Gene Expression and Physiological Analyses to Study Metabolic Shifts in Medicago Truncatula Subjected to Biotic Stresses

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GENE EXPRESSION AND PHYSIOLOGICAL ANALYSES
TO STUDY METABOLIC SHIFTS IN *MEDICAGO TRUNCATULA*
SUBJECTED TO BIOTIC STRESSES
GENE EXPRESSION AND PHYSIOLOGICAL ANALYSES
TO STUDY METABOLIC SHIFTS IN *MEDICAGO TRUNCATULA*
SUBJECTED TO BIOTIC STRESSES

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Plant Pathology

By

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Bachelor of Science in Biology, 2009

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Abstract

Plants are frequently under attack from pests and deploy various defense mechanisms to fight off predators. Many plant defenses are induced following herbivory or pathogen infection. An investment in defense could potentially lead to a diversion of metabolic resources away from primary metabolism, including reduction in plant commitment to photosynthesis. In this thesis, changes in expression levels of representative genes for primary and secondary metabolism were studied using real-time polymerase chain reaction (PCR) in *Medicago truncatula* Gaertner subjected to beet armyworm (*Spodoptera exigua* Hübnner) damage or *Phoma medicaginis* Malbr. & Roum. infection. Photosynthetic yield was also measured using chlorophyll fluorescence to determine if changes in expression level of genes encoding photosynthetic machinery corresponded with changes in yield. In insect-damaged plants, transcripts encoding β-amyrin synthase (βAS), farnesyl pyrophosphate I (FPS1) and a cytochrome P450 monooxygenase (CYP716A12) were induced to levels significantly higher than in non-damaged control plants. Each of these genes is predicted to encode a product directly involved in the synthesis of secondary metabolites, so the strong levels of induction are consistent with activation of plant defenses. However, transcripts encoding protein products directly involved in photosynthesis were significantly suppressed. These included genes for chlorophyll a/b binding protein (CAB), ribulose-1,5-bisphosphate carboxylase small subunit (RubP) and oxygen-evolving enhancing protein (OEE). This observation, along with previous reports in the literature, is consistent with a shift of plant resource commitment away from primary metabolism. Transcript accumulation of genes encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) and chalcone reductase (CHR) were also suppressed in insect-injured plants. In pathogen-inoculated plants, CHR transcripts were induced while those for βAS, FPS1, CYP716A12, DXR, CAB and RubP were suppressed, and the expression level of OEE was unchanged. The CHR enzyme regulates a branch point in the biosynthesis of flavonoids and isoflavoids, which include antimicrobial phytoalexins in the Fabaceae. Its induction following pathogen infection suggests an activation of pathogen-specific responses by the plant. Changes in expression level for all members of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) gene family in *M. truncatula* were also assessed. Differential transcript accumulation for gene family members encoding HMGR, which catalyzes the first committed step in the mevalonate pathway leading to sesqui- and tri-terpenes in plants, is well
established. Transcripts for all five *M. truncatula* HMGR isoforms were induced following insect herbivory. In fungal-inoculated plants, all transcripts were suppressed except HMGR2, which was induced by pathogen infection. The differential expression of the HMGR gene family members in *M. truncatula* demonstrated in this study is consistent with the varied expression profiles of this gene family observed in other plants. Photosynthetic yield was measured via leaf fluorescence as photosystem II operating efficiency. No significant difference in yield was observed among the treatments and all samples had yield values typical for unstressed plants. This was unexpected based on transcript data. Expression levels for photosynthetic genes were greatly suppressed in plants subjected to either treatment while defense genes were induced indicating a metabolic shift. Although photosynthesis measurements showed no change in yield, transcript data showed suppression of photosynthetic genes. This suggests that although photosynthetic-gene transcript levels are reduced, there must still be adequate levels to maintain photosynthesis in the time frame of these experiments. In this study, it was shown that there is not a direct link between molecular activity in the form of transcript accumulation and physiological activity following insect attack and pathogen infection in plants.
This thesis is approved for recommendation to the Graduate Council.

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Introduction

Plant defense

Plants are continually vulnerable to abiotic and biotic stresses that they confront on different levels. Plants are non-mobile, so they cannot escape their mobile attackers. In addition, many live for years whereas most pests are multivoltine, so plants must often endure multiple subsequent attacks. Finally, most plants have numerous species of potential pathogens and herbivorous pests, and so they must have broadly effective defenses (Huber and Bohlmann 2006). Plants are equipped with defenses that can be generalized as either constitutive or inducible. Constitutive defenses include physical and chemical barriers that are always present in the plant such as a waxy leaf surface, thick cuticles, trichomes, and toxins (Bernays and Chapman 1994). Inducible defense mechanisms can be activated upon attack by pests or treatment with chemical elicitors.

Inducible direct defense mechanisms affect the behavior or physiology of the attacking pest. For example, potatoes (*Solanum tuberosum*) produce proteinase inhibitors (PIs) following insect herbivory (Green and Ryan 1972) that have been shown to inhibit animal proteinases as well as bacterial and fungal proteinases (Ryan 1966). Dunse et al. (2010) showed that chymotrypsin activity in gut extracts of the Lepidopteran *Helicoverpa punctigera* was abolished after application of potato type I inhibitors. Furthermore, *H. armigera* larvae that fed upon cotton leaves supplemented with the potato PI *StPin1A*, tobacco (*Nicotiana alata*) PI *NaPI*, or both, weighed 40 to 90% less than the control group. Transgenic cotton plants expressing both *StPin1A* and *NaPI* in a field where *H. punctigera* and *H. armigera* were present exceeded the non-transgenic control plants in boll production and lint weight. Taken together, the data demonstrate the anti-herbivore defensive function of damage-induced PIs.

Another example of a direct inducible defense is observed as altered nutritional values of plant materials. Felton et al. (1989) reported that foliar polyphenol oxidase (PPO) activity in tomato (*Solanum lycopersicum*) increased following fruitworm (*Heliothis zea*) feeding. Herbivore feeding caused tomato PPO to come in contact with its substrate, o-diphenolic compounds such as chlorogenic acid, which are then oxidized and thereby converted to o-quinones. This reaction caused a change in the nutritional
quality of the dietary proteins and resulted in decreased growth rate of the insects. In a subsequent study, beet armyworm (*Spodoptera exigua*) larvae that fed upon dietary proteins treated with chlorogenic acid and PPO also had a severe reduction in growth rate (Felton et al. 1992). Direct inducible defense mechanisms can be very effective against attacking pests, but plants don’t rely on these defenses alone as they use a plethora of mechanisms for security against pests.

Along with direct defense mechanisms, indirect inducible defenses can also be deployed. Indirect defenses do not directly affect the behavior or physiology of the attacking pest; rather, they attract predators or parasitoids of the herbivore. Volatile organic compounds (VOCs) released by plants are an excellent example of indirect defense. For example, corn seedlings (*Zea mays*) subjected to caterpillar feeding released VOCs that attracted the parasitoid wasp *Cotesia marginiventris* (Turlings et al. 1990). The release of these VOCs is systemic, occurring from both injured and uninjured leaves (Turlings and Tumlinson 1992), demonstrating that whole plant signaling occurs following injury. Herbivore ovipositioning can also activate the release of VOCs. Volatiles emitted by field elm trees (*Ulmus minor*) following ovipositioning of the elm beetle (*Xanthogaleruca luteola*) attracted the egg parasitoid *Oomyzus gallerucae* (Meiners and Hilker 2000). The release of VOCs can reduce the herbivore load of a plant by more than 90% (Kessler and Baldwin 2001). Even though plants cannot move away from predators, they can call for help using VOCs in order to reduce their herbivore load.

Extrafloral nectaries (EFN) are produced outside the flower and can serve to attract predaceous insects to protect the plants against herbivores. For example, *Catalpa speciosa* leaves containing EFNs that are damaged by the moth *Ceratomia catalpae* secrete more nectar than do EFNs on undamaged leaves. These nectaries attract predators of herbivores that attack and/or remove *C. catalpae* eggs and larvae. Subsequently, branches with predators have less herbivory and produce more mature fruits than branches without these predators (Stephenson 1982). Clearly plants have several mechanisms with which to protect themselves against herbivores. However, plants are attacked by other pests and use a suite of defenses against these pests as well.

