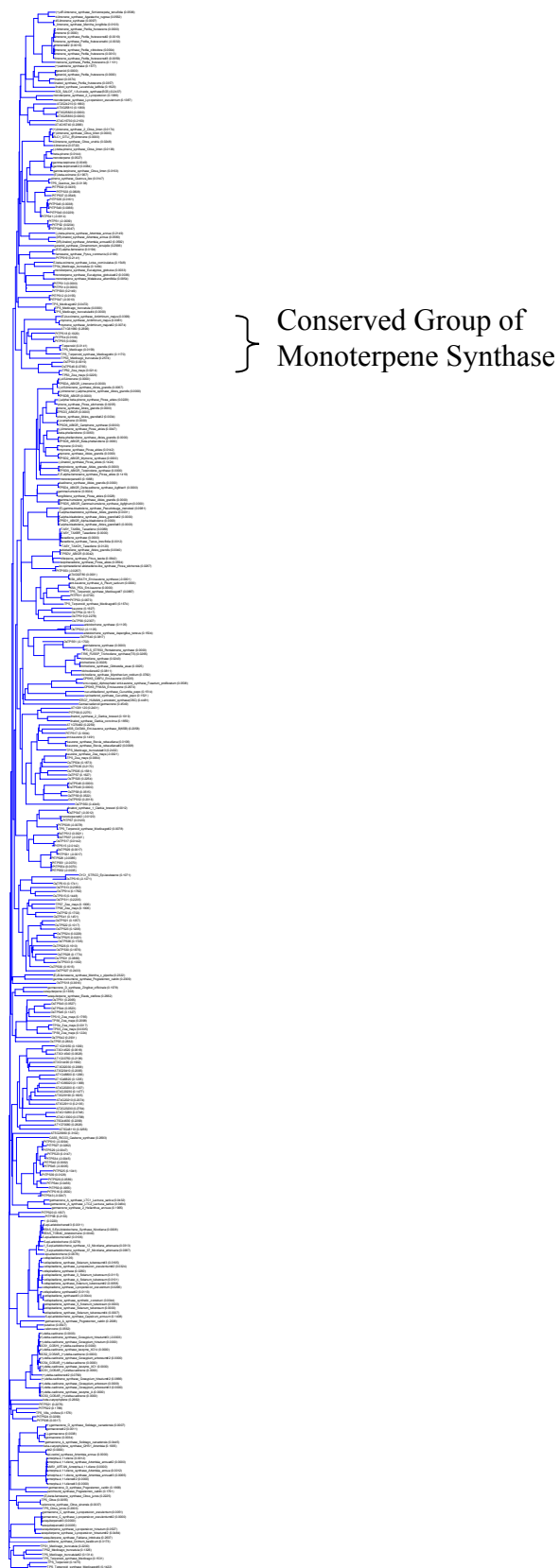


Figure 5.3. Phylogenetic analysis of most *TPS* genes from plant species.

