Differential induction of 3-deoxy-d-arabino-heptulosonate 7-phosphate synthase genes in Arabidopsis thaliana by wounding and pathogenic attack

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ABSTRACT We have isolated cDNAs from two distinct genes encoding 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) in Arabidopsis thaliana. Predicted protein sequences from both genes, DHS1 and DHS2, and a potato DAHP synthase gene are highly related, but none show significant sequence similarity to conserved microbial DAHP synthase proteins. Despite this structural difference, the DHS1 cDNA complements mutations in a yeast strain lacking DAHP synthase activity. DHS1 RNA levels increase in Arabidopsis leaves subjected either to physical wounding or to infiltration with pathogenic Pseudomonas syringae strains. DHS2 RNA levels are not increased by these treatments, suggesting that the DHS1 and DHS2 proteins fulfill different physiological functions. Other enzymes in the Arabidopsis aromatic pathway are also encoded by duplicated genes, an arrangement that may allow independent regulation of aromatic amino acid biosynthesis by distinct physiological requirements such as protein synthesis and secondary metabolism. The presence of amino-terminal extensions characteristic of chloroplast transit peptides on DHS1 and DHS2 suggests that both proteins may be targeted to the chloroplast.

The regulation of aromatic amino acid biosynthesis in plants must accommodate the requirements of both protein synthesis and secondary metabolism. Aromatic amino acids are precursors to a diverse array of plant secondary metabolites, including lignin, anthocyanic pigments, auxin, and antimicrobial phytoalexins (1, 2). These compounds are involved in many processes, including growth control (auxin), physical support and water relations (ligandin), and resistance to infection (phytoalexins), and their synthesis appears to be developmentally and environmentally regulated. The functional diversity of secondary metabolites suggests that the synthesis of each may be under separate regulatory control. The biosynthesis of aromatic amino acids in plants may, in turn, require complex regulatory controls that differ fundamentally from those described for bacteria and fungi.

This report describes the structure and expression of two Arabidopsis thaliana genes encoding 3-deoxy-d-arabino-heptulosonate 7-phosphate (DAHP) synthase (7-phospho-2-dehydro-3-deoxy-d-arabino-heptose-4-phosphate-lyase [pyruvate-phosphorylating]; EC 4.1.2.15), the enzyme catalyzing the first committed step in aromatic amino acid biosynthesis. These genes may have distinct physiological roles, as they are differentially expressed in plants subjected either to physical wounding or to infiltration by virulent and avirulent strains of Pseudomonas syringae. Genes encoding other enzymes in the Arabidopsis aromatic pathway are also duplicated (refs. 3 and 4; K. Niyogi and G.R.F., unpublished work). We suggest that duplication of the genes encoding these enzymes, and possibly all enzymes in the pathway, provides a mechanism to permit independent regulation of aromatic amino acid biosynthesis by different physiological demands, such as protein synthesis and secondary metabolism. The presence of sequences encoding putative chloroplast transit peptides on all Arabidopsis aromatic-pathway genes analyzed to date suggests that differentially regulated aromatic amino acid biosynthesis pathways may exist within the chloroplast.

MATERIAL AND METHODS

Isolation of Arabidopsis DAHP Synthase cDNA Clones. Clones were isolated from a cDNA library (5) by hybridization to a labeled fragment of a potato DAHP synthase gene (6). The probe was prepared by PCR amplification of potato genomic DNA using oligonucleotide primers corresponding to coding-strand nucleotides 16–36 (5′-GCAATGGCTCTTTCAGTACT-3′) and to noncoding-strand nucleotides 903–882 (5′-ACCAAGGGCCTCATCACCTCATC-3′) of AB216 (6). Filters containing phage plaques were hybridized at 58°C in 5× standard saline/phosphate/EDTA (SSPE) and washed at 50°C in 1× standard saline citrate (SSC). cDNA inserts from positive clones were subcloned into pHBluescript KS(−) (Stratagene) and sequences of nested deletions were determined (7). Analyses of DAHP synthase sequences from Escherichia coli [aroF (8), aroG (9), and aroH (10)], Saccharomyces cerevisiae [AR03 (11)], potato (6), and Arabidopsis were performed using the University of Wisconsin Genetics Computer Group programs BESTFIT, IALIGN (12), and MALI (13). The sequence similarity values shown in Table 1 were obtained using the program BESTFIT (12), and the alignment shown in Fig. 1 was made using the program MALI (13).

