

Effects of juvenile hormone on gene expression in the pheromone-producing midgut of the pine engraver beetle, *Ips pini*

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Abstract

Juvenile hormone III (JH III) stimulates biosynthesis of the monoterpene aggregation pheromone component, ipsdienol, in the anterior midgut of the male pine engraver beetle, *Ips pini* (Say). To understand better the hormonal regulation of pheromone biosynthesis in this forest pest, and identify JH III-responsive genes, microarrays were prepared and hybridized to cDNA from midguts of JH III-treated beetles. Expression patterns were confirmed by quantitative real-time RT-PCR. JH III co-ordinately regulated mevalonate pathway genes and many other genes implicated in pheromone biosynthesis. Sex differences in basal levels of mevalonate pathway genes were consistent with their role in male-specific pheromone biosynthesis. This is the first microarray-based study of the developmental and hormonal regulation of insect pheromone biosynthesis.

Keywords: juvenile hormone, pheromone biosynthesis, midgut, ipsdienol, Scolytidae.

Introduction

Many bark beetle species use aggregation pheromones to co-ordinate a mass attack on a host tree, overwhelming the tree's defences. Although biosynthesis of aggregation pheromones in bark beetles was originally thought to simply involve oxidation of host monoterpenes, it is now clear that

de novo biosynthesis is the major source of pheromone (Seybold & Tittiger, 2003). In the pine engraver beetle, *Ips pini* (Say), the monoterpene pheromone component ipsdienol (2-methyl-6-methyleneocta-2,7-dien-4-ol) is synthesized via the mevalonate pathway specifically in the anterior midgut cells of males (Seybold *et al.*, 1995; Hall *et al.*, 2002). Male beetles produce nearly 1% of their body mass per day in volatile pheromone, a significant metabolic load (Tillman *et al.*, 2004).

Phloem feeding by males induces juvenile hormone III (JH III) biosynthesis in the corpora allata (Tillman *et al.*, 1998). This in turn induces the co-ordinate up-regulation of mevalonate pathway gene transcript levels necessary for pheromone biosynthesis (Keeling *et al.*, 2004), the extensive subcellular remodelling of midgut cells (Nardi *et al.*, 2002), and ipsdienol biosynthesis (Tillman *et al.*, 2004). In the absence of feeding, topical application of JH III stimulates pheromone production in male *I. pini* (Tillman *et al.*, 2004) and increases both 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-R) mRNA levels in male *I. pini* (Hall *et al.*, 2002; Tillman *et al.*, 2004; Bearfield *et al.*, 2006) and male Jeffrey pine beetles, *Dendroctonus jeffreyi* Hopkins (Tittiger *et al.*, 2000; Tittiger *et al.*, 2003). HMG-R mRNA levels similarly increase in JH III-treated *Ips paraconfusus* males (Ivarsson *et al.*, 1998; Tittiger *et al.*, 1999; Tillman *et al.*, 2004). These studies support the paradigm that increased JH III titres stimulate *de novo* pheromone production via the mevalonate pathway in the anterior midgut (Seybold & Tittiger, 2003).

Juvenile hormones play many important endocrine roles in insects including regulating development and adult reproductive maturation (Gade *et al.*, 1997). Although several models have been proposed (for reviews, see Davey, 2000; Wheeler & Nijhout, 2003), the mode of action of juvenile hormone remains elusive. With its many roles, there likely is more than one mechanism for JH action, even within one organism or tissue.

JH III-regulated pheromone biosynthesis is a model system to study JH action. The robust response of mevalonate pathway genes in the midgut to JH III suggests this system may contribute to studies of the mechanism of JH action.

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We used a genomics-based strategy to assist our studies of pheromone biosynthesis and JH signalling in bark beetles. A small-scale EST project (curtailed due to high redundancy values) recently recovered representations of 574 tentatively unique genes expressed in the anterior midguts of JH III-treated *I. pini* males (Eigenheer *et al.*, 2003). These include eight mevalonate pathway genes, JH-degrading enzymes, nucleic acid binding proteins, and many others, including 35% with unknown function. We hypothesized that other pheromone-biosynthetic genes would be JH-responsive, similar to the mevalonate pathway genes. We therefore prepared cDNA microarrays from the ESTs and used them to identify JH III-responsive genes in anterior midgut tissue. We found that mRNA levels for 236 of the 574 tentative unique genes were significantly affected following topical application of JH III, providing preliminary identification of putative new targets for future studies on pheromone biosynthesis and JH regulation.

Results and discussion

Microarray analysis and qRT-PCR validation

Feeding male *I. pini* synthesize JH III in their corpora allata (Tillman *et al.*, 1998) and this hormone in turn signals an increase in smooth endoplasmic reticulum in the midgut (Nardi *et al.*, 2002) and the synthesis of copious amounts of ipsdienol (Tillman *et al.*, 2004). We anticipated that this profound change in cellular ultrastructure and metabolism would be accompanied by a notable shift in the expression of a number of genes. The current study used cDNA microarrays to monitor changes in mRNA levels of genes in the anterior midgut following topical application of JH III. Complete microarray hybridization data have been deposited at NCBI GEO, samples GSM25314-GSM25345 and series GSE1483.

As would be expected in cells that undergo extensive subcellular remodelling to actively biosynthesize pheromone (Nardi *et al.*, 2002), the expression of many genes changed over time following JH III treatment. Of the 574 tentatively unique genes on the microarray, 236 were significantly regulated, and 148 of these were regulated $> 2^{\pm 0.5}$ in at least one sample set (Fig. 1). Expression patterns were similar between sexes for most genes. Changes in mRNA levels ranged from sixfold higher to fourfold lower in JH III-treated samples compared with controls.