Plants must cope with pathogens. This is mainly done by resistance which relies on the ability of the plant to recognize the pathogen and initiate defense mechanisms that limit infection. These defense
mechanisms include but are not limited to the hypersensitive response (HR), production of phytoalexins, and production of pathogenesis-related (PR) proteins (Dixon et al. 1994).

The hallmark resistance response in gene-for-gene interactions is the HR. This is characterized by rapid and localized cell death with the likely purpose to limit proliferation and the nutrient supply of pathogens (Dixon et al. 1994). Activation of the HR triggers a whole-plant response known as systemic acquired resistance (SAR) that increases the plant’s broad-spectrum resistance against pathogens (Van Loon 1997). Ross (1961) described acquired resistance using tobacco (Nicotiana tabacum var. Samsun NN). Tobacco plants inoculated with tobacco mosaic virus (TMV) showed resistance in both inoculated and non-inoculated parts when subsequently inoculated with TMV or potato virus X (PVX). Further studies continued to show the induction of SAR and the broad-spectrum resistance that plants acquire. For example, tobacco plants inoculated with TMV showed resistance to the bacteria Pseudomonas tabaci (Lovrekovich et al. 1968). Strobel et al. (1996) showed that cucumber plants inoculated with a HR elicitor protein from Pseudomonas syringae induced SAR against fungal, bacterial and viral pathogens in the plants. Inducing SAR is a way for plants to prime themselves and possibly be able to defend against future attacks more rapidly. Many compounds are associated with SAR such as PR proteins and phytoalexins.

Pathogenesis-related proteins are a large family of plant defense proteins that accumulate following pathogen attack (Van Loon 1997). They were first discovered in the tobacco variety Samsun NN inoculated with TMV. Following inoculation, four proteins accumulated in these plants that were not present in control plants (Van Loon and Van Kammen 1970). Since then, PR proteins have been studied and characterized in various plant systems. They are grouped into families based on molecular weight, amino acid composition, and serological properties (Van Loon 1999). The PR proteins exhibit a wide range of activities, including 1,3-β-glucanase activity (Kauffmann et al. 1987; Kombrink et al. 1988), chitinase activity (Legrand et al. 1987; Kombrink et al. 1988), and proteinase inhibitor activity (Rickauer et al. 1989). Further studies have shown that PR proteins can be antifungal (Mauch et al. 1988; Flores et al. 2002; Niderman et al. 1995), antibacterial (Flores et al. 2002), or antiviral (Park et al. 2004). Ward et al. (1991) showed that in TMV-inoculated tobacco a long list of PR protein genes are coordinately induced
with the onset of SAR. These genes were termed SAR genes by the authors. Although PR proteins are important for pathogen defense, plants produce other defense compounds as well such as phytoalexins.

Phytoalexins are a diverse group of small molecular-weight compounds with antimicrobial activity that are induced after pathogen infection (Hammerschmidt 1999). Müller and Borger (1940) were the first to present evidence of phytoalexin activity in plants. They observed pathogen resistance to several fungal species in potato due to the accumulation of a pathogen-nonspecific substance, which was termed phytoalexin. Since then, many studies have shown that phytoalexins accumulate in plants following a compatible plant-pathogen interaction and that those compounds have broad-spectrum antimicrobial properties (Kuć 1955; Kuć 1957; Müller 1958; Ayers et al. 1976a; Ayers et al. 1976b; Cline et al. 1978; Weinstein et al. 1981; Wotton and Strange 1985; Stolle et al. 1988). Furthermore, enzymes involved in the biosynthesis of phytoalexins are induced by pathogen elicitors (Cramer et al. 1985; Lawton and Lamb 1987).

Experiments with mutant and transgenic plants have confirmed phytoalexin function during pathogen infection. Phytoalexin-deficient (pad) mutants of Arabidopsis thaliana have been isolated that lack camalexin accumulation. Although none of them show a change in resistance against an avirulent strain of Pseudomonas syringae, the mutants pad1, pad2, and pad4 were susceptible to a virulent strain, and would suggest that camalexin may restrict virulent pathogens in A. thaliana (Glazebrook and Ausubel 1994; Glazebrook et al. 1997). Thomma et al. (1999) showed that pad3-1 mutants were more susceptible to the pathogen Alternaria brassicicola than wild type plants, and Roetschi et al. (2001) showed that pad2 mutants were more susceptible to the pathogen Phytophthora porri. Experiments using transgenic plants have also provided evidence that phytoalexins are used in defense. The gene encoding stilbene synthase, an enzyme that produces the phytoalexin resveratrol, has been transferred from grape (Vitis vinifera) to several plant species and studied. Hain et al. (1993) were one of the first to do this when they transferred the gene to tobacco. The transgenic tobacco plants were more resistant to the pathogen Botrytis cinerea. Other transgenic plants producing grape stilbene synthase have also shown greater resistance to pathogens such as tomato to P. infestans (Thomzik et al. 1997) and rice (Oryza sativa) to
*Pyricularia oryzae* (Stark-Lorenzen et al. 1997). Obviously, phytoalexins help plants to defend against a broad range of pathogens.

Whereas VOCs are generally considered a defense mechanism against insects, they also have an impact in plant–pathogen interactions. Cardoza et al. (2002) showed that peanut (*Arachis hypogaea*) plants release VOCs following white mold (*Sclerotium rolfsii*) infection and that these chemicals limit fungal growth. Piesik et al. (2011) showed that wheat (*Triticum aestivum*), oat (*Avena sativa*), and barley (*Hordeum vulgare*) all release VOCs following inoculation with *Fusarium* spp. It is clear that plants have an arsenal of mechanisms they use against pests but one mechanism alone may not be enough to mount an effective defense, so a coordinated strategy must be used.

**Defense signaling molecules**

It is clear that signaling molecules are important for plants to mount a quick, effective response. Endogenous plant signaling molecules such as jasmonic acid (JA) and salicylic acid (SA) have important roles for pest resistance as well as the regulation of induced defense responses. In general, necrotrophic pathogens and herbivorous insects are sensitive to JA-dependent responses, whereas biotrophic pathogens are more sensitive to SA-dependent responses (Glazebrook 2005).

Jasmonic acid is a cyclopentanone-based compound derived from the fatty acid linolenic acid (Vick and Zimmerman 1983, 1984). Jasmonic acid and its methyl ester, methyl jasmonate (MeJA), have roles in plant development such as positively affecting pollen development (McConn and Browse 1996), inducing flowering (Zeng et al. 1999), promoting senescence (He et al. 2002), inhibiting root growth, and inducing vegetative storage protein production (Staswick et al. 1992). They are also involved in plant defense against herbivores and pathogens (Dixon et al. 1994).

Jasmonates have been shown to accumulate in wounded plants (Creelman et al. 1992) and in plants treated with pathogen and herbivore elicitors (Doares et al. 1995a). Studies have also shown that jasmonates induce gene expression of defense genes. For example, treatment with JA and MeJA induces the wound-responsive gene chalcone synthase (Creelman et al. 1992) and MeJA induces genes
encoding some PR-proteins (Xu et al. 1994). Another important defense feature of JA-mediated responses is the ability to prime other plants. Farmer and Ryan (1990) discovered that jasmonates volatized from sagebrush triggered defense gene expression in neighboring tomato plants. Experiments involving mutants provide further evidence of the importance of jasmonates in defense. *Arabidopsis thaliana* mutants deficient in linolenic acid contain small amounts of JA. These mutants are unable to accumulate JA and are rendered susceptible to fungal gnats because they cannot induce JA-responsive genes. Treatment of these plants with exogenous JA reestablishes resistance to the gnats (McConn et al. 1997). The tomato mutant, JL5, is restricted in the synthesis of JA by the inhibition of a step in the biosynthetic pathway. This restriction causes the plants to be more susceptible to damage by *Manduca sexta* moths (Howe et al. 1996). Jasmonates are well established as having an essential role in plant defense.