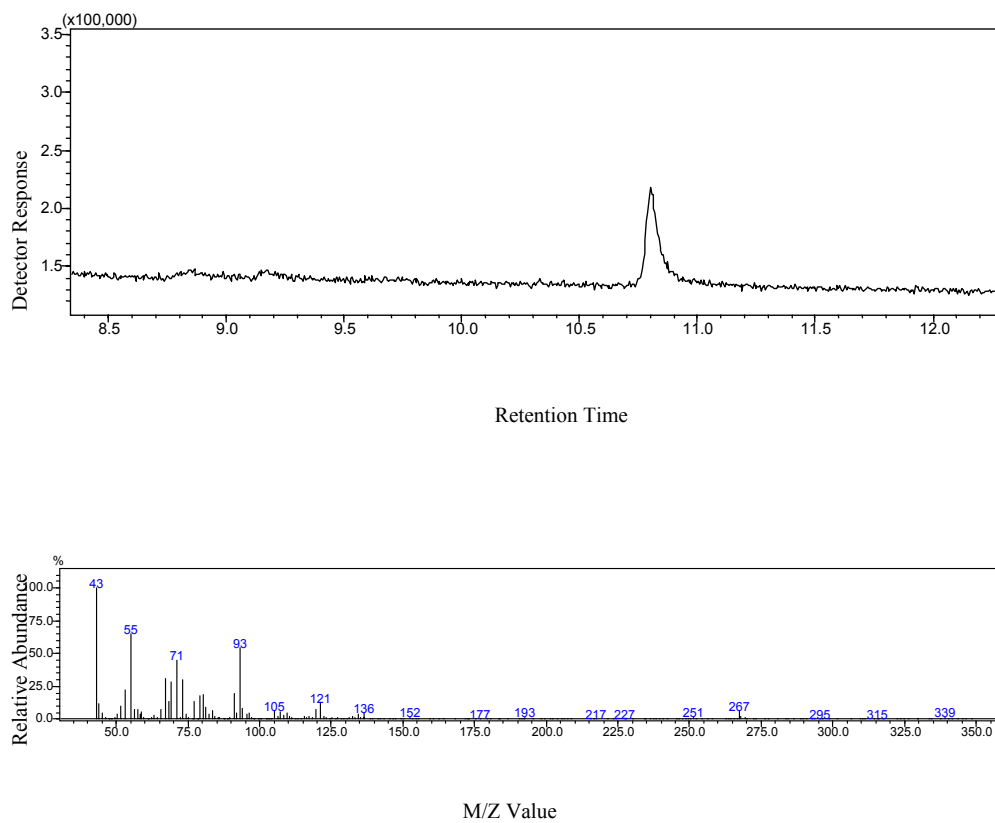
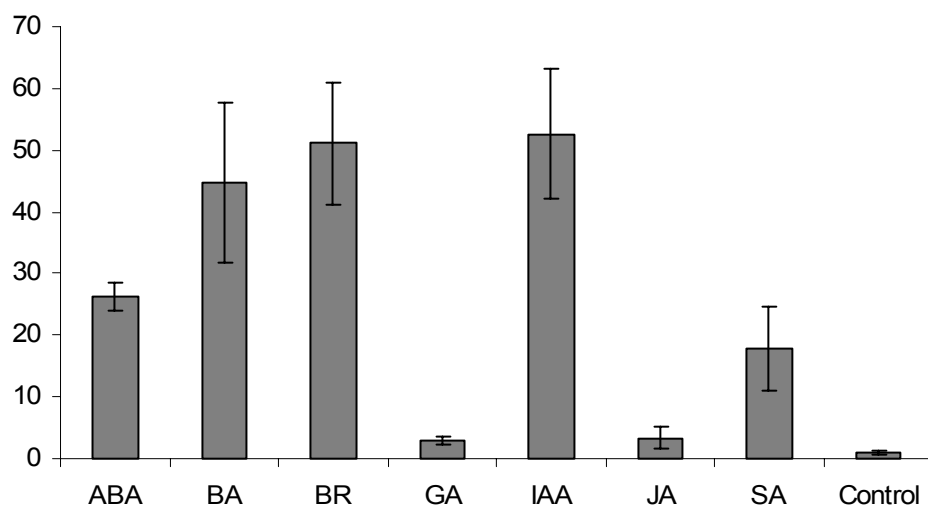
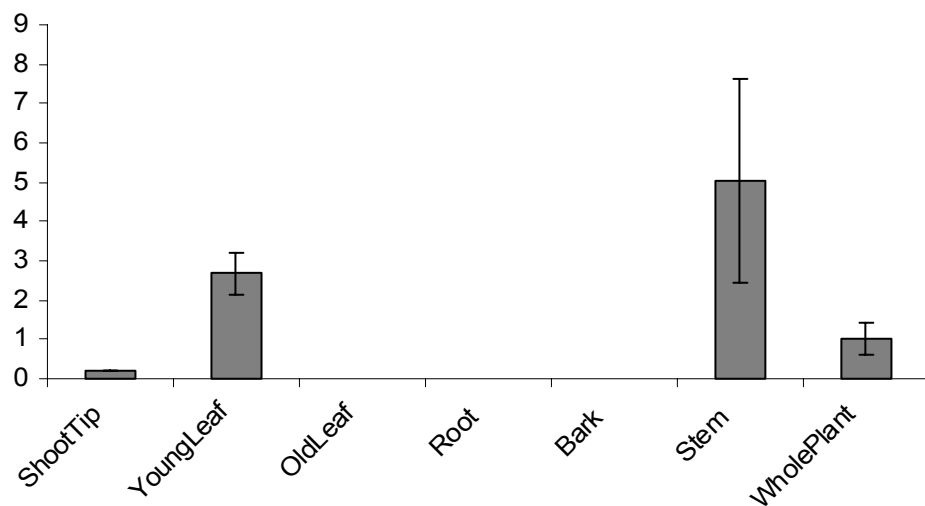


Figure 5.4. Enzyme assay of PtLIS. A. The GC chromatograph showed the retention time of the enzyme assay product and B. the MS ion profile confirms its identity as linalool.



A



B

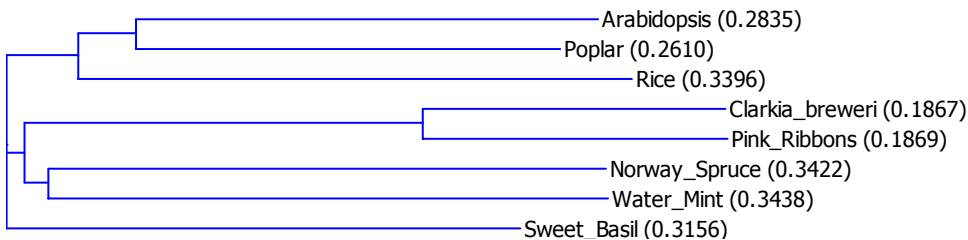
Figure 5.5. Gene expression of *PtLIS* gene. A. Plant hormone induced gene expression. Y axis showed the gene expression ratio of treatment as compared to untreated control sample, and X axis showed the name of the treatment. B.

Figure 5.5. continued.

Tissue specific gene expression pattern. Y axis showed the ratio of tissue specific expression as compared to the control whole plant and X axis showed the tissue type. The blanks indicated no expression detected.

