A study of 3-deoxy-D-*arabino*-heptulosonate 7-phosophate (DAHP) synthase gene (*DHS*) expression in *Xanthium strumarium*, and a partial sequence

Lucy Rubino[1]

[1] Department of Biological Sciences, Fordham University, 441 E. Fordham Rd. Bronx, NY 10458

ABSTRACT

Besides playing an important role in primary metabolism, aromatic amino acids are precursors to many plant defense compounds, such as lignin and alkaloids. 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase is the enzyme that catalyzes the first step of the shikimate pathway which is responsible for the biosynthesis of aromatic amino acids. The gene that encodes this enzyme, *DHS* (specifically *DHS1*), has been shown to be induced by treatment with exogenous jasmonic acid (JA: a defense signaling compound in plants) in *Arabidopsis thaliana*. In this study, *Xanthium strumarium* leaves were treated with control and JA solutions for either 2 or 4 hours prior to extraction of total RNA. RT-PCR with *DHS*-specific and B-actin-specific primers was conducted on RNA samples and the products detected were visualized on 1% agarose gels. Although this study only presents the results of one gel, it appears that JA treatment for four hours induces *DHS* three-fold. The RT-PCR product generated was sequenced and was shown to have 73.5% homology with *A. thaliana DHS*.

Key Words: DAHP synthase; *Xanthium strumarium*; jasmonic acid; *DHS*

INTRODUCTION

To deter the constant onslaught of pathogens and insects, plants have evolved inducible defense systems that "turn on" only in the presence of an elicitor or threat. Jasmonic acid (JA) and its methyl ester (methyl jasmonate) have been implicated as molecules governing plant inducible defense signaling pathways using the following evidence: (1) Mutant plants that lack the ability to synthesize jasmonate are highly susceptible to attack by insect herbivores (McConn *et al.* 1997); (2) JA accumulates in wounded plants (Creelman and Mullet 1995) and in cell cultures treated with elicitors of pathogen defense (Gundlach *et al.* 1992); and (3) Secondary metabolites accumulate in the presence of exogenously supplied JA through *de novo* transcription of genes involved in chemical defense (Gundlach *et al.* 1992, Farmer and Ryan 1990, 1992). It is important to note that although this research focuses on the role of JA, other work has highlighted the importance of other members of the jasmonate family of compounds in inducible plant defenses (Reymond *et al.* 2000 and Stinzi *et al.*2001).

Some of the genes that are induced by exogenous JA application are involved in aromatic amino acid biosynthesis, specifically the shikimate pathway. The shikimate pathway is unique to plants and microorganisms. It is the pathway for biosynthesis of chorismate, which serves as a precursor to the three aromatic amino acids (tryptophan, tyrosine and phenylalanine) and several other compounds of primary metabolism (Herrmann and Weaver 1999). The aromatic amino acids, as well as intermediates of the shikimate pathway, also serve as starting points for synthesis of products of secondary metabolism such as, gallic acid (precursor to defensive hydrolysable tannins) (Ossipov *et al.* 2003), lignin (physical support and water relations) , anthocyanic pigments, auxin

(growth control) and antimicrobial phytoalexins (resitance to infections), among others (Keith *et al.* 1991).

3-deoxy-D-*arabino*-heptulosonate 7-phosophate (DAHP) synthase catalyzes the condensation of phosphoenol pyruvate and erythrose 4-phosphate into 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (Herrmann and Weaver 1999) (Fig. 1). It is also the first enzyme of the shikimate pathway and therefore plays a crucial role in the biosynthesis of the ultimate products of this pathway: proteins and secondary metabolites (Herrmann 1995a). It is also important because the reaction it catalyzes is rate-limiting and it plays a major regulatory role in the pathway (Herrmann 1995b).

The amino acid sequence of plant DAHP synthases show little homology to their microbial counterparts (Keith *et al.* 1991). Therefore it is not surprising that they are regulated differently. The bacterial DAHP synthase genes studied thus far are feedback-inhibited by phenylalanine, tryptophan or tyrosine; whereas plant DAHP synthases are not inhibited by the aromatic amino acids (Herrmann and Weaver 1999). Because no physiological inhibitors of plant DAHP synthases have been identified thus far, Herrmann and Weaver (1999) point out that regulation in plants may occur exclusively at the gene expression level.