As with JA, SA has roles in plant development and defense. For example, it induces flowering (Cleland and Ajami 1974), triggers thermogenesis (Raskin et al. 1989, 1990), and displays allelopathic properties (Shettel and Balke 1983). It is also a modulator of SAR and induces PR protein production resulting in resistance (Ward et al. 1991). Salicylic acid can be derived from phenylalanine via the shikimate-phenylpropanoid pathway (Yalpani et al. 1993; Cuquoz et al. 1998). However, data from SA biosynthesis studies done in potato showed that SA was still produced when this pathway was inhibited suggesting an alternate pathway can be used (Cuquoz et al. 1998). Wildermuth et al. (2001) found that SAR is dependent upon SA synthesized from chorismate by isochorismate synthase. In 1979 White observed that exogenous SA induces resistance in tobacco to TMV. Subsequent studies showed that SA accumulates in plants following inoculation with a pathogen suggesting a role in resistance (Malamy et al. 1990; Métraux et al. 1990; Rasmussen et al. 1991). Parsley (*Petroselinum crispum*) cells treated with SA or the analogues INA (2,6-dichloroisonicotinic acid) and BTH (benzo[1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester) displayed increased sensitivity to fungal elicitors and enhanced transcription of SAR genes (Kauss et al. 1992; Katz et al. 1998). This priming was also observed in whole plant systems. *Arabidopsis thaliana* plants treated with BTH had increased transcription levels of genes encoding the SA biosynthetic enzyme phenylalanine ammonia-lyase when subsequently inoculated with *Pseudomonas*
Transgenic and mutant plants that are either deficient in or have increased SA accumulation give further proof of the importance of SA in defense.

Transgenic tobacco and *A. thaliana* plants have been used in studies to show the significance of SA in plant defense. Transgenic tobacco plants with enhanced SA biosynthesis have significantly higher SA accumulation. These plants also exhibit constitutive expression of PR proteins and display pathogen resistance (Verberne et al. 2000). On the other hand, transgenic tobacco and *A. thaliana* plants expressing the *nahG* gene that encodes the enzyme salicylate hydroxylase are unable to accumulate SA, which renders them susceptible to viral, fungal, and bacterial pathogens (Gaffney et al. 1993; Delaney et al. 1994). *Arabidopsis thaliana* lesions-simulating-disease (*lsd*) mutants constitutively express SAR and have increased SA concentration. When *lsd* mutants are crossed with *nahG* plants, SAR gene expression and disease resistance are suppressed. However, application of SA or one of its analogues restored SAR in the mutants (Weymann et al. 1995). It is clear that SA is crucial in plant defense against pathogens.

Evidence over the years has shown that there is cross-talk between SA- and JA-mediated defense pathways. In general, these two pathways are antagonistic of each other (Schenk et al. 2000; Glazebrook et al. 2003). However, there is some evidence that positive co-regulation may occur in some cases. Gene expression studies with microarrays showed that 168 genes were induced in *A. thaliana* by a fungal pathogen. Several of these genes were also induced by SA and JA with some overlap (Schenk et al. 2000). Gene expression analyses with microarrays have also been done in tobacco with similar results (Heidel and Baldwin 2004). This cross-talk could be a way for plants to have flexibility in their defense response and better regulate that response.

The antagonistic activity between JA and SA pathways has been reported in many studies over the years. Doherty et al. (1988) demonstrated JA-mediated defense pathway inhibition by aspirin in tomato. Further evidence was given by Peña-Cortés et al. (1993) by showing that JA accumulation was inhibited and wound-induced gene expression was suppressed by aspirin in tomato. Tomato plants treated with both SA and JA had significant decreases in JA pathway indicators. For example, proteinase inhibitor accumulation is lower in SA/JA treated plants compared to plants treated with JA alone (Doares et al. 1995b). Polyphenol oxidase activity also decreases in plants treated with both SA and JA (Thaler et
al. 1999). Preston et al. (1999) demonstrated that tobacco plants exhibiting SAR are inhibited in JA-mediated responses which caused them to be more susceptible to *M. sexta* larvae. Furthermore PR protein genes induced by JA are inhibited by SA in tobacco (Niki et al. 1998). Whereas it is clear that JA-mediated pathways are affected by concentration of SA, SA-mediated pathways are affected by JA.

In tobacco, MeJA treatment inhibited SA-induced PR proteins (Niki et al. 1998). Thaler et al. (1999) found that mRNA levels of PR-4 were decreased in JA-treated tomato plants but remained unchanged in plants treated with both SA and JA. In this case, SA-mediated gene expression was abolished in the presence of JA. However, there are some instances in which JA induces SA-mediated responses. For example, SA-induced PR protein genes had increased induction levels in tobacco seedlings treated with both SA and JA (Xu et al. 1994). This synergistic effect was also observed in rice treated with INA and JA (Schweizer et al. 1997).

Clearly, a wealth of evidence shows that JA and SA are critical mediators of plant interactions with pests. In addition, defense responses in plants controlled by these compounds are subject to complex management. The controlled regulation of the JA and SA signaling pathways is critical for plants to focus defenses on the pest at hand, and to direct metabolic resources toward immediate needs.

**Effects of defense on photosynthesis**

Plant response to attack is a complex array of molecular and physiological events. Because of the broad and substantial metabolic impact of induced defenses, their activation can potentially divert resources away from primary metabolism. An important consequence of any such metabolic shift could be a change in photosynthetic capacity. Most studies investigate either molecular changes in plants or physiological changes following their response to attacking pests.

Logemann et al. (1995) used parsley cells to demonstrate a metabolic shift following treatment with fungal elicitors. In this case, primary metabolism genes were down regulated, while defense genes were up regulated. They suggested that this was due to a redirection of resources. Ralph et al. (2006) also demonstrated this metabolic shift in Sitka spruce (*Picea sitchensis*). They showed a suppression of
primary metabolism genes and induction of defense genes following insect herbivory. Neither experiment measured photosynthetic rates to determine if gene expression changes reflected the whole-plant response. In fact, there are few studies of plant responses to pests where measurements of photosynthetic activities have been combined with gene expression data.

An abundance of reports in the literature indicate a negative effect of insect herbivory and pathogen infection on photosynthesis (Roloff et al. 2004; Bonfig et al. 2006; reviewed by Nabity et al. 2009). However, there are several that report results to the contrary. For example, Peterson et al. (2004) reported no change in photosynthetic rates of several legume species following fall armyworm (*Spodoptera frugiperda*) damage. Furthermore some cultivars of alfalfa (*Medicago sativa*) showed no significant change in photosynthetic activity following potato leafhopper (*Empoasca tabae*) feeding (Lamp et al. 2007). Soybean plants damaged by Japanese beetle (*Popillia japonica*), corn earworm (*Helicoverpa zea*) (Aldea et al. 2005), and two-spotted spider mites (*Tetranychus urticae*) (Bueno et al. 2009) demonstrated no change in photosynthetic yield. These types of results have also been observed for non-legumes. Serpentine leafminer (*Liriomyza trifolii*) injury had no effect on potato photosynthesis rates (Bueno et al. 2007). In these examples, photosynthetic yield was measured using gas exchange and calculated based on leaf area. Aldea et al. (2006) reported no change in photosynthetic yield as measured by chlorophyll fluorescence in understory sapling trees damaged by chewing insects or trees infected by a fungal pathogen. However, like the molecular experiments mentioned previously, the physiological experiments only studied one aspect of this response. In order to better understand how these responses affect the plant, a combination of molecular and physiological studies must be done.

Biosynthetic pathways of defense molecules

Of the classes of compounds involved in chemical defense, terpenoids are the most numerous class. They are a class of isoprenoids present in all living organisms and have many functions in growth, development, reproduction, and defense. Plant terpenoids can function as primary metabolites, for example, photosynthetic pigments such as carotenoids, electron carriers such as ubiquinone, membrane
structural components such as sterols, and growth regulators such as gibberellins. Terpenoids can also be secondary metabolites involved in defense in the form of toxins, VOCs and phytoalexins (reviewed by Gershenzon and Kreis 1999). For example, in sunflower (*Helianthus annuus*), sesquiterpene lactones and diterpenes in glandular hairs act as toxins and antifeedants against several insect pests (Gershenzon et al. 1985). The biosynthesis of terpenes is widely studied in yeast, fungi, plants, and mammals. In plants, terpenes can be produced from isopentenyl diphosphate (IPP) derived via the cytosolic mevalonic acid (MVA) pathway or the plastidic 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway (Fig. 1). The early enzymatic steps of both the MVA and DOXP pathways are well defined (Chappell 1995; Gershenzon and Kreis 1999; Hunter 2007; Lichtenthaler 1999; Rohdich et al. 2001). Both pathways result in the formation of the five-carbon compound IPP and its isomer dimethylallyl diphosphate (DMAPP). These compounds are used as building blocks to produce monoterpenes (C\textsubscript{10}), sesquiterpenes (C\textsubscript{15}), diterpenes (C\textsubscript{20}), triterpenes (C\textsubscript{30}), or tetraterpenes (C\textsubscript{40}). The enzyme 1-deoxyxylulose-5-phosphate reductoisomerase (DXR) catalyzes the rate-limiting step of the DOXP pathway (Takahashi et al. 1998). The enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the rate-limiting step in the MVA pathway (Chappell et al. 1995).
An important group of defensive plant terpenes is the saponins. They are molecules with surfactant properties and are based on a triterpenoid structure. All saponins have a sugar chain attached to the aglycone at the C-3 hydroxyl position. The number and identity of sugar moieties vary widely among the different individual saponins (Hostettmann and Marston 1995). These compounds display anti-insect (Herlt et al. 2002; Nozzolillo et al. 1997), antimicrobial (Avato et al. 2006; Oleszek et al. 1990; Soetan et al. 2006), and molluscicidal activity (Ekabo et al. 1996). They can also be toxic to monogastric animals (Oleszek et al. 1999) and negatively impact ruminant digestion (Lu and Jorgensen 1987).