Arabidopsis	:	-----*	20	-----*	40	-----*	60	-----*	80	-----*	:	-	
Poplar	:	-----		-----		-----		-----		-----	:	-	
Rice	:	-----		-----		-----		-----		-----	:	-	
Clarkia_breweri	:	-----	MLSTQSHVDEIKR	-----	GSLNLRLSPSAVDTAWLALI	PDPPDPTR	-----	PMFAKCMHWLVQVQSGMEGFWAADD	-----		:	66	
Pink_Ribbons	:	MQPITKSSSTSSSELEFLVDKVKRESLSSSSSDTQNL	FLSASPYDTAWLALI	PHPHHHHHHG	PMFEKCLQWLHNGT	PGGFWTAAGDNI	-----		-----		:	90	
Norway_Spruce	:	-----		-----		-----		-----		-----	:	-	
Water_Mint	:	-----		-----		-----		-----		-----	:	-	
Sweet_Basil	:	-----		-----		-----		-----		-----	:	-	
Arabidopsis	:	-----	100	-----*	120	-----*	140	-----*	160	-----*	180	:	
Poplar	:	-----		-----		-----		-----		-----		:	
Rice	:	-----		-----		-----		-----		-----		:	
Clarkia_breweri	:	DIITEPVALDCLPATLACLIALKRWGAAPNNIN	KGGLGFERNVEELLRRKGLSDVPRWFTV	TFPLAME	LAIASGLKVPFONLKV	ED	-----		-----		:	155	
Pink_Ribbons	:	SDTDDVTLDCLSTLACLVALKRWQLAFDMI	HKLEFVHRNTERLVMKQ	-----	KPSDVPRWFTV	TFPLAME	LAGASGLRVDS	SENLR	IV	-----	:	178	
Norway_Spruce	:	-----		-----		-----		-----		-----	:	-	
Water_Mint	:	-----		-----		-----		-----		-----	:	-	
Sweet_Basil	:	-----		-----		-----		-----		-----	:	-	
Arabidopsis	:	-----	200	-----*	220	-----*	240	-----*	260	-----*	:	:	
Poplar	:	SKFPNTVDLSFPANTAKR	-----	SIERNVHASVSNPSKO	-----	PHNKTSLEYSHELN	IKRNILSAN	-----		-----	:	86	
Rice	:	KEKPTLPSTTKWSYQOK	-----	OTAFGPTKQYPTINNVIIFVVG	-----	PCTCNCVQGYAEKLOTFKHLRKE	-----		-----		:	97	
Clarkia_breweri	:	ELFENRNTILLREEDKTOYAPLLMFLEALPPSY	WKLLDNLQYLERNLNGDGLQ	-----	SPSATA	AYMATONT	CSYLKSLNTYTYLDGG	-----		-----	:	244	
Pink_Ribbons	:	ELTONRDDILTREYGEKKQYSPLLLLEALPAQSY	VDSDDLKQIIDKKLSSDGLQ	-----	SPSATA	AYMTIGNTC	SYLHSLNCSGNGG	-----		-----	:	267	
Norway_Spruce	:	KSVTFSSMCLTTTYDDGG	-----	VQRREADHFNLDNDNFQSLSTPFG	-----	ATAYHE	AQKLIGEVYVINSILVDEIG	-----		-----	:	113	
Water_Mint	:	WLSLSPSSAASRRSCISSKLYTSSAQETRRSGY	HSVWDFQIQSLDDHYKEEQLE	-----	REELIMYK	LAGAKEA	-----		-----		:	118	
Sweet_Basil	:	PRFSACTPLASAVPESTP	-----	LINGDNSPLKNTQHVR	-----	ERSSKR	EYLLLEETAK	ORNDTES	-----		:	92	
Arabidopsis	:	-----	280	-----*	300	-----*	320	-----*	340	-----*	360	:	
Poplar	:	-----		-----		-----		-----		-----		:	
Rice	:	-----		-----		-----		-----		-----		:	
Clarkia_breweri	:	VPSLYCMDEELQVAVNQLVTEYVPR	-----	KOLLOVQNYKCKRSPFP	PRNALHNVVA	YKDELA	WLLINSHV	PSICW	LIH	-----	:	334	
Pink_Ribbons	:	VPSLYVDDDLHDAVAVNQLVTEYVPR	-----	DHLKQVQNYKCKRSP	-----	KSLYGIAAR	YKDELA	WLLINSHV	PSICW	LIH	:	355	
Norway_Spruce	:	ELITPPNDLLHDAVAVNQLVTEYVPR	-----	DRHKNKRSADYVYVYSEKGGICG	-----	KDSGVN	NTAGL	YVPSLEQ	KE	-----	:	199	
Water_Mint	:	-----		-----		-----		-----		-----	:	-	
Sweet_Basil	:	-----		-----		-----		-----		-----	:	-	
Arabidopsis	:	-----	380	-----*	400	-----*	420	-----*	440	-----*	:	:	
Poplar	:	-----		-----		-----		-----		-----	:	-	
Rice	:	-----		-----		-----		-----		-----	:	-	
Clarkia_breweri	:	NNIEIRHIEKANYMYFDNVLNVR	-----	ETDQARH	YSRNRLCSG	-----	RESHQKOE	MKSAQ	RIKTRG	TS	:	234	
Pink_Ribbons	:	DEIRIDHIEITNYKEFAAVNLNVR	-----	ETDQARH	YSRNRLCSG	-----	RESHQKOE	MKSAQ	RIKTRG	TS	:	247	
Norway_Spruce	:	QNEQACALCTGEKEKVN	-----	ETDQARH	YSRNRLCSG	-----	RESHQKOE	MKSAQ	RIKTRG	TS	:	253	
Water_Mint	:	EHGSDFRKTLEGTKEKVN	-----	ETDQARH	YSRNRLCSG	-----	RESHQKOE	MKSAQ	RIKTRG	TS	:	270	
Sweet_Basil	:	ERGE	-----	FDESETL	DAVRSFSAEEE	-----	E	-----		-----	:	231	
Arabidopsis	:	-----	460	-----*	480	-----*	500	-----*	520	-----*	540	:	
Poplar	:	-----		-----		-----		-----		-----		:	
Rice	:	-----		-----		-----		-----		-----		:	
Clarkia_breweri	:	LMEEAASDALMMGKSSHHRLSR	LNHLLOELKSLTLR	SVYRNSEH	KKWKRER	-----	LCDG	GRKRTYCY	YARAASTL	CS	:	513	
Pink_Ribbons	:	LMEEAASDALMMGKSSHHRLSR	LNHLLOELKSLTLR	SVYRNSEH	KKWKRER	-----	LCDG	GRKRTYCY	YARAASTL	CS	:	532	
Norway_Spruce	:	LMEEAASDALMMGKSSHHRLSR	LNHLLOELKSLTLR	SVYRNSEH	KKWKRER	-----	LCDG	GRKRTYCY	YARAASTL	CS	:	358	
Water_Mint	:	LMEEAASDALMMGKSSHHRLSR	LNHLLOELKSLTLR	SVYRNSEH	KKWKRER	-----	LCDG	GRKRTYCY	YARAASTL	CS	:	347	
Sweet_Basil	:	LMEEAASDALMMGKSSHHRLSR	LNHLLOELKSLTLR	SVYRNSEH	KKWKRER	-----	LCDG	GRKRTYCY	YARAASTL	CS	:	308	
Arabidopsis	:	-----	560	-----*	580	-----*	600	-----*	620	-----*	:	:	
Poplar	:	-----		-----		-----		-----		-----	:	-	
Rice	:	-----		-----		-----		-----		-----	:	-	
Clarkia_breweri	:	VRHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Pink_Ribbons	:	VRHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Norway_Spruce	:	VRHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Water_Mint	:	VRHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Sweet_Basil	:	VRHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Arabidopsis	:	-----	640	-----*	660	-----*	680	-----*	700	-----*	720	:	
Poplar	:	-----		-----		-----		-----		-----		:	
Rice	:	-----		-----		-----		-----		-----		:	
Clarkia_breweri	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Pink_Ribbons	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Norway_Spruce	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Water_Mint	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Sweet_Basil	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Arabidopsis	:	-----	740	-----*	760	-----*	780	-----*	800	-----*	:	:	
Poplar	:	-----		-----		-----		-----		-----	:	-	
Rice	:	-----		-----		-----		-----		-----	:	-	
Clarkia_breweri	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Pink_Ribbons	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Norway_Spruce	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Water_Mint	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Sweet_Basil	:	YVHLAKAAIVVYADLPDPE	-----	SRNDELNTDAV	MEVEG	-----	LRHS	IEBALDDV	NRILK	CFQKHK	IKNNPHIL	YETFS	619
Arabidopsis	:	-----	820	-----*	840	-----*	860	-----*	880	-----*	:	:	
Poplar	:	-----		-----		-----		-----		-----	:	-	
Rice	:	-----		-----		-----		-----		-----	:	-	
Clarkia_breweri	:	FTIPFQVHFKKPRYAKNPOQ	EATTSAAATSSAAPTSDQYASGGL	-----		-----		-----		-----	:	813	
Pink_Ribbons	:	FTIPFQVHFKKPRYAKNPOQ	EATTSAAATSSAAPTSDQYASGGL	-----		-----		-----		-----	:	871	
Norway_Spruce	:	TVIEHVPL	-----		-----		-----		-----		:	623	
Water_Mint	:	YV	-----		-----		-----		-----		:	606	
Sweet_Basil	:	PULSS	-----		-----		-----		-----		:	574	