In the context of 'regulated' meaning genes that respond to JH III treatment without distinguishing between direct (primary) responders and indirect (downstream) responders as defined by Ashburner *et al.*, (1974), JH III-regulated genes sorted into two broad clusters: those that were generally up-regulated and those that were down-regulated (Fig. 1). Four subgroups were distinguishable in the broad cluster of generally up-regulated genes: genes that were initially down-regulated, but end with elevated transcripts (Group I, three

genes); rapid and robust responders (Group II, 20 genes); intermediate responders (Group III, 23 genes); and late responders (Group IV, 28 genes). The 'early' genes in Group II include seven of the eight mevalonate pathway genes represented on the array. For generally down-regulated genes, there was a small group (Group V, five genes) that responded early to JH III, while the majority was down-regulated late after treatment (Group VI) with the exception of one gene (IPG024D01) that was initially up-regulated before being down-regulated. Genes in Group II may represent primary responders to JH III, while genes in the other groups may respond indirectly to JH III via unknown signals likely triggered by upstream events. Further experiments are needed to establish which genes are primary responders.

When sorted into functional categories as per Eigenheer *et al.* (2003), most of the up-regulated genes were involved in metabolism, transport or 'unknown' (Fig. 2). This is consistent with the induction of ipsdienol biosynthesis in midgut cells, and indeed, mevalonate pathway genes appear co-ordinately induced by JH III (see below). It is also interesting that most of the JH III-regulated cell-structure genes were also up-regulated, which may reflect the intracellular reorganization that accompanies pheromone biosynthesis (Nardi *et al.*, 2002). Interestingly, JH treatment similarly increases the rough endoplasmic reticulum of mosquito midguts (Rossignol *et al.*, 1982).

The effects of JH III at later time points may have resulted from secondary factors. For example, down-regulation of cell energy and translation genes was most evident in males at the 16 h time point (Fig. 1, Groups V and VI), when starved male beetles had used significant resources for ipsdienol production. Many of the metabolic genes that were induced late in males were putative glycoside hydrolases (Fig. 1, Groups I and V). These were initially down-regulated, but had higher mRNA levels later, suggesting their induction as metabolic stores become depleted. Pheromone-biosynthesizing beetles are usually feeding, so the incoming meal presumably compensates for this. Microarray analysis of the effects of feeding for 24 h on the expression of 384 genes in male *I. pini* midguts shows that feeding also reduces rRNA levels (similar to JH III treatment) but not genes in the energy category (Tittiger *et al.*, 2005). The apparent decrease in mRNAs for energy producing genes as well as rRNAs, which are sorted into the translation category along with tRNAs and translation-associated enzymes (Fig. 2), may thus indicate the metabolic state of the cells, and not necessarily direct effects of JH III.

The trends in the microarray data were confirmed by qRT-PCR of 13 selected genes and eight mevalonate pathway genes (Table 1, Figs 3 and 4). The most strongly and early responding of these genes were IPG010G08, encoding a protein of unknown function, and IPG005F08, a putative mitochondrial carrier protein. In qRT-PCR