In *A. thaliana*, DAHP synthase can be produced from two distinct genes (*DHS1* and *DHS2*) with different induction characteristics (Keith *et al.* 1991). *DHS1* transcripts have been shown to increase in response to wounding (Keith *et al.* 1991 and McConn *et al.* 1997), pathogenic attack (Keith *et al.* 1991 and Henstrand 1992), light (Henstrand 1992) and application of exogenous jasmonate (McConn *et al.* 1997). *DHS2* transcripts have been shown to remain stable or slightly decline in response to wounding (Keith *et*

al. 1991). Deduced protein sequences of *DHS1* and *DHS2* transcripts in *Arabidopsis thaliana* are similar except in their N-terminal regions (Keith *et al.* 1991). Immunological and enzyme studies have shown DAHP synthase to be induced by wounding in the Solanaceae (Dyer et al. 1989) and by a fungal elicitor and light in parsley (*Petroselinum crispum*) cell cultures (McCue and Conn 1990).

Xanthium strumarium (common cocklebur) is a widespread weed that is of economic importance because it competes with soybean and peanut crops (Tranel 2001). This study specifically examines the levels of expression of the *DHS* suite of genes in *X. strumarium* treated with JA and control solutions for varying lengths of time. Primers were not designed to differentiate between the genes within the *DHS* suite because there is no sequence information for these genes in *X. strumarium*. Primer sequences were based on the region of an *A. thaliana* sequence of the *DHS1* gene that exhibited the highest homology across multiple plant species. I hypothesized that the JA treatment would induce levels of the *DHS* transcripts, and that induction would be higher at the 2 than the 4 hour treatment.

MATERIALS AND METHODS

Plant material and jasmonic acid treatment

Xanthium strumarium, common cocklebur, seeds were collected from one parent and planted in a greenhouse with a temperature control system set to average around 25˚C. After germination, plants were watered every three days with deionized water and were grown under natural light until the day of the experiment. Plants chosen for the experiment were at least three weeks old and had a minimum of five true leaves.

Individual leaves were treated with either control (0.1% aqueous solution of Triton-X 100) or jasmonic acid (0.01% jasmonic acid and 0.1% Triton X-100) solutions for either 2 or 4 hours before leaves were harvested. Immediately after harvesting, approximately 50 mg sub-samples of individual leaves were cut and then flash frozen in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kits (Qiagen). RNA was eluted uisng 40 µL warm RNase-free water. Each sample of extracted RNA was run on an agarose gel to confirm the presence of rRNA bands and compare relative quantities of RNA.

Quantification of mRNA transcripts

Multiple pairs of primers were designed based on *Arabidopsis thaliana DHS1* mRNA sequence (NM 120162; gi: 30692575). Since *X. strumarium* is not closely related to *A. thaliana*, primers were designed in regions of the *DHS1* gene that exhibited relatively high homology across multiple plant species including *Lactuca sativa* (lettuce), *Morinda citrifolia* (morinda), and *Fagus sylvatica* (European beech). Of the plant species that have a recorded sequence for the *DHS* gene, lettuce is the most closely related to *X. strumarium* because it belongs to the same family, Asteraceae.

The primers that consistently gave the expected RT-PCR products were Ex3F2.1(5'CTGGTCTCTACTATGATTG3') and Ex4R2.1 (5'GGTGTTTCCATGCATTGGATC3') and these were used in all subsequent experiments. These primers span a 335 bp intron that lies between exons three and four in the *A. thaliana* sequence of the *DHS1* gene. The expected RT-PCR product is approximately 220 base pairs in length. B-actin primers were used as a control gene for comparison.