A



B

Figure 5.6. continued.

Figure 5.6. Analysis of all LIS identified. A. Multiple sequence alignment of linalool synthases as indicated in the figure. The conserved DDXXD domain is also marked; B. Phylogenic analysis of the linalool synthase genes as shown in A.

Chapter VI. Conclusions and Perspectives

I. Conclusions

In the previous chapters, functional and comparative genomics approaches were employed to discover and characterize five rice *TPS* genes and one poplar *TPS* gene. The biological function of these genes was studied with respect to plant indirect defense from both molecular and ecological levels. The insect and jasmonic acid induced genes and pathways were surveyed with global gene expression profiling, which helped to define the molecular mechanisms of rice defense against herbivorous insects as well as the role of *TPS* genes in the process. Moreover, the regulation of *TPS* genes during the diurnal cycle was studied to further elucidate the molecular mechanisms of insect-induced response in plants. The ecological function of the *TPS* genes was studied with the Y-tube bioassay, which helped to define the tritrophic interaction among rice, rice fall armyworm, and its parasitoid, *Cotesia marginiventris*. The evolution of *TPS* gene function and the *TPS* gene family was investigated with respect to the evolution of plant defense mechanisms and gene family expansion. Our research has led to these nine major conclusions.

First, *TPS* genes and volatile terpenoids are important components of rice indirect defense. As shown in Chapter II, rice (*Oryza sativa japonica*) plants treated with the generalist herbivorous insect fall armyworm (*Spodoptera frugiperda*) emitted large amount of volatile organic compounds, with volatile terpenoids accounting for more than 70% of the total volatile emission. In terms of number of compounds, more than 20 volatile terpenoids were found among

the about 35 different volatile organic compounds. In fact, linalool was emitted at a significantly higher amount than any other compounds in the profile. The volatile organic compounds released by armyworm-damaged rice can attract a parasitoid of fall armyworm, *Cotesia marginiventris*, which indicated their important function in the tritrophic interaction. Moreover, a Y-tube bioassay with plants to which linalool was applied showed the strong capacity of linalool to attract the *Cotesia*, which suggested a role for linalool in indirect defense in rice.