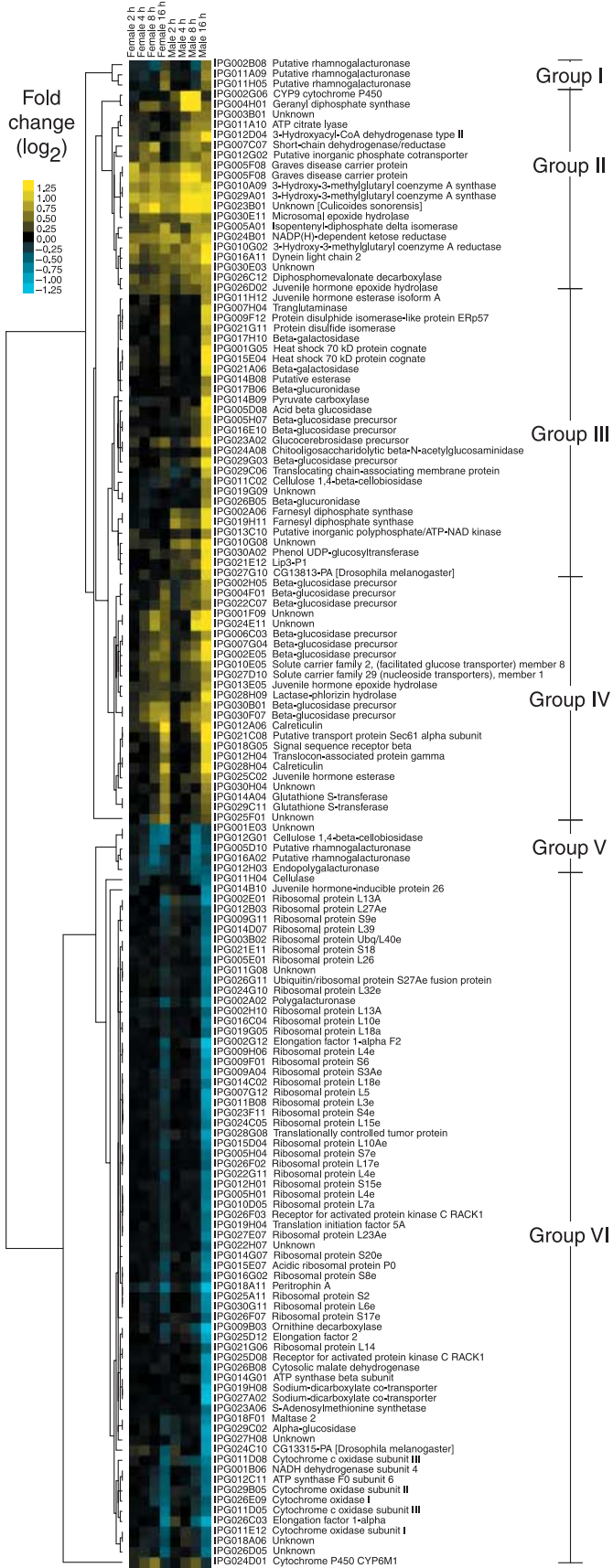


Figure 1. Clustering of significantly JH III-regulated genes with expression differences $> 2^{0.5}$ in at least one treatment. Each column represents the average relative expression ratios (log₂ JH III/acetone) for a specific time point. $n = 4$ biological replicates at each time point, 30 midguts/treatment per array. Putative identity/function shown are the best annotated Blastx hits of the EST, or the contig it represents.

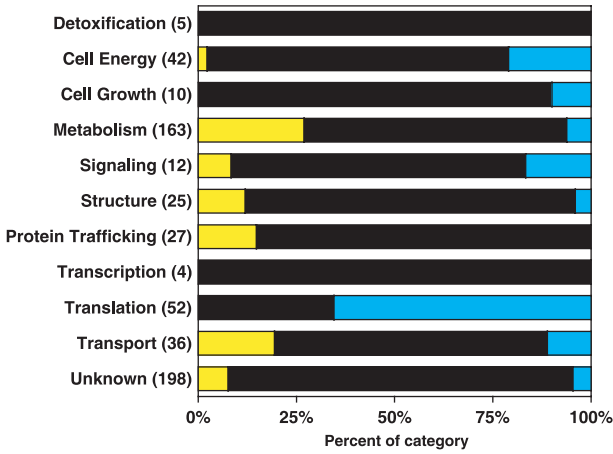


Figure 2. Functional categories of genes significantly regulated $> 2^{\pm 0.5}$ by JH III. Overall trend from all treatments shown: yellow, up-regulated; black, unregulated; blue, down-regulated. Numbers in parentheses indicate the number of genes in each category on the microarray.

analysis, both these genes were up-regulated approximately fourfold at 2 h and peaked at approximately 32-fold at 8 h. The qRT-PCR data show many strong correlations (both negative and positive) in expression patterns among the analysed genes (Fig. 5) suggesting that JH III co-ordinately regulates the processes necessary for pheromone biosynthesis and excretion.

Pheromone-biosynthetic genes

Of the eight mevalonate pathway genes represented on the microarray, only acetoacetyl-CoA thiolase (AACT) was not significantly up-regulated. The early genes in the pathway clustered together and appeared co-ordinately induced by JH III in both sexes (Fig. 1). Farnesyl diphosphate synthase (FPPS) and geranyl diphosphate synthase (GPPS) did not cluster with this group and had more male-specific expression patterns. qRT-PCR confirmed that all eight were significantly up-regulated in at least one time point in males (Fig. 4). These data are consistent with the significant increase in carbon flowing through the mevalonate pathway and into ipsdienol biosynthesis in male midguts. The gene for FPPS did not sort into the same microarray cluster as the other mevalonate pathway genes because it was induced by JH III in males, but repressed in females. The function of the JH III responses in female midguts is not known. The observed inductions were not an artefact of our experimental procedure because feeding similarly down-regulates FPPS and up-regulates the remaining mevalonate