Reverse transcription polymerase chain reaction was performed using a OneStep RT-PCR Kit (Qiagen). Each reaction contained a total volume of 15 µL consisting of 3 µL 5x RT buffer, 0.6 µL dNTP, 0.6 µL enzyme mix, 0.75 µL forward primer (10 pmol), 0.75 µL reverse primer (10 pmol), 1.5 µL extracted RNA (estimated concentration of 13 ng/µL) and 7.8 µL dH_2O, plus a 12 µL layer of mineral oil. The pre-cycle reaction temperatures were 50˚C for 30' and 95˚C for 15'. This was followed by either 30 (B-actin) or 47 (*DHS*) cycles of 94˚C for 20"; 56˚C for 30"; and 72˚C for 30". After the cycles there was a final extension at 72˚C for 7' and then samples were stored at 4˚C until they were run on a gel. A 6 µL sample of each reaction was run on a 1% agarose gel and band intensity was analyzed using Quantity One (v 4.2.2 Bio Rad) software.

Sequencing cDNA

Sequencing was conducted directly on pooled RT-PCR products that had been shown to contain the expected band. An AmpliCycle Sequencing Kit (Perkin Elmer) was used to sequence the RT-PCR product by a modified Sanger Dideoxy sequencing method. Sequence reactions had a total volume of 8 µL and contained 0.8 µL cycling mix, approximately 10 fmol template, 0.1 µL α33-ATP, either forward or reverse primer, dH_2O, and 2 µL respective dideoxynucleotide; with a top layer of 12 µL of mineral oil. The PCR reaction consisted of a 94ºC hold for 2′ followed by 35 cycles of 94ºC for 30″; 58ºC for 30″; and 72ºC for 1′. The samples were stored at 4ºC until analyzed on a sequencing gel. The same primers that were used in RT-PCR (Ex3F2.1 and Ex4R2.1) were used in the sequencing reaction. The forward and reverse sequences were united to give a complete sequence of the 219 bp RT-PCR product. A BLAST search identified

genes with homology to this sequence and alignments were conducted using Clustal-W MacVector (Accelrys Inc).

RESULTS

DHS primers designed using an *A. thaliana* mRNA sequence produced a band of the expected size (220 bp) for RNA (as opposed to genomic DNA which would have produced a band at 555 bp) (Fig. 2). B-actin primers were also effective in producing a band of predicted length for B-actin mRNA. Band intensities from the 1% agarose gel were analyzed and B-actin levels were not found to be constant across all four treatments (Table 1). Because of this discrepancy, band intensities of the *DHS* gene product needed to be adjusted to reflect the differences in total mRNA. When control treatments were set to 100%, it was seen that the *DHS* gene product was induced three-fold at the 4 hour exposure, but was not induced appreciably at the 2 hour exposure (Fig. 3).

The sequence of the 220 bp RT-PCR product produced by the *DHS* primers was compared to the NCBI database of nucleotide sequences using a BLAST search. The sequence exhibited the highest homology with other plant DAHP synthase genes. In the region of the *DHS* gene sequenced, the highest level of homology (75.3%) was illustrated in the comparison to the *DHS2* gene of European beech (*Fagus sylvatica*), followed by 73.5% homology with *DHS* in *A. thaliana,* and 71% homology with the *DHS1* gene of lettuce (*Lactuca sativa*). A Clustal-W alignment of the sequenced region of *X. strumarium DHS* with the *DHS* genes of the three plant species mentioned above can be seen in Figure 4.

DISCUSSION

In agreement with other studies (Farmer and Ryan 1990, 1992, Gundlach *et al.* 1992, and McConn *et al.* 1997), I have illustrated that JA can induce the mRNA transcript of a protein that is involved in plant defense. It is somewhat surprising, however, that an appreciable induction of the transcript was only observed in the 4 hour exposure. Keith *et al.* (1991) found that *DHS1* mRNA transcript levels in *A. thaliana* increased about 3- to 5-fold reaching a maximum at 1.5 hours after wounding. It is possible that the effect of wounding on *DHS* transcripts reaches a peak induction earlier than JA treatment does. When a plant is treated with JA, the compound needs to pass through the cuticle of the leaf (or enter in a gaseous form through the stomata) and this may take more time to produce an effect than the immediate impact of wounding.