In general, little is known about the enzymes and biochemical pathways involved in saponin biosynthesis. It is known that the first step is the cyclization of 2,3-oxidosqualene. The cyclization of this compound in plants can result in cycloartenol, the first committed product in sterol biosynthesis, or β-
amyrin, the first product in saponin biosynthesis. This step is mediated by cycloartenol synthase (CAS) or β-amyrin synthase (βAS), respectively (Fig. 2). The triterpenoid backbone then undergoes modifications such as oxidation, substitution, and glycosylation that are mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases, and other enzymes (Hostettmann and Marston 1995). An up-regulation of βAS would indicate a shift to secondary metabolite production, specifically saponin biosynthesis, in plants. Previous work has shown that βAS transcript levels in *M. truncatula* are induced by meJA and SA and that this correlates with enhanced saponin concentrations (Suzuki et al. 2002).

Figure 2. Schematic of the saponin and sterol biosynthetic pathway.

Flavonoids are a diverse group of secondary metabolites involved in many functions such as ultra-violet light protection (Li et al. 1993), aluminum toxicity resistance (Kidd et al. 2001), pollen fertility (van der Meer et al. 1992), regulation of auxin transport (Jacobs and Rubery 1988), and antimicrobial activity (Wang et al. 1989). Antimicrobial activity occurs through the deployment of phytoalexins. Bonde et
al. (1973) found that vestitol and sativan were induced and accumulated in leaves of *Lotus corniculatus* in response to inoculation with the fungus *Exserohilum turcicum*. Many phytoalexins of the Fabaceae are isoflavonoids synthesized through a branch of the phenylpropanoid pathway (Fig. 3) (Reviewed by Aoki et al. 2000).

Figure 3. Schematic of isoflavonoid biosynthetic pathway. Abbreviations: CHS: chalcone synthase; CHR: chalcone reductase; CHI: chalcone isomerase; IFS: isoflavone synthase; IFR: isoflavone reductase.

Although HMGR is the most studied enzyme of the MVA pathway in plants, a considerable amount remains to be learned about its regulation. As stated earlier, HMGR catalyzes the rate-limiting step in the MVA pathway. This enzyme is highly regulated and is subject to control at many levels, from transcriptional to post-translational (Nieto et al. 2009). The number of genes encoding HMGR in plants varies among species. It is encoded by at least two distinct genes in *A. thaliana*, three in *Hevea*.
*brasiliensis*, and three in tomato (*Solanum lycopersicum*). Differential expression of HMGR gene families in plants could play an important role in regulation of HMGR activity (Stermer et al. 1994). For example, transcript levels of members of the potato HMG gene family can be either up- or down-regulated in tubers by wounding, bacterial and fungal pathogens, arachidonic acid, or methyl jasmonate (Korth et al. 1997). Following elicitor treatment, mRNA levels of hmg1 in potato were strongly suppressed but transcript levels for hmg2 and hmg3 were enhanced (Choi et al. 1992). In *H. brasiliensis*, hmg1 was induced by ethylene while hmg3 was constitutively expressed (Chye et al. 1992). The presence of these gene families is consistent with the hypothesis that different isoforms of HMGR are involved in separate subcellular pathways for terpenoid biosynthesis. Therefore, induction of defensive terpene metabolites is possibly subject to complex, fine-tuned regulation of specific gene-family members encoding biosynthetic enzymes in response to different types of biotic stress.

**Measuring photosynthetic yield**

Chlorophyll fluorescence imaging provides a non-invasive and non-destructive means to study photosynthetic performance and stress in plants (Baker 2008; Oxborough 2004). When light energy is absorbed by chlorophyll molecules, it has three possible fates: photochemical quenching, heat dissipation or chlorophyll fluorescence. These three processes are in competition with each other, therefore an increase in the efficiency of one will result in a decrease in the other two (Maxwell and Johnson 2000).

When measuring fluorescence, it is important to differentiate it from the absorbed light. This is easily done due to fluorescence having a longer wavelength than excitation light (Chaerle et al. 2009), so fluorescence yield can be measured by subjecting a leaf to light of a known wavelength and measuring the amount of light re-emitted at longer wavelengths (Maxwell and Johnson 2000).

When a plant is dark-adapted, the electron carrier $Q_A$ in photosystem II (PSII) is maximally oxidized (open state) and minimal fluorescence ($F_o$) can be measured. If these plants are exposed to a saturating light pulse, $Q_A$ will be maximally reduced (closed state) and maximal fluorescence ($F_M$) can be measured and used to indicate changes in photochemical quenching (Baker 2008). Reducing $Q_A$
suppresses photochemical quenching which leaves only non-photochemical quenching (chlorophyll fluorescence and heat dissipation). Heat dissipation occurs relatively slowly, so if a short burst of saturating light (≈1 s) is used, then any changes in chlorophyll fluorescence are due to changes in photochemical quenching and not heat dissipation. Changes in fluorescence can also be due to variations in photosystem I (PSI). However, at room temperature, changes in fluorescence essentially come from PSII alone (Schreiber et al. 1995).

The PSII maximum efficiency (photosynthetic yield) is calculated from chlorophyll fluorescence measurements. The PSII maximum efficiency is the efficiency that light absorbed by PSII can be used to drive photochemistry when all PSII centers are in the open state. It is calculated as $F_V/F_M$ where $F_V$ is the variable fluorescence in dark-adapted plants and is calculated as $F_M - F_O$. The PSII maximum efficiency for non-stressed plants has been calculated to be approximately 0.83. Chlorophyll fluorescence can also be measured in light-adapted plants where light-adapted fluorescence signal ($F'$) and maximal fluorescence ($F'_M$) are measured. A prime (') notation indicates that the measurement was taken from a plant that has been exposed to photosynthetically-driving light. With these measurements, PSII operating efficiency can be calculated. The PSII operating efficiency also calculates the efficiency at which light absorbed by PSII is used to drive photochemistry when all PSII centers are open. It is calculated as $F_{q'/F'_M}$ where $F_{q'}$ is calculated as $F'_M - F'$ (Baker 2008; Oxborough 2004). Whether dark-adapted or light-adapted plants are used, measurement of chlorophyll fluorescence is a technique widely used in plant physiology studies as a way to measure photosynthetic yield in a non-invasive manner.

**Medicago truncatula – A model plant**

The legume family Fabaceae includes important agricultural and commercial crops because of their use in human and animal diets and their contribution to global nitrogen fixation. This family includes species such as soybean (*Glycine max*), which provides a major source of protein and oil for both humans and animals, and alfalfa (*Medicago sativa*) that serves as an important forage and soil conditioning crop (Graham and Vance 2003). *Medicago truncatula* (barrel medic) is a close relative of
alfalfa. There are several hundred reported ecotypes of *M. truncatula* which include commercial varieties such as Jemalong, Cyprus and Ghor. Some of these cultivars are grown in rotation with cereal crops in certain regions of Australia (Barker et al. 1990). It is chosen as a model legume because of its prolific nature, its small diploid genome (~5x10^8 bp), self-fertilization, ease of genetic transformation, rapid generation time and availability of genome sequence data (Tivoli et al. 2006).