In addition to characterizing the volatile organic compounds, the enzymes responsible for volatile terpenoid biosynthesis were also identified and characterized. Three genes including *OsLIS*, *OsTPS37* and *OsTPS38*, were shown to be responsible for most of the volatile terpenoids produced by fall armyworm damaged rice. These genes were significantly up-regulated in response to fall armyworm treatments, which indicated their biological function in plant defense against herbivorous insects.

Second, herbivorous insect damage caused coordinative changes in plant defense genes and pathways in rice. These up-regulated metabolic genes and pathways included those involved in up-stream terpenoid biosynthesis, phenolic compound biosynthesis, lignin biosynthesis and others. A general shift in secondary metabolism was observed as expected. However, the biological and biochemical functions of many enzymes remain undefined. Besides the

metabolic pathways, many components in the signal transduction pathways were also up-regulated, and these genes included WRKY transcriptional factors and protein kinases/phosphatases. Moreover, plant defense proteins such as protease inhibitors were also among the induced genes. The research represented one of the first examinations of transcriptome signature of rice response to insects using microarray technology.

A detailed examination of terpene biosynthesis related pathways revealed the coordinative up-regulation of the mevalonate pathway, the non-mevalonate (MEP) pathway, and *TPS* genes, which ensure that the production of terpenoid compounds will not be limited by the availability of substrates. Generally speaking, the up-regulation of *TPS* genes is only part of the overall coordinative responses against herbivorous insects in rice plants that serve as indirect components of the defense processes. The overall responses include both direct and indirect components, which could lead to synergetic effects of reducing the fitness of herbivorous insects. The global gene expression profiling helped to elucidate the molecular mechanisms of ecological interactions among plant, herbivorous insects and natural enemies.

Third, jasmonic acid induced responses involved a time-dependent production of volatile terpenoids. The quantity and composition of the volatile compounds changed at different time points after the treatments. In case of monoterpene biosynthesis, limonene emission first increased within two h upon jasmonic acid

treatment, and then linalool began to be emitted approximately four hour after the treatment. Sesquiterpenes were induced even later beginning at around six hour after JA treatment. The time-dependent composition and content changes of volatile terpenoids reflected the differential regulation of volatile biosynthesis and emission for different terpenoids.

Fourth, global gene expression profiling revealed that jasmonic acid induced transcriptome changes were similar to, but slightly different from those of insect treatments. The early transcriptomic response toward jasmonic acid in rice was surveyed with a half-genome DNA microarray. Four h after jasmonic acid treatment, genes in several defense relevant pathways were up-regulated, including mevalonate pathway, non-mevalonate pathway, phenolic compound biosynthesis. Moreover, genes in the growth and development pathways such as the cytokinin pathway were down-regulated. The early jasmonic acid induced transcriptomic response was similar to the insect induced transcriptomic response. Despite the similarity, only one *OsTPS* gene was up-regulated, which indicates that the up-regulation of *TPS* genes may occur in a later stage of jasmonic acid response. This possibility was also supported by the time-dependent volatile emission, in which limonene was the major volatile compound induced four hours after the jasmonic acid treatment.

Fifth, jasmonic-acid induced *OsLMS* genes might have a defense function. The jasmonic acid-induced *OsTPS* gene in the microarray experiment was

characterized as *OsLMS1*. A homolog gene with high similarity was also characterized to be limonene synthase, and was named *OsLMS2*. Both genes were up-regulated by the jasmonic acid treatment in a time-dependent fashion, with *OsLMS2* more responsive to the JA treatment with more than 20 fold induction of gene expression. The genes were also shown to be up-regulated by insect treatments and other biotic and abiotic stress treatments, which indicated multiple functions in defense and stress responses.

Sixth, the jasmonic acid and insect induced responses in rice followed a diurnal cycle dependent pattern for both volatile emission and gene expression. For both jasmonic acid and insect treated rice, terpenoid volatile emission and expression of *TPS* genes were significantly lower during the night time compared to that of the daytime.

Seventh, the global gene expression profiling revealed differential regulations of defense related genes at night compared to the daytime. Only one third of the genes up-regulated by the insect treatment at night was also induced at day time. Moreover, two key committed step genes in mevalonate and non-mevalonate pathways were regulated at a diurnal cycle-dependent way.

Eighth, comparative genome analysis revealed the rapid evolution of the *TPS* gene family. Most of the *OsTPS*, *PtTPS*, and *AtTPS* genes evolved after the divergence between monocot and dicot and after the divergence between the

herbaceous annual species and perennial woody species. The rapid and dynamic evolution of the *TPS* gene family allowed rapid evolution of new product profiles for plants to adapt to the ever-changing environments.

Ninth, despite the dynamic evolution of the *TPS* gene family, I identified a conserved group of *TPS* genes with deep evolutionary origin. Biochemical characterization of one *PtTPS* gene in the group indicated its activity as *PtLIS*. We expect that the conserved gene structure and function was preserved during the dynamic evolution of *TPS* gene family due to the selection pressure of herbivorous insects.

Overall, in this thesis, I used an integrated functional genomics approach to discover important *TPS* genes involved in plant indirect defense, and further studied the function, regulation, and evolution of these genes.