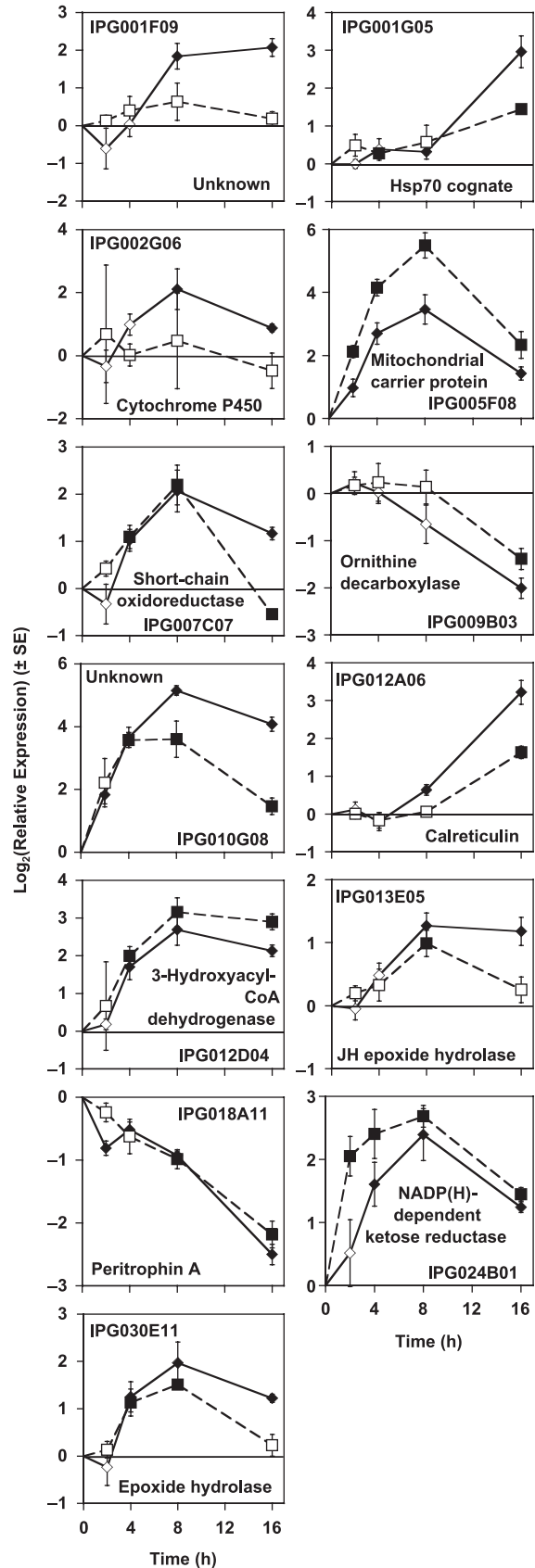


Figure 3. qRT-PCR validation of microarray results. Expression (\log_2) is relative to acetone-treated beetles at each time point. Males, solid line with diamond symbol; females, dashed line with square symbol. Filled-in symbols are significantly different than acetone-treated beetles, *t*-test, $P < 0.05$, $n = 4$, 30 midguts/rep.

Table 1. Information for ESTs validated by qRT-PCR. Abbreviations shown for mevalonate pathway genes

Clone ID	GenBank. acc. no.	Putative gene identity	Best BLASTx hit (searched September 2005)	E-val	qRT-PCR Primers Forward (5'-3') Reverse (5'-3')	Amplicon size (bp)
IPG001F09	CB408160	Unknown	Unknown	–	ttgtctgcgtttggcagtt catcaactgggcacgatttca	68
IPG001G05	CB408167	Heat shock 70 kDa protein cognate	Heat shock 70 kDa protein cognate [<i>Bombyx mori</i>]	2E-70	cttattcatgcgtggcgat tgcgacataggaagcgtaat	89
IPG002G06	CB408249	CYP9 cytochrome P450	CYP9 cytochrome P450 [<i>Drosophila mettleri</i>]	7E-93	ttcaactccacatccatcaact taatgtcagccctcagctttcttg	83
IPG004H01	CB408408	Geranyl diphosphate synthase (GPPS) ^a	GPPS [<i>Ips pini</i>]	0	cggtgtcctcatggaatgtt ggaagagaaggttgcggagaa	104
IPG005A01	CB408590	Isopentenyl diphosphate isomerase (IPPI)	IPP:DMAPP isomerase [<i>Dendroctonus jeffreyi</i>]	4E-77	gaaataccggaacgagagaaga tgcccagctcgtagtcaaa	82
IPG005E02	CB408614	Cytoplasmic actin	GA17886-PA [<i>Drosophila pseudoobscura</i>]	0	gccgttctccatcaatcgt ttttgctcgggttcatcac	100
IPG005F08	CB408622	Mitochondrial carrier protein	PREDICTED: similar to ENSANGP0000008222, partial [<i>Apis mellifera</i>]	8E-63	ttgtgaggagttgctgtaa tccaaggggtcactgctgtt	52
IPG007C07	CB408556	Short-chain dehydrogenase/reductase	ENSANGP00000021773 [<i>Anopheles gambiae</i> str. PEST]	3E-34	ctgaccacgacctgaaaacca ttgccactgccaacaaatg	51
IPG009B03	CB408275	Ornithine decarboxylase	PREDICTED: similar to ENSANGP00000020224 [<i>Apis mellifera</i>]	5E-48	cgaactcaaaatgaagatcaaacg catactcctgataaccgacctgta	88
IPG010A09	CB408343	3-Hydroxy-3-methylglutaryl CoA synthase (HMG-S) ^b	3-Hydroxy-3-methylglutaryl coenzyme A synthase [<i>Ips pini</i>]	6E-69	tgcaatgaccgggaagaca ttcctgaggttgatgtgtaacg	84
IPG010G02	CB408376	3-Hydroxy-3-methylglutaryl CoA reductase (HMG-R) ^c	3-Hydroxy-3-methylglutaryl coenzyme A reductase [<i>Ips pini</i>]	1E-121	ccctgggaagttaactggtgct taggtttggtggtgggattg	88
IPG010G08	CB408379	Unknown	Unknown	–	ctggaagcgaaaatcgaaag tcgttgctgattatggtgta	87
IPG012A06	CB408637	Calreticulin	PREDICTED: similar to calreticulin [<i>Apis mellifera</i>]	5E-44	tgaagtggcctgctgtt cccatgaatcgtgtgtaatt	96
IPG012D04	CB408666	3-Hydroxyacyl-CoA dehydrogenase	CG7113-PA [<i>Drosophila melanogaster</i>]	8E-31	ggaagttggcgggtgcatg ctgtatagtcgccaggtgtgag	94
IPG013E05	CB408068	JH epoxide hydrolase	PREDICTED: similar to juvenile hormone epoxide hydrolase II [<i>Apis mellifera</i>]	5E-71	tgccacctcaagccacta gcagccaaccaacaggacta	71
IPG013H02	CB408094	Acetoacetyl-CoA thiolase (AACT)	GA21576-PA [<i>Drosophila pseudoobscura</i>]	0	tgaagatactctgctgggaat acactggtccatctcagggtt	91
IPG018A11	CB408858	Peritrophin A	ENSANGP00000015393 [<i>Anopheles gambiae</i> str. PEST]	5E-8	ccgtcaggacaatccaaac gaacaaacctcaggacaatgc	119
IPG019H11	CB408986	Farnesyl diphosphate synthase (FPPS) ^d	Farnesyl pyrophosphate synthase [<i>Ips pini</i>]	1E-87	gggaaatgggaacggacat ttggctggagtgacctt	81
IPG024B01	CB407830	NADP(H)-dependent ketose reductase	NADP(H)-dependent ketose reductase [<i>Bemisia argentifolii</i>]	3E-75	gagcggcgattatcatctttg tctgaggagtgccacagaag	54
IPG026C12	CB407651	Mevalonate 5-diphosphate decarboxylase (MPDC)	ENSANGP00000013738 [<i>Anopheles gambiae</i> str. PEST]	7E-41	tcacatctgtcgaaaacaatt agcgtacaccagacaggcatag	81
IPG030E03	CB407561	Mevalonate kinase (MK)	Unknown ^e	–	cactgctgtatccccattg accgcctttctaccacatca	91
IPG030E11	CB407470	Epoxide hydrolase	Microsomal epoxide hydrolase [<i>Trichoplusia ni</i>]	3E-48	cctgccattccaacctctt tgcccagctactccactatg	90