Chewing insects and a fungal pathogen were the biotic stresses used this research. The insect species was *Spodoptera exigua* (beet armyworm) and the fungus was *Phoma medicaginis*. *Spodoptera exigua* is a lepidopteran species that has a broad host range which includes plants from the family Fabaceae. Larvae of *S. exigua* are highly mobile moving within and between plants (Vickerman and Trumble 1999) and cause extensive defoliation (Kolodny-Hirsch et al. 1997). *Phoma medicaginis* is a necrotrophic fungal pathogen that infects both *M. sativa* and *M. truncatula*. It is the causal agent of spring black stem and leaf spot (Ellwood et al. 2006).

Even though *M. truncatula* is not grown as a crop in the U.S., it can give insight into how legume species in general will respond to biotic stresses. Information gathered from this plant can be extrapolated to other closely related agriculturally important legumes such as alfalfa. If growers and pathologists have knowledge of how a plant will respond to a biotic stress, reaction time in crop management will be quicker and result in more efficient management.

**Objectives**

The overall goal of this study is to assess metabolic shifts in *M. truncatula* treated by chewing insects (*S. exigua*) or infected with a pathogen (*P. medicaginis*) and to determine whether changes in gene expression patterns reflect physiological outcomes. Connecting these alterations in physiology to changes in gene expression will increase our ability to estimate whole-plant responses to attack and provide estimates of the impact of attack on higher levels of biological organization such as yield loss (Nabity et al. 2009). Preliminary data (K. Korth, unpublished) has indicated that following lepidopteran feeding on leaves, *M. truncatula* responds with a substantial decrease in transcript levels for genes
encoding proteins involved in photosynthesis. This same observation has been made in other plant species. Some have suggested that this is indicative of a major metabolic shift away from primary metabolism toward production of defensive compounds via secondary metabolism. Previous measures of total photosynthesis (via CO$_2$ uptake) in response to both changing light and CO$_2$ levels did not indicate any change in photosynthetic yield following severe caterpillar damage (Gomez, unpublished data).

Objective 1:

Compare gene expression for candidate genes involved in both primary and secondary metabolism will be compared in untreated plants, $S. exigua$-damaged plants, and $P. medicaginis$-infected plants. Gene expression patterns will be assayed by real-time reverse-transcription PCR.

Objective 2:

Measure photosynthetic yield of plants exposed to the treatments in objective 1 will be measured using light-adapted chlorophyll fluorescence imaging.

Objective 3:

Determine the differential expression of HMGR genes in $M. truncatula$ in response to $S. exigua$ damage and $P. medicaginis$ infection and phylogenetic tree indicating gene relationships.

Materials and Methods

Pest and plant maintenance:

$Medicago truncatula$ cv. Jemalong (A17) seeds were scarified in concentrated sulfuric acid for five minutes. The seeds were then rinsed with distilled water and germinated between moist filter paper in the dark at room temperature for three days. Seedlings were transplanted into round pots (4.5 inches in diameter and 3.75 inches tall) in Sunshine LC1 mix potting medium (SunGro Horticulture Distribution,
Inc., Bellevue, WA) and placed in a growth chamber at 24°C with a 16:8 (light:dark) hour photoperiod at a photosynthetic photon flux of 175 µmol m$^{-2}$ s$^{-1}$. Fertilizer (Miracle-Gro® water soluble all-purpose plant food, Scotts Miracle-Gro Products, Inc., Marysville, OH) was administered weekly at a rate of one tablespoon per gallon of water. A biological larvicide (Gnatrol® WDG, Valent BioSciences Corporation, Libertyville, IL) was administered weekly at a rate of 1.7 grams per gallon of water. Six- to eight-week old plants were used in experiments.

Eggs of *S. exigua* were initially obtained from Bio-Serv (Frenchtown, NJ) and subsequent generations have been maintained in the lab on beet armyworm artificial diet which contains agar and wheat-germ based feed mixture (Bio-Serv, Frenchtown, NJ) at approximately 22°C. To prepare insects for feeding treatments, third-instar larvae were allowed to feed on six- to eight-week-old *M. truncatula* plants for at least 24 hours prior to initiation of experimental treatments.

A culture of *P. medicaginis* was obtained from Drs. Richard Dixon and Bettina Devours at the Samuel Roberts Noble Foundation (Ardmore, OK). The isolate was from alfalfa and was originally provided by the American Type Culture Collection (ATCC No. 16929, Manassas, VA). It was maintained by subculturing on V8 agar medium [prepared with 100 mL V8 juice, 8.45 g Bacto agar (Becton, Dickinson, and Company, Sparks, MD), 1 g CaCO$_3$ (Fisher Chemical, Fisher Scientific, Fair Lawn, NJ), and 400 mL deionized water]. Cultures were grown at room temperature in the dark. Spores were collected from the subculture and stored in the lab in a 30% glycerol-spore stock solution at -80°C. To prepare spore suspensions for plant inoculations, cultures were initiated by adding one drop of glycerol-spore stock suspension to a V8 medium agar plate with a sterile transfer pipette and incubating at room temperature in the dark for two weeks. Spores were collected from the plates by adding 3 mL deionized water per plate, rubbing a bent glass rod across the surface, and transferring the resulting spore suspension to a 15 mL culture tube. The spore suspension was diluted 1:5 with deionized water and quantified with a haemocytometer. For application to plants, the spore suspension was diluted with deionized water to a final concentration of $5 \times 10^5$ spores/mL in 0.01% Tween 20 solution.
**In silico analysis of genes:**

The DFCI *M. truncatula* Gene Index (MtGI) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago) was used to identify candidate genes for primary metabolism and secondary metabolism. A comparison of expressed sequence tags (ESTs) found in libraries from developing leaves and from insect-damaged leaves was used to identify transcripts that are potentially differentially present in the two libraries. Tentative consensus sequences (TCs) predominantly found in the insect herbivory library would be predicted to encode proteins induced by insect feeding, whereas TCs predominantly present in the developing leaf library would be predicted to encode proteins that are suppressed by insect feeding, and perhaps related to the growth and development of the plant (primary metabolism).

**PCR primer design:**

The TCs for primary and secondary metabolism genes were analyzed using BLASTn against the NCBI database (http://blast.ncbi.nlm.nih.gov). Forward and reverse PCR primers were designed for each cDNA sequence using the oligoanalyzer on the Integrated DNA Technologies database (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/)(Table 1).
Table 1. Primer sequences with GenBank accession numbers and closest BLASTn hit for *Medicago truncatula*. Abbreviations: CAB: chlorophyll a/b binding; RuBP: ribulose-1,5-bisphosphate carboxylase; OEE: oxygen-evolving enhancer; βAS: beta-amyrin synthase; FPS1: farnesyl pyrophosphate synthase; CHR: chalcone reductase; CYP716A12: cytochrome P450 monoxygenase CYP716A12; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; MtTub: *Medicago truncatula* α-tubulin.

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>GenBank Accession No.</th>
<th>Sequence (5' to 3')</th>
<th>Closest BLASTn Hit</th>
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<td>CAB Forw</td>
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<td>CTTCAGTTATGGCTTGCTG</td>
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<td>CAB Rev</td>
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<td>RuBP Forw</td>
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<td><em>Medicago truncatula</em> chromosome 6 (Ribulose bisphosphate carboxylase small chain)</td>
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<td>OEE Forw</td>
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<td><em>Medicago truncatula</em> chromosome 5 (Oxygen-evolving enhancer protein)</td>
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<td>OEE Rev</td>
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<tr>
<td><strong>Secondary Metabolism</strong></td>
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<td><em>Medicago truncatula</em> mRNA for beta-amyrin synthase</td>
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<td>HMGR5 Rev</td>
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<td><strong>Housekeeping Gene</strong></td>
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<td>MtTub Rev</td>
<td></td>
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</tr>
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</table>
Fungal inoculations:

*Medicago truncatula* plants were inoculated with *P. medicaginis* with a hand-held compressed air sprayer and applied in a chemical fume hood until runoff (Fig. 4A). Control plants were mock inoculated with 0.01 % Tween 20 solution. Three biological replicates were sprayed for each treatment. Plants were placed in a dew chamber (Fig. 4B) for 32 hours with the following settings: 19°C wall, 30°C water and 40°C air. They were subsequently moved to a growth chamber set to 21°C and 100 % humidity. Five days after inoculation, plants were placed in the greenhouse in clear plastic cages covered with a piece of light fabric to allow air exchange (Fig. 4C). Six days after inoculation (DAI), leaf tissue was collected and chlorophyll fluorescence measurements were taken as described below.