II. Perspectives

Despite the significant progress in this research, unanswered questions remain about the function and evolution of *TPS* genes in rice and poplar. Future research can be carried out from the following perspectives.

Biochemical function of TPS genes

The biochemical function of several *OsTPS* genes and one *PtTPS* gene has been identified. However, a majority of *TPS* genes in both species remain to be characterized. We do not know about either of their biological functions. The biochemical and biological functions are actually relevant to one another. *TPS* genes often fulfill their biological functions with their products. Moreover, understanding their biological functions will allow better design of gene discovery strategies for gene cloning and characterization. Further characterization of biochemical functions of *TPS* genes will help to understand their biological functions. Since many *OsTPS* and *PtTPS* genes cannot be cloned by RT-PCR due to the low or no expression, a genomic DNA transient expression strategy may help the biochemical identification of *TPS* genes (Wu et al., 2005).

The study of *TPS* biochemical function will not only help to understand their biological functions, but also help to study the evolution of *TPS* genes and their biochemical activities. The same *TPS* gene from different species, or even different cultivar from the same species, could account for different product

profiles (Kollner et al., 2004). TPS enzymes have high flexibility for their product specificities, and the first step to study such flexibility is to study the biochemical functions of ortholog or paralog genes (Iijima et al., 2004; Katoh et al., 2004; Tholl, 2006; Hyatt et al., 2007). In rice, the terpenoid volatiles were emitted in a cultivar-specific way, which indicated that the TPS biochemical function could be different in different cultivars (Lou et al., 2005; Lou et al., 2005; Lou et al., 2006; Cheng et al., 2007). As shown in Figure 6.1, the indica cultivar Zhe733 has much lower amount of terpenoid volatile produced as compared to Nipponbare when challenged by rice fall armyworm. In order to further understand the diversity of volatile terpenoid emission and the evolution of such diversity, more *TPS* genes from different cultivars of rice need to be cloned and characterized for their biochemical functions. Moreover, computational structure modeling of the same *TPS* genes from different cultivars with different product profile will shed light into the evolution of *TPS* gene functions and plant defense against herbivorous insects.

Biological function of TPS genes

The biological functions of *TPS* genes in plants needs to be further studied. We have confirmed the indirect defense functions of rice *TPS* genes. However, previous research from different disciplines indicated a variety of functions of terpenoid volatiles ranging from plant disease resistance to abiotic stress response (Dudareva et al., 2006). The importance of each aspect of *TPS* gene functions has been debatable, in other words, some considered plant indirect

defense role as the major function of terpenoid volatiles, whilst others considered terpenoid volatiles have many different biological functions (Dudareva et al., 2006).

In order to address these questions, I carried out volatile and gene expression profiling of terpenoid volatiles and *TPS* genes under different conditions. As shown in Figure 6.2, gene expression and volatile emission was analyzed under wounding, BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methylester), Ala (alamethicin), and insect treatment. BTH treatment was used to mimic the salicylic acid response, and Ala treatment was used to mimic the fungi response. From the volatile profiling, linalool seemed to be induced in an insect and jasmonic acid specific way. Since jasmonic acid is largely considered as the plant hormone involved in insect induced responses, the pattern indicates that linalool has more defined insect defense functions as compared to other terpenoid volatiles (Liechti and Farmer, 2006; Liechti et al., 2006). The *OsLIS* gene expression followed the same pattern as volatile emission. In fact, the volatile terpenoid profiling generally correlated well with the gene expression of the *TPS* genes producing these terpenoids, which indicated the importance of the transcriptional level regulation in volatile emission control. Moreover, the correlation could also be used to discover new *TPS* genes with unknown biochemical functions. Besides *OsLIS*, the products of *OsTPS42* and *OsTPS44* were also analyzed. The products of *OsTPS42* were emitted in insect and jasmonic acid treatments only, which correlated with the insect and jasmonic

acid specific gene expression pattern for *OsTPS42*. However, the major products of *OsTPS44* showed up under different treatments, which correlated with the gene expression pattern of *OsTPS44*. Considering the phytoalexin roles of many sesquiterpenes, *OsTPS44* may be involved in pathogen defense as well.

The same scenario is true for OsLMSes. Limonene has been found to be emitted under most of conditions; however, it was also found to be emitted at a higher level under insect, jasmonic acid and blast fungi *M. grisea* treatments. The gene expression of the two OsLMSes was also inducible by insect, JA and other treatments. In fact, MPSS data indicated that the two OsLMSes respond differently to *Xanthomonas oryzae* and *M. grisea* treatment, which indicates their distinct roles in bacteria and fungi defenses. Overall, we have characterized the function of terpenoid volatiles and *TPS* genes in rice indirect defense against herbivorous insects, however, the exact biological functions of *TPS* genes might not be limited to indirect defense. Further studies need to be carried out to define the exact biological functions of these volatile terpenoid compounds and *TPS* genes.

Genetic studies represent one of the most definitive approaches to study gene function. My gene discovery work enabled further study of the *TPS* gene function through mutant studies. Since insertional mutants for most of the *TPS* genes are not available, I therefore propose to further study these gene function through RNAi (Hirochika et al., 2004). The knockout mutants can be subject to

different bioassays including insect, pathogen and fungi treatments. If the mutants have different phenotypes as compared to control plants, the biological function of the gene can be further defined. For example, if fall armyworm treated *OsLIS* mutants result in less linalool production as well as less capacity in attracting the parasitoid *Cotesia*, the biological and ecological function of *OsLIS* gene in indirect defense can be further confirmed. Beside knock-out analysis, over-expression key *TPS* genes in indirect defense will also help to define their ecological functions (Schnee et al., 2006). Genetic study based on gene function study will be the reasonable next step after the biochemical characterization of gene functions (Schnee et al., 2006).

