Development of a Biomarker for Jasmonate Pathway Function in *Fraxinus*

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Abstract

At present, North American ash species (genus *Fraxinus*) are under attack by Emerald Ash Borer (*Agrilus planipennis* Fairmaire; Coleoptera: Buprestidae; EAB), an aggressive, invasive insect native to southeastern Asia, whereas native Asian ash species are comparatively resistant to this phloem-feeding insect. To date, little research has been done to determine the basis for susceptibility of North American ash species to EAB. It is possible that Asian species are resistant because of metabolites they produce. The broad objective of my research is to compare metabolite profiles of North American and Asian ash species in order to better understand what makes the Asian ash species resistant to EAB. Insect feeding will be simulated by wounding and application of methyl jasmonate (MJ), a method often used in other insect pest-tree host systems. Following treatment, tissue samples will be harvested for metabolic profiling via various biochemical techniques. However, we do not know how much of a delay there is between imposition of the treatment and the plant's response, or if the molecule imparting resistance is distributed systemically. In order to ensure that we are sampling the right tissues and at the right time, a biomarker is being developed to determine when the jasmonate response pathway is up-regulated. This will allow us to predict when and where a response to simulated insect feeding is occurring. Preliminary development of this biomarker is focusing on a gene in the jasmonate pathway. Future work will integrate molecular and biochemical methods to predict candidate genes that may be involved in imparting resistance to EAB.

Introduction

We know very little about the biochemical and molecular biological effects of EAB on *Fraxinus*, which presents challenges for defining experimental parameters for research meant to expand our knowledge in these areas. While we know physiologically how EAB kills ash trees (the wood-boring larvae effectively strangle a tree by feeding on its phloem), we know nothing about the effects of this herbivory on the defense pathways of *Fraxinus*. How quickly does a tree respond? Are the effects local or systemic? Induced or constitutive? Direct or indirect? Experiments must be designed in order to answer these questions, but we need to ensure that our sampling for these experiments occurs within an appropriate timeframe, and that we sample appropriate tissues.

A molecule that indicates a particular biological state is generally termed a *biomarker*. For instance, in medicine, a biomarker may indicate presence of a disease. For this project, a biomarker will be useful for identifying when the defense pathway regulated by jasmonic acid (JA) is activated in *Fraxinus*. Any of the genes in the jasmonate pathway (Figure 1) have the potential to serve as a biomarker. We are initially focusing on jasmonic acid carboxyl methyltransferase (*JMT*), which is the enzyme responsible for catalyzing the transformation of JA to MJ. *Arabidopsis* plants treated with MJ show a 12-fold increase of *JMT* in comparison to wild-type plants (Zimmermann *et al.*, 2004). Because we are planning to use MJ to simulate herbivory in our future studies, *JMT* is a good candidate for development as a biomarker.

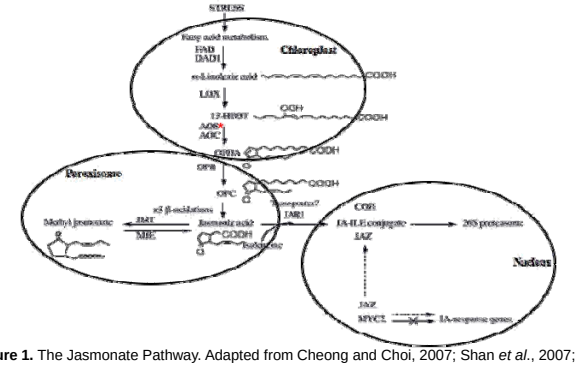


Figure 1. The Jasmonate Pathway. Adapted from Cheong and Choi, 2007; Shan *et al.*, 2007; Thines, 2007; and Turner, 2007. * = First committed step in pathway. 13-HPOT, 13-hydroperoxy-α-linolenic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1, an F-box protein; DAD1, defective anther dehiscence; FAD, fatty acid desaturase; JA, jasmonic acid; JA-ILE, jasmonate-isoleucine; JAR1, jasmonate resistant-1; JAZ, jasmonate ZIM-domain; JMT, jasmonic acid carboxyl methyltransferase; LOX, lipoxygenase; MJE, methyl jasmonate esterase; MYC2, basic helix-loop-helix leucine zipper transcription factor; OPC, 12-oxophytoenoic acid; OPDA, 12-oxo-phytodienoic acid; OPR, OPDA-reductase.

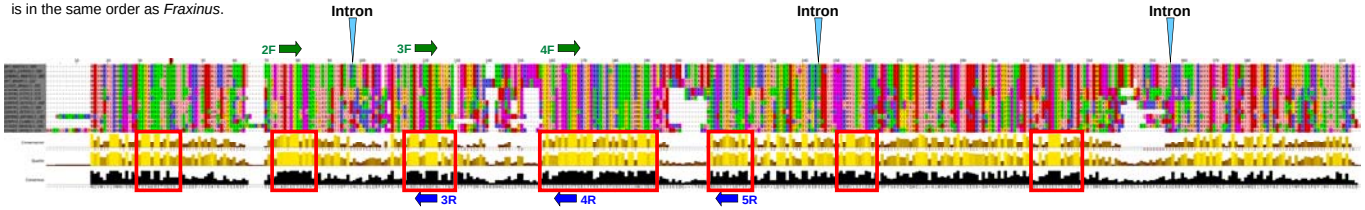
Methods

Very little sequence information for *Fraxinus* exists. While a few *Fraxinus* genes have been annotated in GenBank, none are genes known to be involved in any defense pathways. To find our potential biomarker, the *JMT* gene, in *Fraxinus*, the following steps have been or will be undertaken:

1. Found the *Arabidopsis JMT* gene's protein sequence in GenBank.
2. Used the *Arabidopsis* JMT protein sequence to BLAST GenBank for similar protein sequences in other organisms.
3. Aligned the top 15 most-similar protein sequences using the Jalview alignment program (Clamp *et al.*, 2004).
4. Identified the conserved regions of JMT across species.
5. Designed degenerative primers for the conserved regions of JMT for conducting the polymerase chain reaction (PCR).
6. Currently testing degenerative primers in *Fraxinus* and *Arabidopsis* tissues using touchdown PCR.