Figure 4. A. Inoculation technique used for infection of *M. truncatula* with *P. medicaginis* (5 x 10⁵ spores/mL in 0.01 % Tween 20 solution). B. *M. truncatula* plants in dew chamber. C. Caging system of *M. truncatula* plants treated with insects.
Insect treatments:

Twenty third-instar *S. exigua* larvae per plant were placed on *M. truncatula* plants. The insect treated plants were placed in the greenhouse in clear plastic cages. Control plants without insect treatment were also placed in cages in the greenhouse. There were three biological replicates for insect-treated and control plants. Twenty-four hours after insect placement, leaf tissue was collected and chlorophyll fluorescence measurements were taken as described below.

Tissue collection:

Fifteen insect-damaged leaves (fig. 5A) were collected from each of the insect-treated plants. Fifteen leaves showing symptoms of *P. medicaginis* infection (fig. 5B) were collected from each of the plants inoculated with *P. medicaginis* but not treated with insects. Fifteen random leaves were collected from each of the non-inoculated and mock-inoculated plants that were not treated with insects. In each case, only fully expanded mature leaves were collected. The leaves were immediately chilled in liquid nitrogen and stored at -80°C until analysis.

Figure 5. A. *Spodoptera exigua* larva feeding on *M. truncatula*. B. *Phoma medicaginis* symptoms on *M. truncatula* line A17.
**Gene expression studies:**

RNA isolation was performed with TRI Reagent ® (Molecular Research Center, Inc.). Total RNA was quantified with a BioSpec nano spectrophotometer and cDNA was synthesized with iScript™ cDNA synthesis kit (BioRad). Concentration of cDNA was quantified with a BioSpec nano spectrophotometer and adjusted to 50 ng/µL with deionized water. Real-time reverse transcriptase (RT)-PCR reactions were carried out with Power SYBR ® Green PCR master mix (Applied Biosystems) with 20 µL reaction volumes. Final concentration for forward and reverse primers was 1 µM and final cDNA concentration was 100 ng per reaction. Applied Biosystems StepOnePlus™ real time PCR systems was used to run reactions with the following protocol: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s and 55°C for 1 min. Three technical reps were run for each biological rep.

Relative gene expression ratios for each gene were calculated using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) where $\Delta\Delta Ct$ is calculated as:

\[
\Delta\Delta Ct = \Delta Ct_{\text{Treated}} - \Delta Ct_{\text{Untreated}}
\]  

Equation 1

\[
\Delta Ct = Ct_{\text{Target}} - Ct_{\text{Reference}}
\]  

Equation 2

The mean Ct was calculated for each biological rep (e.g. control 1, control 2, control 3, insect-damaged 1, etc.). For control samples only, the mean of the mean Ct was calculated and this value was used in equation 2 which resulted in one $\Delta Ct$ value for each control. For treatment samples (e.g. insect-damaged and fungal-inoculated), the mean Ct was used in equation 2 which resulted in three $\Delta Ct$ values for each treatment. The $2^{\Delta\Delta Ct}$ value was then calculated. The mean $2^{\Delta\Delta Ct}$ was calculated for treatment samples and compared to the control. Statistical analysis was performed using a one-sample $t$ test ($\alpha = 0.05$) with GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

**Photosynthesis studies:**

Photosynthetic yield was calculated for each treatment with chlorophyll fluorescence measurements. Plants were dark-adapted for 20-30 minutes to achieve a steady state. The plants were
then light-adapted for five minutes (Aldea et al. 2006) at a photosynthetic photon flux of approximately 200 µmol m\(^{-2}\) s\(^{-1}\) and chlorophyll fluorescence was measured with the Hansatech Fluorescence Monitoring System FMS 2 (Fig. 6). The minimum fluorescence in the light-adapted state (F\(^{'}\)) was recorded. Maximum fluorescence (F\(_{M}'\)) was recorded following a 1 s saturating light pulse. Photosystem II operating efficiency was calculated as the quotient F\(_{q}'\)/F\(_{M}'\) where F\(_{q}'\) is calculated as F\(_{M}'\)-F\(^{'}\) (Baker 2008; Oxborough 2004). Three biological replications for each treatment were measured. Four measurements per plant were taken and the mean was calculated for each plant. This value was used to calculate the mean PSII operating efficiency for each treatment. Statistical analysis was performed using a one-sample t test (\(\alpha = 0.05\)) with GraphPad Prism version 5.04 for Windows.

Figure 6. Measuring chlorophyll fluorescence based on leaf area using a Hansatech Fluorescence Monitoring System FMS 2 in *M. truncatula*.

**HMGR characterization:**

To analyze genetic relationships of the *M. truncatula* HMGR gene family, the nucleotide sequences for five *M. truncatula* (EU302813, EU302814, EU302815, EU302816, and EU302817), two *A. thaliana* (NM106299 and NM127292), three *S. tuberosum* (U51986, U51985, and L01400), three *G. max*
(XP003517117, XP003534226, and XP003545556), Pisum sativum (pea, AF303583), and Glycyrrhiza uralensis (Chinese liquorice, JF461267) HMGR genes were analyzed for putative translation products via the SIXFRAME program (Rice et al. 2000). The resulting predicted protein sequences were analyzed with MEGA 5.0. Bootstrap analysis of 500 replicons was carried out with the maximum likelihood method (Tamura et al. 2011). Potato and A. thaliana HMGR gene families were used in this analysis because these gene families have been well characterized. Soybean, pea and Chinese liquorice were chosen as representative legume species. The nucleotide sequences of the M. truncatula HMGR isoforms were aligned with ClustalW (http://www.ngbw.org/) and used in gene-specific PCR primer design.

Results and Discussion

Biotic stress affects gene expression in Medicago truncatula

Candidate genes involved in primary metabolism and secondary metabolism were chosen based on the ratio of ESTs in the insect herbivory library compared to the developing leaf library (Table 2). When M. truncatula plants were subjected to S. exigua damage and P. medicaginis infection, transcript levels of genes encoding enzymes that catalyze reactions in several well characterized defense pathways were significantly induced, while genes encoding enzymes directly involved in production of photosynthesis machinery were significantly suppressed (Fig. 7). Gene expression experiments were repeated with similar results (data not shown).

In insect-damaged plants, beta-amyrin synthase (βAS) transcript levels were significantly induced (4.289 ±0.3412 fold difference). The enzyme encoded by this gene catalyzes the first committed step in anti-herbivore triterpene saponin biosynthesis. These results support previous reports in the literature. Suzuki et al. (2002) reported an induction of the gene encoding βAS in M truncatula cells following MeJA treatment. Furthermore Broeckling et al. (2005) reported an accumulation of the βAS protein in MeJA-treated M. truncatula cells. Considering that MeJA induces wound-responsive genes (Creelman et al. 1992), the induction of βAS by insect damage would indicate a shift to defense gene expression in plants. The gene encoding the enzyme farnesyl pyrophosphate synthase 1 (FPS1) was also significantly induced
(2.4 ±0.111 fold difference). This is also in agreement with what has been previously reported. For example, insect damage in rice (Tao et al. 2003) and the application of insect-induced VOCs to corn (Farag et al. 2005) resulted in increased gene expression levels of FPS. Farnesyl pyrophosphate synthase acts upstream of βAS, so the induction of both of these transcripts suggests a shift to secondary metabolism. The gene encoding the cytochrome P450 monooxygenase CYP716A12 was significantly induced (5.881 ±0.9424 fold difference) as well. This might also be indicative of defense pathways being induced because some cytochrome P450 enzymes are involved in the modification of βAS in the saponin biosynthetic pathway (Suzuki et al. 2002).

Transcript levels of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) were suppressed (0.3091 ±0.01705 fold difference) which is consistent with previous work. Gene expression levels of DXR were suppressed in *M. truncatula* following 24 hours of insect herbivory (Bede et al. 2006). Expression levels of chalcone reductase (CHR) were also significantly suppressed (0.2257 ±0.07742 fold difference). This enzyme catalyzes a branching step in the flavonoid and isoflavoid biosynthetic pathway which includes antimicrobial phytoalexins in legumes and may not be involved in herbivore defense. The gene expression results for insect-damaged *M. truncatula* also support the *in silico* analysis in which secondary metabolism ESTs were predominantly found in the insect herbivory library (Table 1).