The sequence of the 219 bp *DHS* RT-PCR product was found to have the highest homology with other plant *DHS* genes, which supports the conclusion that the *DHS* bands in Figure 2 are indeed *DHS* mRNA bands. Initially, I hypothesized that the *X. strumarium,* sequence would share the highest homology with the most closely related species, *L. sativa* (lettuce). However, this was not the case. The highest homology was exhibited in the comparison with European beech (75.3%). It is possible that if the entire *X, strumarium DHS* gene were sequenced, it would show the highest homology with lettuce. It is difficult to draw conclusions based only on a single partial sequence.

It is also interesting to note that of the three genes that most closely matched the *X. strumarium* partial sequence, one is *DHS1,* one is simply called *DHS*, and the other is *DHS2*. It is likely then, that the sequence I have generated represents both *DHS1* and *DHS2* transcripts in *X. strumarium*, if they both exist in this plant. If the bands generated

in Figure 2 represent both *DHS1* and *DHS2* transcripts, it could obscure the true pattern of induction and explain why no induction was observed at the 2 hour treatment. Further research must be done to be able to design primers that can distinguish between the two transcripts in *X. strumarium* before the induction patterns of each can be analyzed separately. However, it is interesting that even without being able to distinguish between the two transcripts, an induction pattern was still observed.

It appears that each RNA sample contained varying levels of mRNA because of the variable intensities (Table 1) of the B-actin bands in Figure 2. Future work will focus on methodology to generate more consistent levels of mRNA across all treatments. Because the induction conclusion is based on a single gel (Fig. 2), the results must be repeated to see if there is a statistically significant difference in expression of the *DHS* gene in control and JA-treated plants. I also hope to clone the RT-PCR product and sequence again to confirm current sequence results.

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TABLE

Table 1 Intensity of bands from gel depicted in Fig. 1 as quantified using a densitometric analysis of the image using QuantityOne software package (v4.2.2 Bio Rad).

gene	treatment	exposure (hrs)	intensity
B-actin	C	2	61.06
B-actin	J	2	115.01
B-actin	C	4	93.83
B-actin	J	4	76.55
DHS	C	2	42.52
DHS	J	2	97.12
DHS	C	4	45.09
DHS	J	4	119.44

FIGURES

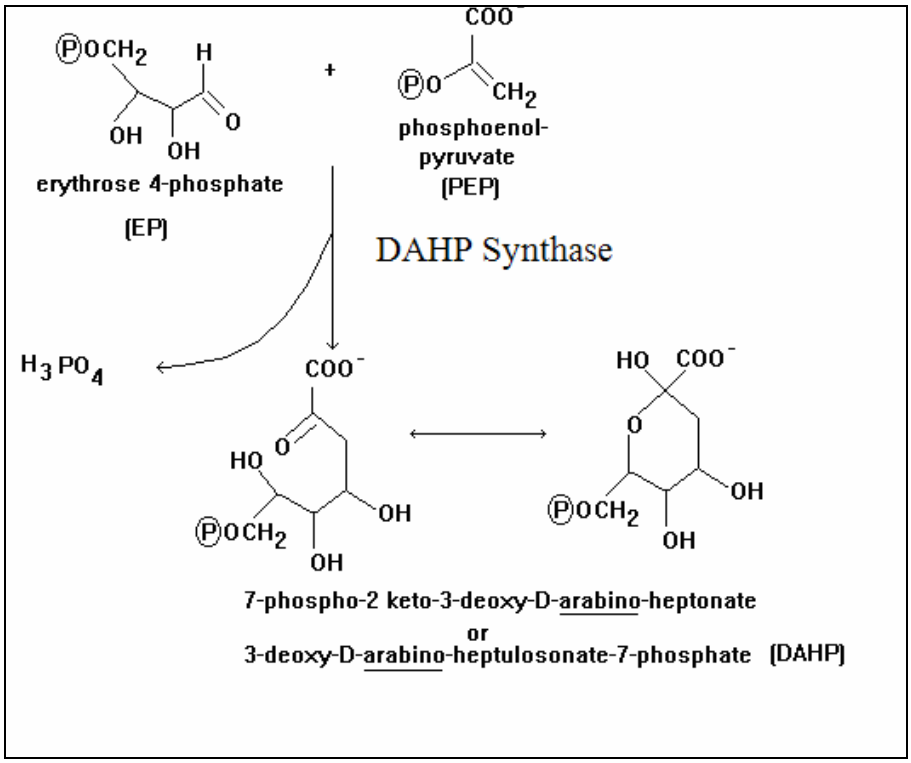

Figure 1 The enzyme encoded by the *DHS* gene, DAHP synthase, catalyzes the first step of the shikimate pathway (aromatic amino acid biosynthesis) as depicted above.