Results

Figure 2. Alignment and consensus sequence for the top 15 BLAST hits for *Arabidopsis* JMT protein sequence. Location of the introns based on the *Arabidopsis JMT* genomic sequence are as indicated. Amino acids are color-coded based on physicochemical properties (Table 1B). Red boxes indicate highly conserved regions of the protein sequence across species. Green and blue arrows represent regions used for forward and reverse degenerate primer design, respectively. The name of each primer is given near the corresponding arrow. Alignment includes *Arabidopsis* and two other mustards; rice; two members of the order Solanales, including *Petunia*; two plants from the order Gantianales, including *Stephanotis*; three grape sequences; one member each from the orders Laurales and Myrtales; and snapdragon, which is in the same order as *Fraxinus*.



Seven conserved regions were identified in the JMT protein sequence. Three forward and three reverse sets of degenerative primers were designed for use in PCR amplification. In addition, we are using a poly(T)$_{26}$ reverse primer. These primers are being tested in *Fraxinus* and *Arabidopsis* tissues in nine different combinations using touchdown PCR.

Table 1. The 20 amino acids, their single-letter codes, and the DNA codons representing each (**A**). Each of the 64 possible three-letter combinations of nucleotides encodes an amino acid or a stop codon. Note that often several codons may code for a single amino acid; for example, six different nucleotide combinations code for leucine. Due to this degeneracy of code, it is not possible to predict a single, specific DNA sequence from a protein sequence; instead, a number of possible DNA sequences might represent the same protein sequence. Color of each amino acid corresponds to its physicochemical properties (**B**); those with similar properties share a common color.

A

Second Position	T		C		A		G	
	Code	Amino Acid	Code	Amino Acid	Code	Amino Acid	Code	Amino Acid
T	TTT	Phenylalanine (F)	TCT	Serine (S)	TAT	Tyrosine (Y)	TGT	Cysteine (C)
	TTC		TCC		TAC		TGC	
	TTA	Leucine (L)	TCA		TAA	Stop	TGA	Stop
	TTG		TCG		TAG		TGG	Tryptophan (W)
C	CTT		CCT	Proline (P)	CAT	Histidine (H)	CGT	Arginine (R)
	CTC		CCC		CAC		CGC	
	CTA		CCA		CAA	Glutamine (Q)	CGA	
	CTG		CCG		CAG		CGG	
A	ATT	Isoleucine (I)	ACT	Threonine (T)	AAT	Asparagine (N)	AGT	Serine (S)
	ATC		ACC		AAC		AGC	
	ATA		ACA		AAA	Lysine (K)	AGA	Arginine (R)
	ATG	Methionine (M)	ACG		AAG		AGG	
G	GTT	Valine (V)	GCT	Alanine (A)	GAT	Aspartic Acid (D)	GGT	Glycine (G)
	GTC		GCC		GAC		GGC	
	GTA		GCA		GAA	Glutamic Acid (E)	GGA	
	GTG		GCG		GAG		GGG	

B

Physicochemical Property
- Hydrophobic
- Aromatic
- Basic
- Acidic
- Hydrophilic
- Special
- Cysteine

Amino acid sequence:	D	L	P	S	N	D	F
Possible nucleotide sequences:	GAT C	TTC C T A	CCT C T A G	AGC C T A G	AAT C	GAT C	TTT C
768 degeneracies =	2	x 6	x 4	x 6	x 2	x 2	x 2

After using hints for reducing degeneracy level:

48 degeneracies =	2	x 2	x 1	x 3	x 2	x 2	x 1

Final primer sequence:

5' GAY CTY CCI AGV AAY GAY TT- 3'

Figure 3. Example of degenerate forward primer design for the third conserved region of the JMT protein alignment (3F in Figure 2). Possible codon sequences for each amino acid in the seven amino acid-residue sequence are shown. A degenerate primer pool must contain 768 forward primers to cover all possible sequence combinations for this short amino acid sequence. Using hints for decreasing degeneracy level (*e.g.*, using a *Fraxinus* codon bias table), the level of degeneracy for this primer was reduced to only 48-fold, a level much more likely to result in PCR amplification. The 48-primer pool was ordered using the abbreviations given in the final primer sequence: "Y" = T or C; "I" = inosine, a neutral base that pairs with A, G, T, or C; and "V" = A, C, or G.

Discussion

So far, we have been unsuccessful in amplifying gene products from either *Fraxinus* or *Arabidopsis* genomic DNA or cDNA. This could be due to primer design, PCR conditions, or choice of tissue. Troubleshooting methods currently underway include checking primer design, using nested primers, and testing a variety of PCR conditions. We also plan to use the current primers in tissue from an organism that is more closely related to *Fraxinus* than is *Arabidopsis*; snapdragon would be a great example, as it is in the original protein alignment and the same order as *Fraxinus*. Finally, we may try designing primers for another MJ-responsive gene in the jasmonate pathway (for instance, *LOX* or *AOS*). The key is to identify a gene we can amplify and ultimately use as a biomarker; as long as it is MJ-responsive, it does not matter which gene we utilize.

References

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