^aFull-length sequence is available for GPPS, GenBank accession no. AY953508.

^bFull-length sequence is available for HMG-S, GenBank accession no. AY902193.

^cFull-length sequence is available for HMG-R, GenBank accession no. AF304440.

^dFull-length sequence is available for FPPS, GenBank accession no. AY953507.

^eFurther sequencing identified this as mevalonate kinase (unpublished).

pathway genes in female midguts (Keeling *et al.*, 2004). Although the fold changes were similar between sexes, females initially had significantly lower transcript levels (see below). Thus even after induction, transcript levels in the female midgut were still less than or equal to those from an untreated male, which does not produce pheromone. However, females do produce a small amount of pheromone

after mating (Pureswaran *et al.*, 2000), so the competence of female midguts to respond to JH III may facilitate later pheromone production.

The co-ordinate regulation of most of the mevalonate pathway genes suggests that some of the other genes in the same cluster may be involved in pheromone biosynthesis, either directly as enzymes or else indirectly as transport

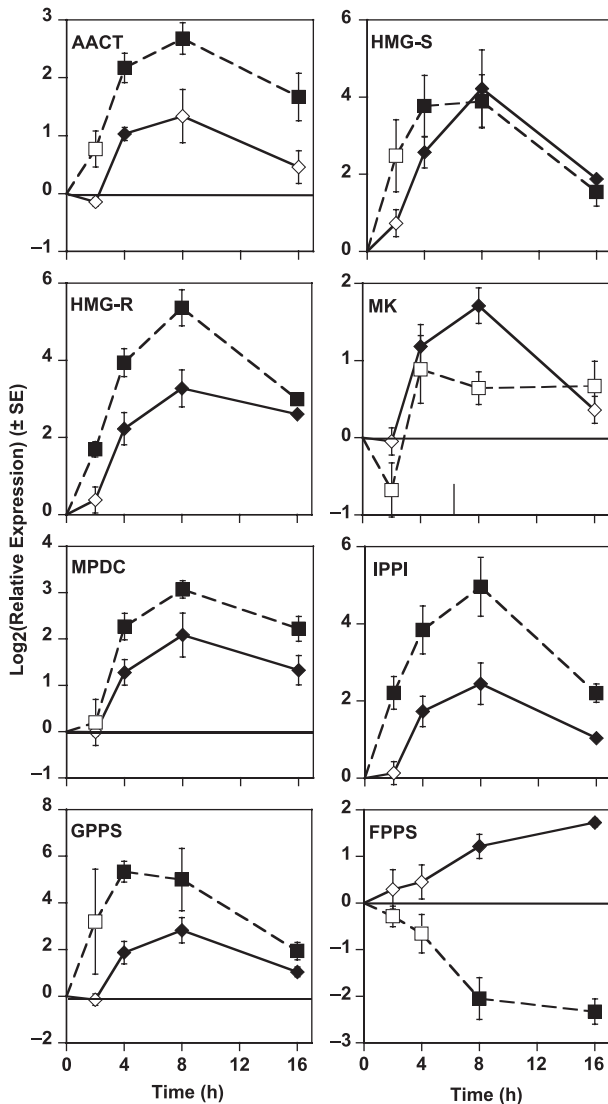


Figure 4. qRT-PCR analysis of JH III-induced changes in expression of mevalonate pathway genes. Expression (\log_2) is relative to acetone-treated beetles at each time point. Males, solid line with diamond symbol; females, dashed line with square symbol. Filled-in symbols are significantly different than acetone-treated beetles, *t*-test, $P < 0.05$, $n = 4$, 30 midguts/rep.

proteins or modulators of cell organization. Identifying putative pheromone biosynthetic genes based solely on JH III response may not be reliable because induction of many of the mevalonate pathway (pheromone biosynthetic) genes was not male-specific. An earlier study of starved *I. pini* midguts indicated that mevalonate pathway mRNAs are more abundant in males than females (Keeling *et al.*, 2004). We repeated this analysis with a larger sample size and confirmed that the relative basal levels for all the mevalonate pathway genes were significantly higher in males compared with females, ranging from 3.5-fold for mevalonate kinase (MK) to 400-fold for GPPS (Fig. 6). GPPS in particular is critical to directing mevalonate pathway products into

pheromone production. The induction of mevalonate pathway genes in females by JH III would have little significance for pheromone biosynthesis because basal GPPS transcript levels were so much lower than males. Male beetles seem developmentally primed to rapidly synthesize pheromone upon entering the phloem, mediated by a feeding-induced increase in JH III biosynthesis. Thus, combining microarray data with additional data may provide a more accurate guide to identifying pheromone-biosynthetic genes and their regulation.