Evolution of TPS genes and terpenoid volatile based defense

Further understanding of the evolution of *TPS* genes will help to elucidate the mechanisms and evolution of terpenoid based indirect defense and multi-trophic interactions. Our research revealed the rapid evolution of the *TPS* gene family and the potential selection pressure for the gene family evolution. Further research is needed to understand the evolution of the *TPS* gene family and gene functions. As discussed before, *TPS* genes can be cloned from multiple cultivars and studied for their biochemical and gene regulations. Moreover, the *TPS* gene orthologs from multiple species can be studied for their enzyme activity evolution with computational modeling. Previous research in maize indicated enzyme activity evolution of *TPS* genes as an important aspect of the evolution of tritrophic interactions (Hoballah et al., 2002; Kollner et al., 2004). It should

be noted that the study of orthologs needs to be carried out in closely relevant species due to the rapid evolution of the TPS gene family. For example, maize *TPS10* is an ortholog of *OsTPS44*, and structure analysis of the two genes will reveal how different product specificities has evolved.

Besides the biochemical activity evolution, the changes in gene expression also need further study. As shown in Figure 6.1, we found no terpenoid volatile emission for indica cultivar Zhe733 under the insect treatments. Sequence analysis revealed that the TPS genes in indica should be functional. The differential expression regulation thus was speculated to be responsible for differentially induced volatile emission.

It would also be interesting to study whether any common or major terpenoid compounds are used in multiple ecosystems as info-chemicals. Previous study has indicated the importance of linalool as an indirect defense signal in wild tobacco (Kessler and Baldwin, 2001). I also found the *LIS* gene to be conserved among rice, *Arabidopsis* and poplar, which led to several questions. Is linalool a common compound in indirect plant defense? Is there any common compound for indirect defense at all? Which compound was more frequently used by plants in different ecosystems? Is there a correspondence between insect species and linalool like compounds? A comprehensive survey of volatile emission for different plants under different herbivorous insects may help to answer these

questions and allow us to better understand the molecular evolution of plant defense against herbivorous insects.

Overall, this research has led to further understanding of *TPS* gene function in plant defense, the regulatory mechanisms of plant defense against insects, and the evolution of the *TPS* gene family. However, the molecular evolution of terpenoid volatile based plant indirect defense needs to be further studied with respect to the evolution of biochemical function of TPS genes, the development of differential regulatory mechanisms of gene expression, and importance of specific compounds in a wide range of ecosystems.

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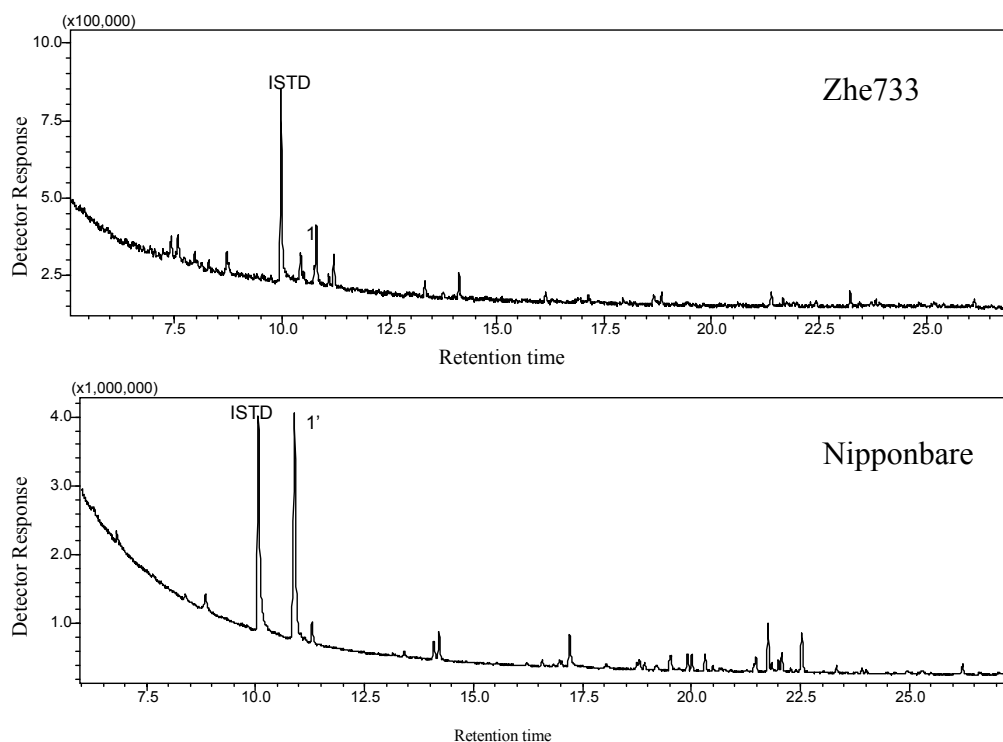


Figure 6.1. The volatile profiling of insect treated *indica* rice (Zhe733) as compared to that of *japonica* rice (Nipponbare). The *indica* volatile profile is quite different from that of *japonica*. Both 1 and 1' represented linalool peak in Zhe733 and Nipponbare, respectively. Significant differences for the amount of linalool and other terpenoid products can be found between the two cultivar as shown in the figure.