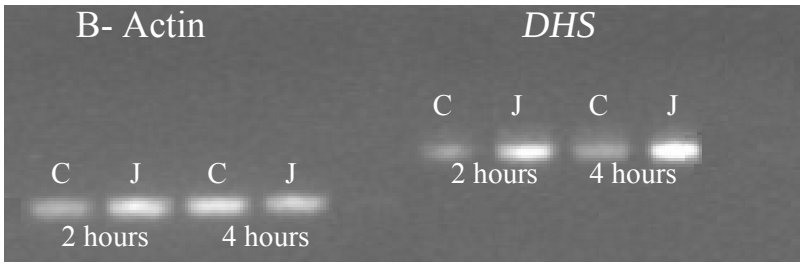

Figure 2 RT-PCR products for B-actin primers and *DHS* primers. B-actin samples were subjected to 30 PCR cycles and *DHS* samples were subjected to 47. Individual leaves on *X. strumarium* plants were exposed to either **C** (control: 0.1% aqueous solution of Triton-X 100) or **J** (jasmonic acid: aqueous solution of 0.01% jasmonic acid and 0.1% Triton X-100) treatments for either 2 or 4 hours prior to RNA extraction.

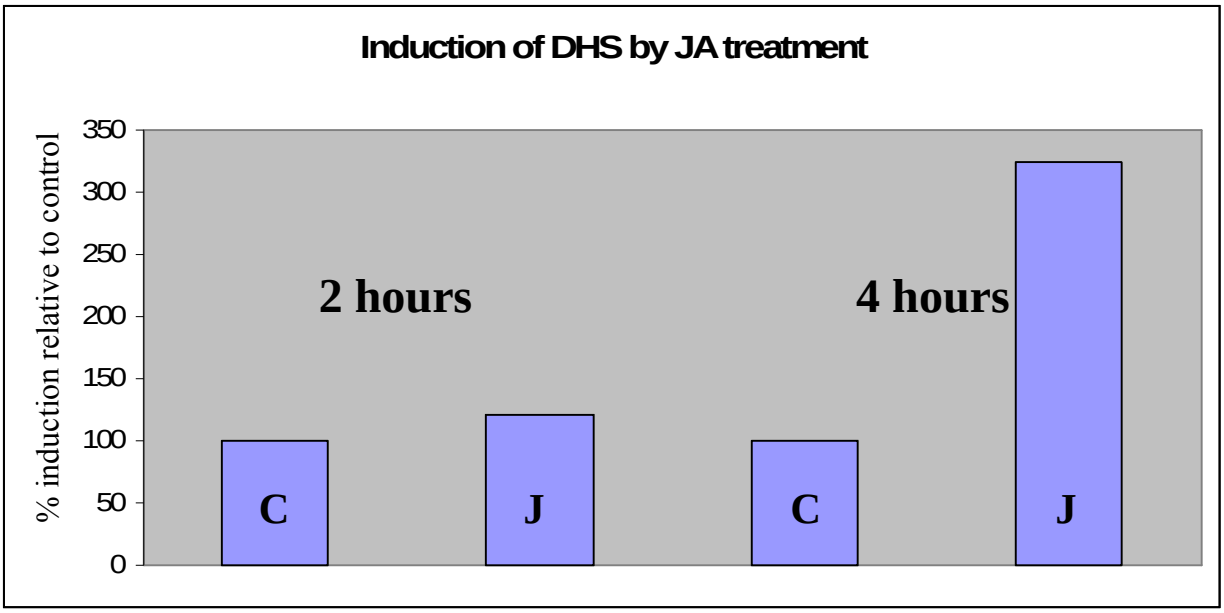

Figure 3 Relative percent induction of the *DHS* transcript relative to the control treatment. Intensities were adjusted to account for differences in B-actin levels for each treatment. **C** = control treatment and **J =** jasmonic acid treatment. Control transcript levels were set to 100% and the relative induction due to JA treatment was calculated.