We also compared basal expression levels of the 13 other JH III-regulated genes examined by qRT-PCR (Fig. 6). Three of these, putative cytochrome P450 CYP9 (IPG002G06), mitochondrial carrier protein (IPG005F08), and 3-hydroxyacyl-CoA dehydrogenase (IPG012D04), had relative basal expression levels between sexes comparable with the mevalonate pathway genes, implying their involvement in pheromone production. In particular, the cytochrome P450 had a nearly 500-fold higher basal expression level in males compared with females, was induced only in males, and clusters beside the EST for GPPS (Fig. 1). These data are consistent with a possible role for IPG002G06 in the pheromone biosynthetic pathway. Although the final steps of ipsdienol biosynthesis have not been firmly established, it is thought that geranyl diphosphate produced by GPPS in the mevalonate pathway is converted to myrcene by a monoterpene synthase and then hydroxylated to ipsdienol by a cytochrome P450 (Seybold *et al.*, 2000). A JH III- and feeding-inducible myrcene synthase activity has recently been detected in cell-free extracts of male, but not female, *I. pini* beetles (Martin *et al.*, 2003). Although a recognizable myrcene synthase was not identified in the ESTs, the enzyme may be represented by one of the unknowns in Group II (Fig. 1). IPG002G06 and other P450s are currently being functionally characterized for their roles in pheromone biosynthesis (P. Sandstrom, unpublished data).

Summary

Most previous studies of JH III-regulated gene expression are in the context of one or a few genes (Arif *et al.*, 2002; Keiser *et al.*, 2002; Sempere *et al.*, 2003; Tillman *et al.*, 2004). To our knowledge, this is only the second microarray analysis of the response of a target tissue to a juvenile hormone or JH analogue (Terashima & Bownes, 2005) and is the first microarray-based approach to study the hormonal regulation of pheromone biosynthesis. Although our sample of the *I. pini* genome was relatively small (only 574 genes), it clearly demonstrated a broad-based response to JH III in midgut cells. The shift in metabolic activity towards producing high amounts of the monoterpene pheromone was most evident in the co-ordinate up-regulation of mevalonate pathway genes and was accompanied by a reduction in translation. Other responsive genes may play roles

	IPG001F09	IPG001G05	IPG002G06	IPG004H01	IPG005A01	IPG005F08	IPG007C07	IPG009B03	IPG010A09	IPG010G02	IPG010G08	IPG012A06	IPG012D04	IPG013E05	IPG013H02	IPG018A11	IPG019H11	IPG024B01	IPG026C12	IPG030E03	IPG030E11	
IPG001F09					0.50					0.36				0.29								0.35
IPG001G05	0.29			-0.38	-0.25	-0.25						0.50				-0.38						
IPG002G06	0.54			0.58									0.31									
IPG004H01	0.26			0.75	0.26	0.29			0.38	0.31			0.40					0.26				
IPG005A01			0.73	0.96		0.77	0.69	0.35	0.74	0.59				0.45	0.46			0.57	0.36			0.72
IPG005F08			0.65	0.88	0.88		0.72	0.31	0.83	0.47				0.34	0.56			0.43	0.46			0.71
IPG007C07	0.50		0.60	0.59	0.55	0.46		0.28	0.52	0.58	-0.37			0.30	0.25			0.58				0.59
IPG009B03	-0.40	-0.57							0.38	0.34	-0.49			0.30		0.69		0.35				
IPG010A09			0.40	0.44	0.40	0.52				0.32				0.34								
IPG010G02	0.51		0.69	0.79	0.81	0.62	0.73		0.34		0.32		0.38	0.44	0.55			0.29	0.55	0.26		0.70
IPG010G08	0.59		0.66	0.65	0.62	0.55	0.67		0.57	0.81		-0.34		0.40				0.49	0.29			0.53
IPG012A06	0.40	0.89						-0.64								-0.61	-0.33	-0.25				
IPG012D04	0.61		0.73	0.77	0.76	0.59	0.60		0.39	0.91	0.85			0.30				0.32	0.29			
IPG013E05	0.76	0.26	0.62	0.46	0.48	0.31	0.51		0.26	0.75	0.78	0.34	0.81		0.50			0.32				0.44
IPG013H02			0.57	0.60	0.61	0.73			0.51	0.46	0.50		0.59	0.32				0.81	0.32			0.51
IPG018A11	-0.29	-0.75						0.59			-0.87						0.38					
IPG019H11	0.43	0.33	0.25		0.25		0.31	-0.26		0.53	0.38	0.46	0.48	0.61		-0.44						-0.49
IPG024B01	0.37		0.79	0.71	0.72	0.65	0.76			0.71	0.60		0.58	0.53	0.38							0.59
IPG026C12	0.43		0.69	0.66	0.67	0.67	0.42		0.49	0.70	0.74		0.83	0.62	0.84			0.47			0.42	0.47
IPG030E03			0.44	0.64	0.57	0.70	0.31		0.59	0.38	0.55		0.47	0.25	0.51			0.32	0.50			
IPG030E11	0.53		0.80	0.74	0.76	0.63	0.84		0.27	0.86	0.74		0.77	0.64	0.49		0.32	0.89	0.65	0.34		

Figure 5. Correlations between gene expression profiles from qRT-PCR analysis. Top-right section, female data; bottom-left section, male data. r^2 values shown are for statistically significant linear regressions, $P < 0.05$. Negative signs in front of r^2 values indicate a negative correlation. Yellow cells indicate a significant positive correlation, blue cells indicate a significant negative correlation, and black cells indicate no significant correlation.