Blot Hybridization of Arabidopsis DNA and RNA. Arabidopsis genomic DNA was digested with restriction enzymes (New England Biolabs), electrophoresed in a 0.7% agarose gel, and blotted to nitrocellulose membranes. Hybridization probes were prepared by random oligonucleotide labeling (Prime Time kit, IBI) of full-length DHS1 and DHS2 cDNAs. Blots were hybridized in 5× SSPE/5× Denhardt’s solution/0.1% SDS at 68°C and washed in 0.1× SSC/0.1% SDS at 65°C (14). Total RNA was isolated from Arabidopsis plants by miniprep procedures (15, 16). RNA was electrophoresed in formaldehyde/1% agarose gels (14) and blotted to nitrocellulose or GeneScreen (New England Nuclear) membranes. Hybridization was performed in either 50% formamide/5× SSPE/0.1% SDS/5× Denhardt’s solution at 42°C (14) or 0.5 M Na2HPO4, pH 7.2/7% SDS/1% bovine serum albumin at 60°C (17). Blots were washed in 0.2× SSC/0.1% SDS at 55°C. Autoradiographs were scanned with a computing densitometer (Mo...
molecular Dynamics, Sunnyvale, CA) to obtain approximate values for changes in transcript levels.

**Complementation of Yeast DAHP Synthase Mutation.** *S. cerevisiae* strain YB7K (MATα, aro3-2, aro4-1, ural3-1, Gal+) was transformed with plasmids as described (18).

**Wounding and Bacterial Infiltration of Arabidopsis Plants.** Three-week-old plants were wounded by two methods: (i) rosette leaves were diced with scissors and kept in a darkened humidified chamber or (ii) leaves were scored with a metal file and left attached to the plant. Both methods produced the same patterns of DAHP synthase gene expression. Bacterial infiltration of *Arabidopsis* plants was achieved as described (19).

**RESULTS**

**Structure of Arabidopsis DAHP Synthase Genes.** *Arabidopsis* DAHP synthase cDNA clones *DHS1* and *DHS2* were identified by cross-hybridization to a potato DAHP synthase gene fragment (6). As shown in Fig. 1 and Table 1, protein sequences deduced from *DHS1*, *DHS2*, and potato DAHP synthase cDNAs are largely identical, except in their amino-terminal portions. These divergent regions are characteristic of chloroplast transit peptides; they are rich in serine, threonine, and proline residues and carry a net positive charge (20). Southern blot analysis of *Arabidopsis* genomic DNA hybridized to *DHS1* and *DHS2* cDNA probes revealed cross-hybridization between *Arabidopsis* DHS genes (Fig. 2A). Neither probe hybridized consistently to other genomic fragments under low-stringency conditions (data not shown). Restriction fragment length polymorphism mapping revealed that *DHS1* and *DHS2* genes are linked, separated by ≈27 centimorgans; these data extend the existing map of chromosome 4 (Fig. 2B).

The polypeptide sequences deduced from the *Arabidopsis* and potato DAHP synthase genes display a high degree of similarity, but none of these genes shows significant sequence homology to the highly conserved DAHP synthase protein sequences of *E. coli* or *S. cerevisiae* (Table 1). Attempts to identify *Arabidopsis* genes homologous to microbial DAHP synthase genes by low-stringency hybridization and PCR techniques (22) were not successful.

**Complementation of Yeast DAHP Synthase Mutations.** To demonstrate that the *Arabidopsis* genes encode functional DAHP synthase enzymes, we used the *DHS1* cDNA to complement mutations in yeast strain YB7K. YB7K is incapable of growth on synthetic complete medium lacking phenylalanine, tryptophan, and tyrosine, because mutations of the two yeast DAHP synthase genes, *ARO3* and *ARO4*. The full-length *DHS1* cDNA insert was placed under the control of the inducible GAL1 promoter in the yeast expression vector pDAD2 and transformed into YB7K. Complementation of the *aro3* and *aro4* mutations in YB7K by the GAL-DHS1 construct is expected to result in growth on medium containing galactose but lacking aromatic amino acids (Gal, -Aro). Initially, however, transformants containing the GAL-DHS1 construct did not display complementation. We observed complementation by the DHS1 cDNA in segregants of the original transformants after subsequent selections. Individual transformants carrying the various pDAD2-derived plasmids were grown under uracil selection in liquid galactose medium to induce the GAL1 promoter, and 10^6 cells were plated on galactose medium lacking uracil and aromatic amino acids. No growth was observed on plates containing cells carrying the pDAD2 vector alone or the GAL-DHS1 (antisense) construct. However, 100–200 slowly growing colonies appeared when cells containing the GAL-DHS1 construct were plated. Growth was due to the expression of the *DHS1* cDNA, as the same segregants were unable to grow on -Aro medium containing glucose, which represses *GAL1* promoter activity (Fig. 3).