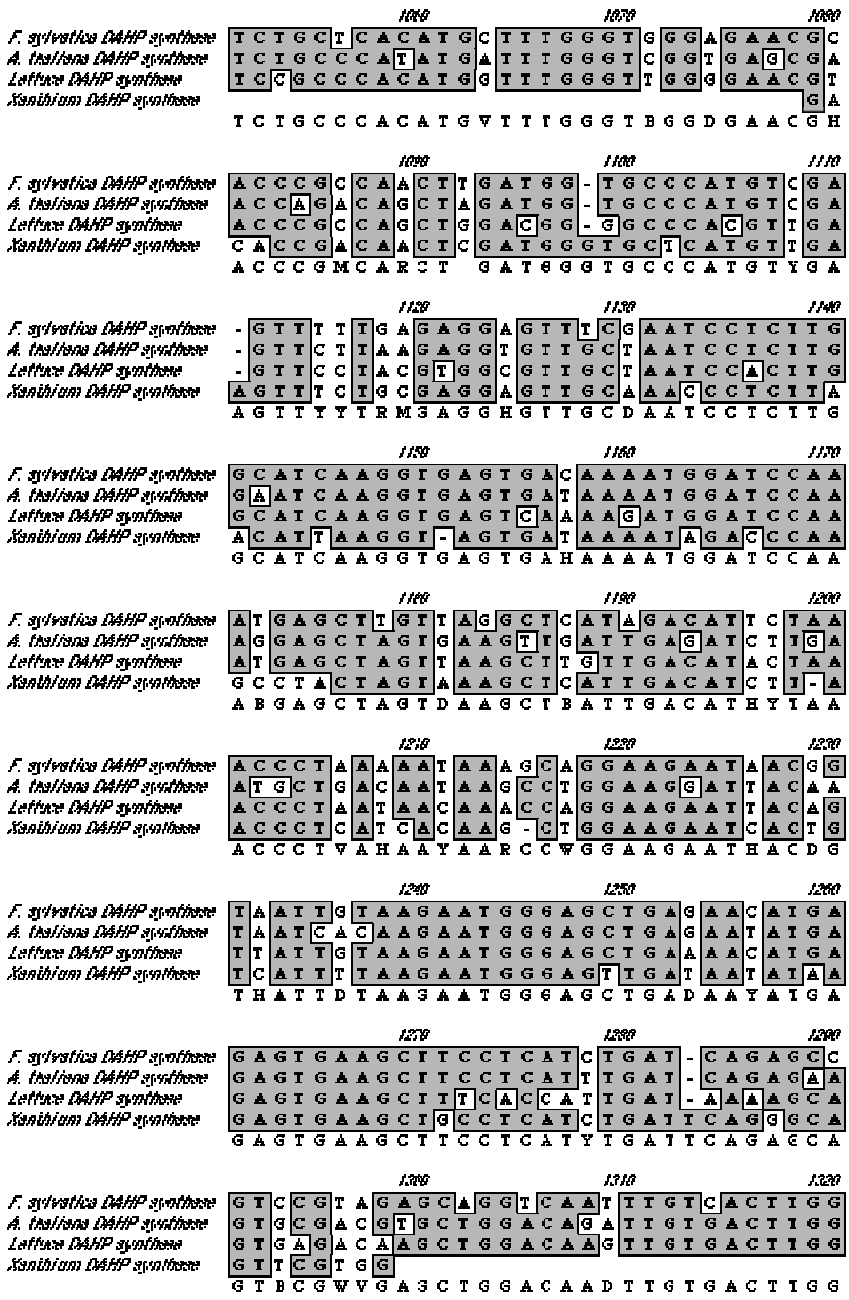

Figure 4 Clustal-W alignment of RT-PCR product (*Xanthium* DAHP synthase) with three other sequences of the *DHS* gene from three different plant species.