Expression levels for the primary metabolism genes encoding chlorophyll a/b binding protein (CAB), ribulose-1,5-bisphosphate carboxylase small subunit (RubP), and oxygen-evolving enhancing protein (OEE) were significantly suppressed in insect-damaged plants (0.3474 ±0.07830 fold difference, 0.1875 ±0.03536 fold difference, and 0.2343 ±0.1367 fold difference, respectively). As been formally reported, herbivory suppresses primary metabolism genes in plants (Logemann et al. 1995; Ralph et al. 2006). In these reports, it was indicated that a shift from primary metabolism to secondary metabolism resulted from the herbivory. The data presented in Fig. 7 agrees with the previous findings. This data shows a shift from primary metabolism in the form of photosynthesis to insect-specific and pathogen-specific defense.

Because plants are also attacked by pathogens, changes in gene expression levels in *M. truncatula* following *P. medicaginis* infection were analyzed. During pathogenesis, CHR transcripts
significantly induced (9.129 ±2.921 fold difference) six DAI. The CHR enzyme catalyzes the branching step in antimicrobial phytoalexin biosynthesis in Fabaceae. Its induction following pathogen attack suggests an induction of a pathogen-specific defense pathway and is consistent with what has been reported in the literature regarding the induction of CHR in *M. sativa* after inoculation with various pathogens (Salluad et al. 1995). Transcript levels for βAS, FPS1, CYP716A12, DXR, CAB, and RubP were significantly suppressed (0.6196 ±0.002545 fold difference, 0.7845 ±0.07279 fold difference, 0.3645 ±0.2141 fold difference, 0.4644 ±0.05030 fold difference, 0.6055 ±0.02461 fold difference, and 0.5817 ±0.1339 fold difference, respectively) six DAI with *P. medicaginis* whereas expression levels for OEE were unchanged (1.218 ±0.1109 fold difference). The suppression of photosynthetic genes and induction of pathogen-specific defense genes would also indicate a shift from primary metabolism to secondary metabolism at the gene level.
Table 2. Classification of TCs in *M. truncatula* for primary metabolism and secondary metabolism genes. Abbreviations: TC: tentative consensus sequence; EST: expressed sequence tag; H: insect herbivory library; L: developing leaf library; CAB: chlorophyll a/b binding; RuBP: ribulose-1,5-bisphosphate carboxylase; OEE: oxygen-evolving enhancer; βAS: beta-amyrin synthase; FPS1: farnesyl pyrophosphate synthase; CHR: chalcone reductase; CYP716A12: cytochrome P450 monooxygenase CYP716A12; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase.

<table>
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</tbody>
</table>

\(^a\) Number of ESTs in the TC is derived from all libraries that have been analyzed.

\(^b\) The ratios were normalized based on the total number of ESTs in the insect herbivory library (9889) and developing leaf library (8336). The EST number from the insect herbivory library (H) was multiplied by 0.843.
Figure 7. Relative gene expression of *M. trucatula* A17 leaf tissue subjected to *S. exigua* damage (gray bars) and *P. medicaginis* infection (white bars) when compared to undamaged and mock-inoculated tissue, respectively. Asterisk above a column indicates a P value ≤ 0.05 (n = 3). Error bars indicate SD. Abbreviations: βAS: beta-amyrin synthase; CHR: chalcone reductase; CYP716A12: cytochrome P450 monooxygenase CYP716A12; FPS1: farnesyl pyrophosphate synthase; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; CAB: chlorophyll a/b binding; RuBP: ribulose-1,5-bisphosphate carboxylase; OEE: oxygen-evolving enhancer.

**Biotic stress does not affect photosynthetic yield**

Photosynthetic yield, when measured as PSII operating efficiency ($F_{q'}/F_{M'}$), did not significantly change among treatments when compared to the controls (Fig. 8A). The PSII operating efficiency was about 0.8, which is the normal value for non-stressed plants (undamaged: 0.7654 ±0.05957, *S. exigua*: 0.7708 ±0.05442, mock-inoculated: 0.7763 ±0.06635, and *P. medicaginis*: 0.8251 ±0.02161). To confirm measurements were taken correctly and the machine was working properly, a second experiment was performed. Chlorophyll fluorescence was measured in plants subjected to low light conditions (PAR 100
μmol m<sup>-2</sup>s<sup>-1</sup>). These plants were immediately transferred to intense light conditions (PAR 1870 μmol m<sup>-2</sup>s<sup>-1</sup>). Because an immediate transfer from low light to intense light is known to reduce photosynthetic yield within minutes (Powles 1984), chlorophyll fluorescence was measured 10 minutes after exposure to intense light. There was a significant difference between the mean PSII operating efficiencies of the two treatments when statistically analyzed using a one-sample t test (α = 0.05) with GraphPad Prism version 5.04 for Windows (Fig. 8B). Because these showed a difference, the results in Fig. 8A are valid. Chlorophyll fluorescence measurements were repeated in independent experiments with similar results (data not shown).

The results in Fig. 8A do not agree with some of the data reported in the literature. Zangerl et al. (2002) reported a suppression of photosynthesis activity measured by gas exchange and chlorophyll fluorescence in wild parsnip 24 hours after cabbage looper damage. It was suggested that resources were allocated to the synthesis of defense-related compounds upon herbivory damage causing a decrease in photosynthesis. This would give credence to the hypothesis that there is a metabolic cost for defense in plants by diverting resources away from primary metabolism to defense. However, plants need to balance resource allocation in order to successfully defend against pests and maintain ample levels of primary metabolism products. The data in Figs. 7 and 8 indicate that M. truncatula was able to minimize the metabolic cost of defense by maintaining normal levels of photosynthesis while defending against pests.
Figure 8. PSII operating efficiency of *M. truncatula* A17 plants. A. Plants subjected to *S. exigua* damage and *P. medicaginis* infection. Mean values compared to undamaged and mock-inoculated plants, respectively. B. Plants subjected to low light (100 µmol m$^{-2}$ s$^{-1}$) and intense light (1870 µmol m$^{-2}$ s$^{-1}$) conditions. Asterisk above a column indicates a P value ≤ 0.05 (n = 3). Error bars indicate SD.

The results for the gene expression and physiological analyses emphasize the need to pair these two types of analysis. The photosynthesis data did not agree with the transcript data. Expression levels of genes encoding photosynthetic proteins were significantly suppressed following insect damage and fungal infection but photosynthetic yield was unaffected by both. This would suggest that there is a delayed response between gene expression activity and physiological activity. Plants must have a way to maintain photosynthetic yield even though transcript levels of primary metabolism genes are reduced. These data also suggest that sufficient levels of photosynthetic gene transcripts must still be present in insect-damaged and fungal-inoculated plants in order for them to maintain adequate levels of photosynthesis in the time frame that these measurements were taken.

**HMGR is differentially expressed in *Medicago truncatula***

The HMGR gene family in *M. truncatula* was differentially expressed between *S. exigua*-damaged and *P. medicaginis*-inoculated tissue with the exception of HMGR2 (Fig. 9). All five members of
this gene family were induced following insect attack (HMGR1: 28.01 ±6.785 fold difference, HMGR2: 2.914 ±0.09974 fold difference, HMGR3: 7.055 ±0.6590 fold difference, HMGR4: 7.584 ±0.6512 fold difference, and HMGR5: 6.001 ±1.623 fold difference). The induction of HMGR genes in insect-treated *M. truncatula* is consistent with the MVA pathway producing insect defense-related products, such as terpene glycosides, i.e., saponins, and sesquiterpene VOCs such as caryophyllene (Arimura et al. 2008) and is consistent with the induction of other insect-defense related genes (Fig. 7). In pathogen-inoculated plants, expression levels for genes encoding HMGR1, HMGR3, HMGR4 and HMGR5 were suppressed (0.4225 ±0.1829 fold difference, 0.2735 ±0.02762 fold difference, 0.3766 ±0.1180 fold difference, and 0.3112 ±0.2289 fold difference, respectively) while HMGR2 was induced (3.867 ±1.126 fold difference). Gene expression analysis was repeated in an independent experiment with similar results (data not shown).