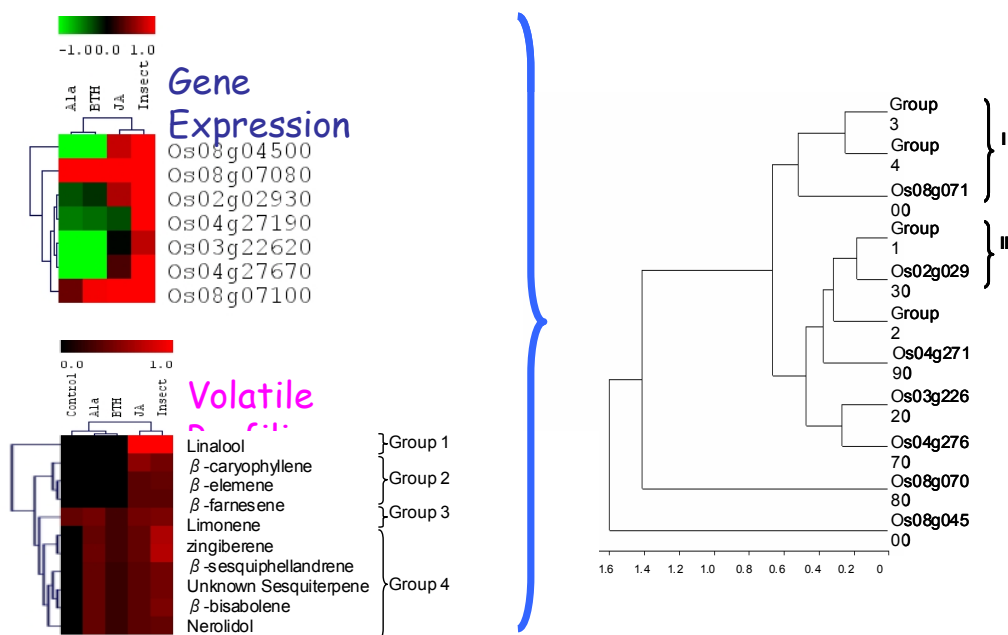


Figure 6.2. The correlation of gene expression and volatile profiling under multiple treatments. The gene expression panel presented the cluster of logarithm 2 transformed gene expression level as shown by the color schema on the top of the cluster. The volatile profiling panel presented the volatile emission level as shown by the scale on the top of the cluster. The quantification is based on relative amount of the octanol internal standard amount. The volatile profile and the gene expression data were standardized and clustered based on the distance among the genes and volatiles. The cluster on the right derived the gene to volatile relationship based on the cluster of standardized volatile profiling and gene profiling data.

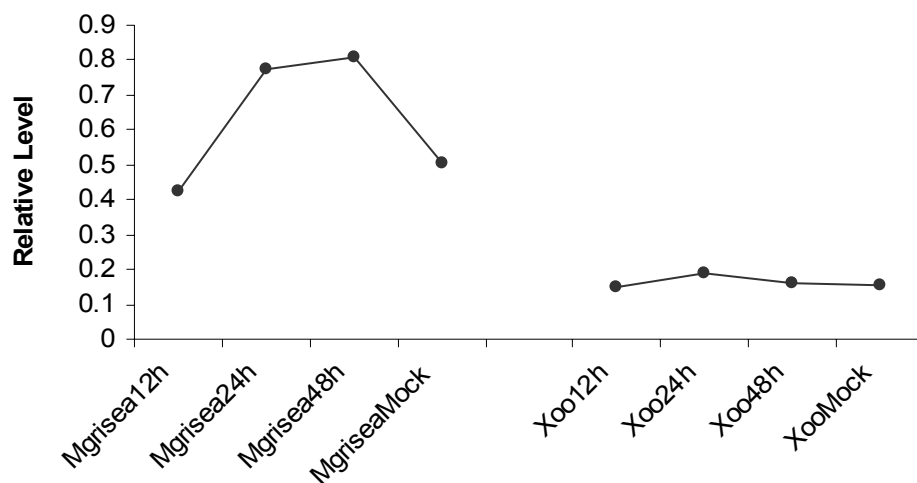


Figure 6.3. Level of limonene under *M. grisea* and *Xoo* treatment. The Y axis represented the relative peak area of limonene as compared to internal standard, and the X axis showed the different treatment indicating the number of hours after the *M. grisea* or *Xanthomonas oryzae* treatment.

Vita

Joshua S. Yuan was born in 1973 in Nanjing, Jiangsu Province, China. He finished his early education in Jilin City, Jilin Province, China. He obtained his bachelor degree in 1997 from Fudan University, Shanghai, China. He obtained his M.S. degree in 2001 at the University of Arizona, Tucson, AZ, with Dr. David W. Galbraith. He worked in BASF Plant Sciences LLC to establish the genomics platforms for the company, and Gallo Center at UCSF (University of California, San Francisco) as microarray core manager, subsequently. He married Susie Yuan Dai in June, 2002. Due to the immigration status, he had to give up an opportunity to pursue the Ph.D. study at the Scripps Research Institute in 2002, and came to Tennessee for a Genomics Scientist/Research Associate position in 2004. His job responsibility is to manage the UTIA Genomics Hub. He became a part-time Ph.D. student at UT in 2004, where he has also been involved in different kinds of research projects involving bioenergy research, plant indirect defense, low temperature germinability, and bioinformatics. He received his PhD in 2007.