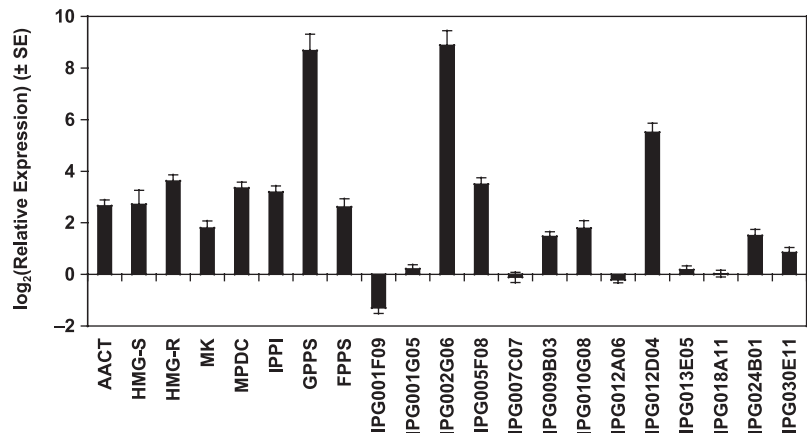


Figure 6. qRT-PCR analysis of relative basal mRNA levels between sexes (males/females). See Table 1 for annotation of these genes. Expression (\log_2) for acetone-treated males relative to acetone-treated female beetles, $n = 16$ (all time points combined), 30 midguts/rep. All differences between sexes are significant except for IPG001G05, IPG007C07, IPG012A06, IPG013E05, and IPG018A11 (t -test, $P < 0.05$).

in pheromone transport, subcellular reorganization, or support of the cells' new role. Some processes may have gone undetected due to the relatively low representation of genes. For example, very few known signalling genes are included in our ESTs (Eigenheer *et al.*, 2003), and we did not detect a JH III-mediated change among them; therefore, this study did not provide a direct link into the signalling mechanism of JH. However, it did reveal several new JH III-responsive genes that may be helpful in future studies on the mode of action of JH in this tissue. For example, one of these, IPG010G08, a gene with unknown function that

responds rapidly to JH III, is currently undergoing extensive characterization (Bearfield *et al.* 2006).

Experimental procedures

Tissue samples

Logging slash of Jeffrey pine (*Pinus jeffreyi* Grev. & Balf.) infested with *I. pini* were obtained from Ward Creek, CA, USA (39°08'41"N, 120°12'01"W, 2048 m) and placed in a greenhouse to allow emergence of adult beetles (Browne, 1972). Emerged beetles were typed and sexed following Wood (1982) and kept at 4 °C on moist paper towels. Beetles were immobilized by chilling and then

treated topically on the ventral surface of the abdomen with either 10 µg (+/–) JH III (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 µl acetone or acetone (control). They were then incubated in the dark at room temperature in groups of 10 in 60 ml plastic containers (Dixie) for 2, 4, 8 or 16 h. Following incubation, beetles were immersed in water and the anterior midguts excised under a stereo microscope. Midguts were gently purged of their contents and then immediately frozen in liquid nitrogen and stored at –80 °C. Thirty midguts were pooled for each experimental unit and each treatment/control time point pair had four biological replicates. Treatment and control beetles for a replicate pair were cohorts and were incubated simultaneously.

Microarray preparation

Five hundred and seventy-four tentatively unique genes were previously identified in an *I. pini* EST project (GenBank accession numbers CB407466–CB409136) (Eigenheer *et al.*, 2003). EST sequences can also be found at NCBI by the clone IDs used here. If multiple ESTs occurred for a gene, a specific clone was chosen to represent the longest clone of a cluster-contig. Selected clones were grown overnight from glycerol stocks in 200 µl Luria Broth containing 100 µg/ml ampicillin. A portion of each culture was heat inactivated and then amplified using vector specific primers. Amplicons were purified with Millipore Montage PCR96 filter plates (Millipore, Billerica, MA, USA) on a Qiagen 3000 BioRobot (Qiagen Inc., Valencia, CA, USA). A sample of each product was run on a 1% agarose gel to assess yield and purity. Those with poor yield or with multiple products were re-grown and re-amplified, or else alternate clones amplified. PCR products were lyophilized and then re-suspended in Micro Spotting Solution Plus (Arrayit, TeleChem International Inc., Sunnyvale, CA, USA) and spotted on to SuperAmine microarray substrates (Arrayit) using a Virtek SDDC2 Microarrayer (Virtek Vision International Inc., Waterloo, ON, Canada) with a 1 × 8 array of SMP5B Stealth Micro Spotting pins (Arrayit) printing subarrays of 10 × 8 spots with 550 µm spacing. Spotting was replicated in triplicate with replicate spots offset 6 mm, producing 3 × 8 subarrays on each substrate. DNA was bound to substrate by first rehydrating the array, flash drying on a hot plate, cross-linking with 120 mJ of UV light (Stratagene UV Stratalinker, 1800, Stratagene, La Jolla, CA, USA), and then baking for 60 min at 80 °C. Complete details of the genes represented on this microarray platform are available at NCBI GEO (Edgar *et al.*, 2002), accession no. GPL575.