The strong induction of transcripts for HMGR2 in Phoma-inoculated plants indicates the production of pathogen-specific defense compounds the MVA pathway in *M. truncatula*. The induction of MVA pathway products in pathogen defense has been reported for other plant species. Sesquiterpenoid VOCs have been found to be used in pathogen defense in peanut and cereal crops (Cardoza et al. 2002; Plesik et al. 2011). Toome et al. (2010) reported willow plants inoculated with leaf rust emitted increased amounts of VOCs as well. The use of VOCs in pathogen defense in *M. truncatula* has not been reported. However, the induction of the HMGR2 gene by pathogen inoculation in this plant might indicate a use for VOCs in *M. truncatula* in pathogen defense. Antimicrobial phytoalexins can be produced via the MVA pathway in some plant species such as potato (Brindle et al. 1988) and maize (Huffaker et al. 2011). Terpenes or terpenoid moieties play important roles in anti-microbial or anti-insect activities (Reviewed by Gershenzon and Dudareva 2007). For example, the triterpenoid saponins are typically not classified as phytoalexins, although they have been shown to suppress growth of both insects (Herlt et al. 2002; Nozzolillo et al. 1997) and fungal pathogens (Mandal et al. 2005) . Likewise, terpene biosynthetic pathways likely provide the prenyl groups that decorate numerous antimicrobial compounds (Yazaki et al. 2009).
The differential expression of this gene family is consistent with reports from other plant species as well. In potato, tuber wounding, elicitor treatment and pathogen infection resulted in differential expression of the three HMGR gene family members (Yang et al. 1991). Choi et al. (1992) also demonstrated this in potato, where transcript levels of hmg1 were suppressed following elicitor treatment whereas transcript levels for hmg2 and hmg3 were induced. *Arabidopsis thaliana* has two members of the HMGR gene family that are differentially expressed as well. For example, mRNA for Hmg1 is expressed in all tissues, but Hmg2 is only expressed in seedlings, roots and inflorescences (Enjuto et al. 1994). Experiments with HMGR-deficient mutants in *A. thaliana* showed a reduction of triterpenoids by 65% in *hmg1* mutants and 25% in *hmg2* mutants when compared to wild type (Ohyama et al. 2007). These results, along with the results of this study, indicate the importance of HMGR in plant defense and that the differential regulation of this gene family does occur. This differential gene expression possibly allows plants to fine-tune defense responses by accumulating HMGR transcripts that are specifically responsive to certain stimuli, and possibly dedicated to specific end-products.
Figure 9. Relative gene expression of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) gene family transcripts in *M. truncatula* A17 leaf tissue subjected to *S. exigua* damage (gray bars) and *P. medicaginis* infection (white bars) when compared to undamaged and mock-inoculated tissue, respectively. Asterisk above a column indicates a P value ≤ 0.05 (n = 3). Error bars indicate SD.

To further characterize this gene family the nucleotide sequences of the *M. truncatula* HMGR gene family were aligned with ClustalW (Fig. 10). Most of the sequences were conserved throughout. This would agree with the close relationship of these isoforms reported in the unrooted phylogram (Fig. 11). The phylogram was constructed using the maximum likelihood method which compared the *M. truncatula*, *P. sativum*, *G. uralensis*, *A. thaliana* and *S. tuberosum* HMGR gene families. The *M. truncatula* HMGR gene family was more closely related to HMGR genes from other legumes species than to the *A. thaliana* and potato gene families. Legumes produce antimicrobial phytoalexins via the isoflavonoid biosynthetic pathway. Other plant families produce these compounds via the MVA pathway, in which HMGR catalyzes the rate limiting step. This could explain why the legume HMGR genes grouped together in a large clade that was separate from potato and *A. thaliana*. The *M. truncatula* HMGR1 and
HMGR2 genes were grouped together in a clade, which indicates they are more closely related to each other than the other *M. truncatula* HMGR genes. This is interesting since the gene expression data showed that these two were the most differentially expressed (Fig. 9). The *M. truncatula* HMGR5, *G. max* HMGR(b), *P. sativum* and *G. uralensis* HMGR genes grouped in a separate clade with no other *M. truncatula* HMGR genes. This would indicate that HMGR5 is the ancestral gene from which the other *M. truncatula* HMGR genes diverged.

| HMGR1       | ATGGACGTTCAGCAGA-----AAATAA--G-------------------CAAAAATGAGAAGAAGAA   |
| HMGR2       | ATGGGGCGTTCAGCAGA-----AAATAA--G-------------------CAAAAATGAGAAGAAGAA   |
| HMGR3       | ATGGACGTTCACCGGA-----GACACGTCAACCGCTGCTCCGAAAATAATTCAAAA   |
| HMGR4       | ATGGAGTTCACCGGCA-----AAATAT--ATTCTGACTCTCCAGCAGAAACACAGAAGAA   |
| HMGR5       | ATGGAGCCTCGCGGAGACTTAAAGCCTTCTCCACCGGGAAACACCTCAGTTCA   |

| HMGR1       | C-CAA-AGGCAGCAGGATTC-------ACAGTCAACACACCTTTCTCTTCA-------   |
| HMGR2       | C-CAA-AAGAAGCAGGAGATTC-------ACAGTCAA---------------------TA-------   |
| HMGR3       | C-AAAGACAAAAAGTCTGTTCT---ACAGTCAA---------------------TCTCTCA-------   |
| HMGR4       | C-CAA-A--AGGCACAGAATTC-------ACTGTCAAA---------------------CTTCTTCA-------   |
| HMGR5       | CTAACAAAAGGAGTCTTACAATCTCTTACACCAACACCCGGAACACCTCAGTTCA   |

| HMGR1       | ----------------------CTGTATTTAACAAGCGCTTTTTCTCTCTCTCTCCGCGC   |
| HMGR2       | ----------------------CTGTACTAAACAATGCTTTTTCTCTCTCTCTCTCCGCGC   |
| HMGR3       | ----------------------CTTACTAAACAAAGCTTTTTCTCTCTCTCTCTCCGCGC   |
| HMGR4       | ----------------------CTTTACTGAAACAAACTTTTTCTCTCTCTCTCCGCGC   |
| HMGR5       | CAGACTTGTTTCTCTGCTGTCACTAACGCTTTCTCTCTCTCTCTCCGCGC   |

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Figure 10. *Medicago truncatula* 3-hydroxy-3-methylglutaryl coA reductase (HMGR) gene family nucleotide sequence alignment. Alignment was done using clustalW. Forward and reverse primer sequences for each gene are indicated in boxes.

Figure 11. Unrooted phylogenetic analysis of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) gene families of *Medicago truncatula*, *Glycine max*, *Pisum sativum*, *Glycyrrhiza uralensis*, *Arabidopsis thaliana*, and *Solanum tuberosum*. Nucleotide sequences for each gene were translated using the SIXFRAME program and the resulting protein sequences were analyzed. Phylogenetic analysis was done by MEGA 5.0. Bootstrap analysis of 500 replicons was carried out with the maximum likelihood method. GenBank accession numbers are indicated for each sequence.
In this research, changes of transcript accumulation in response to insect herbivory or pathogen infection were explored among genes encoding defense- and photosynthesis-related proteins. Photosynthetic yield was also measured to determine if changes reflect patterns of gene expression. Expression levels for photosynthetic genes were greatly suppressed in plants subjected to either treatment, while defense genes were induced, which suggests a possible metabolic shift. Photosynthesis measurements showed no change in yield, which conflicted with transcript data. This suggests that although photosynthetic-gene transcript levels were reduced, there must still be sufficient levels present to maintain photosynthesis in the time frame of these experiments. It was shown that there is not a direct link between molecular activity in the form of transcript accumulation and physiological activity following insect attack or pathogen infection in plants.

The HMGR gene family in *M. truncatula* was also studied. Differential expression of transcripts for the five HMGR gene-family members was observed in insect-damaged and pathogen-infected plants. This is consistent with differential accumulation of transcripts encoding HMGR isoforms in other plant species. It also supports the hypothesis that plants use differential expression of HMGR isoforms for enhanced specificity in regulation of the MVA pathway and its end-products. For example, HMGR2 gene transcripts were induced by pathogen infection in *M. truncatula*, and therefore further work could focus on determining if the protein isoform encoded by this gene plays a role in biosynthesis of end-products specifically targeted toward invading pathogens.