Microarray hybridization

Total RNA was extracted from midguts using QIAshredder and RNeasy Mini kits (Qiagen) and quantified spectrophotometrically. Total RNA (approximately 40 µg) was reverse transcribed with oligo(dT)15 (Promega, Madison, WI, USA), Superscript III RT (Invitrogen, Carlsbad, CA, USA), and dNTPs (Promega) containing aminoallyl-dUTP (Sigma, aminoallyl-dUTP/dTTP ratio of 2 : 3). After alkaline hydrolysis of the RNA, clean up with a Microcon-30 (Millipore), and lyophilization, the aminoallyl-dUTP cDNA was reacted with Cy3 or Cy5 monofunctional reactive dyes (Amersham Biosciences, Piscataway, NJ, USA) for 60 min. Cy3 and Cy5 dyes were swapped equally between treatment and control samples among the four replicates. Unreacted dyes were removed from the labelled cDNA by a QIAquick spin column (Qiagen). The fluorescently labelled cDNAs from treatment and control samples were combined together with 20 µg denatured herring sperm DNA (Promega) and 20 µg poly(dA) (Amersham), lyophilized, and then

resuspended in 20 µl hybridization buffer [50% formamide, 5 × sodium citrate/chloride buffer (SSC), and 0.1% sodium dodecyl sulphate (SDS)]. Immediately prior to hybridization, microarrays were washed for 5 min in 0.1% SDS, 5 min in 2 × SSC, denatured 3 min in boiling water, placed into ice-cold ethanol for 5 min, dried by centrifugation, incubated for 60 min at 42 °C in prehybridization buffer (5 × SSC, 0.1% SDS, and 1% BSA), washed 5 min in water, 5 min in 2-propanol, and then dried by centrifugation. The hybridization mixture was denatured at 95 °C for 5 min, centrifuged at 16 000 ×g for 5 min, and then immediately applied to the microarray preheated to 42 °C. The microarray was then covered with a HybriSlip (22 × 40 mm, Sigma) and immediately placed into a preheated, humidified hybridization chamber and placed in a 42 °C oven. After 16–20 h, microarrays were removed from the hybridization chamber and immediately washed with agitation at room temperature for 5 min each in 2 × SSC with 0.03% SDS, 1 × SSC, and 0.2 × SSC, and then dried by centrifugation.

Microarray data analysis

Microarrays were scanned at 5 µm resolution with a ScanArray 4000 array scanner and analysed with ScanArray and QuantArray software (Packard Bioscience, PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA, versions 3.1 and 3.0, respectively). Median signal (adaptive spot algorithm) and background intensities were obtained for both dyes for each microarray element. Data were background subtracted, globally and locally (across microarray surface, span = 0.5, trim = 0.1) normalized, log₂ transformed, converted to mean log (intensities) and log (ratios), and then locally normalized across element signal intensity (span = 0.7, trim = 0.1) using online SNOMAD tools (Colantuoni *et al.*, 2002). To account for outliers, the elements in triplicate on the four replicated microarrays were temporarily treated as 12 independent measurements and a Q-test (96% confidence) was performed (Dean & Dixon, 1951). If an element tested as an outlier, its value was replaced with the average value of the two other replicate elements on the same microarray. At most, only one of the 12 measurements was excluded. The triplicate measurements on an individual microarray were averaged and the genes significantly regulated by treatment in the four biological replicates were determined using the SAM method of Tusher *et al.* (2001) with a false discovery rate of three genes at each time point. Those genes showing SAM-significant regulation by JH III of at least 2^{+0.5} were put into a self-organizing map and then clustered (correlational, uncentred, average linkage clustering) with Cluster (Eisen *et al.*, 1998) and displayed with Java Treeview freeware (Saldanha, 2004).

Analysis of gene expression by qRT–PCR

Relative gene expression levels in midguts were determined for several genes with qRT–PCR using the ΔΔCT method (Livak & Schmittgen, 2001). Both JH III-treated vs. acetone-treated at each time point, and acetone-treated males vs. acetone-treated females (relative basal) values were calculated. Genes were chosen to represent the mevalonate pathway genes and the major groups found by clustering. A portion of each RNA sample extracted for the microarray experiments was reverse transcribed for qRT–PCR using random primers and Superscript III Reverse Transcriptase (Invitrogen). Cytoplasmic actin (IPG005E02), which appeared unaffected by feeding or JH III treatment in microarray experiments (Tittiger *et al.*, 2005 and this study), was used as the endogenous control. Primers with minimal potential for primer-dimer formation

were identified using Vector NTI software (version 7.1, Invitrogen) from candidate primers suggested by Primer Express software (Applied Biosystems, Foster City, CA, USA) and then synthesized (Integrated DNA Technologies, Coralville, IA, USA). Genes examined by qRT-PCR and their primers are listed in Table 1. Most sequences used to design qRT-PCR primers were contigs or singlets from the EST project (Eigenheer *et al.*, 2003). HMG-R sequence has previously been reported (Hall *et al.*, 2002). In addition, Bearfield *et al.* (2006) provided HMG-S and Gilg *et al.* (2005, and unpublished) provided GPPS and FPPS sequences. qRT-PCR data were acquired on an ABI Prism 7000 Sequence Detection System using qPCR Mastermix Plus for SYBR Green I (Eurogentec, San Diego, CA, USA) and universal thermocycler conditions according to the manufacturer's protocol (Applied Biosystems) in 25 µl reactions. Template and primer concentrations were optimized, and amplification efficiencies determined for each gene. Melting curves for each template/primer pair were examined for non-specific amplification. Control templates prepared without reverse transcriptase indicated insignificant genomic DNA contamination.

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