Subsequent tests indicated that these Aro+ cells were segregants expressing sufficient *Arabidopsis* DAHP synthase activity to complement the yeast *aro3* and *aro4* mutations. First, segregants that lost the GAL-DHS1 plasmid became Aro– on galactose. Second, the Aro+ phenotype was not a consequence of chromosomal mutation in the yeast strain, because segregants that lost the plasmid (Ura+ , Aro–) again produced Aro+ revertants at a frequency of roughly 10–6 when retransformed with the GAL-DHS1 construct. Third, the Aro+ phenotype did not result from plasmid rearrangements, because plasmids isolated from the Aro+ segregants had the same properties as the original plasmid. The simplest explanation of our observations is that the Aro+ segregants arose by increased plasmid copy number. In agreement with this explanation, the Aro+ segregants were extremely unstable under nonselective conditions, segregating a high proportion of Ura+, Aro– cells. We assume that these Aro+ segregants, which still retained the plasmid, simply had lowered copy number.

**DHS1 and DHS2 Are Expressed Differently in Response to Wounding.** Intact *Arabidopsis* leaves contain approximately equal steady-state levels of the cDNA encoded by *DHS1* and *DHS2* RNAs (Fig. 4A). After wounding, however, DHS1 RNA levels increased about 3- to 5-fold, reaching a maximum at ≈1.5 hr. The level of DHS2 RNA appeared to decline during the same time period. The level of transcripts encoding phenylalanine-
monia lyase (PAL), the first enzyme in the phenylpropanoid pathway, also increased about 2- to 3-fold after wounding. The difference between our results and those of Ohl et al. (26), who observed induction of PAL RNA 1 hr after wounding Arabidopsis plants, may be due to differences in the manner and severity of the wounding procedure. Not all amino acid biosynthetic pathways respond to wounding, because transcripts from ALS, the single-copy Arabidopsis gene encoding acetolactate synthase (25), an enzyme in the isoleucine–valine pathway, showed the same pattern of expression as DHS2 (Fig. 4A). The decline of DHS2 and ALS RNA levels reflects general RNA degradation in wounded tissues. By 4 hr after wounding, the RNA samples were obviously degraded, as evidenced by increased mobility of hybridizing RNA in all samples. Induction of DAHP synthase RNA levels in wounded tissue has been observed in potato (27), although the number of potato DAHP synthase genes and their individual expression patterns have not been described.

**Induction of DHS1 RNA Levels by Pathogenic Attack.** DHS1 RNA levels were also induced about 3-fold in Arabidopsis plants challenged with bacterial pathogens. The virulent strain _Pseudomonas syringae pv. maculicola_ ES4326 (Psm ES4526) multiplies 10^4- to 10^5-fold within 96 hr in infiltrated Arabidopsis leaves, producing chlorotic lesions (19). In contrast, the avirulent strain _P. syringae pv. tomato_ 1065 (Pst MM1065) does not multiply in infiltrated Arabidopsis leaves. Another avirulent strain, ES4326/pMMXR1, was created by transfer of a cloned avirulence (avr) gene from strain Pst MM1065 into strain Psm ES4526 (19). This strain induces a hypersensitive response in infiltrated Arabidopsis leaves (19). DHS1 RNA levels increased 3-fold 6 hr after Arabidopsis plants were infiltrated with the avirulent strains Pst MM1065 or ES4326/pMMXR1 (Fig. 4B), whereas plants infiltrated with the virulent strain Psm ES4326 showed an increase 24 hr after infection. DHS2 RNA levels did not show this induction following bacterial infiltration. Mock-infected plants showed no significant alteration of DHS1 or DHS2 RNA levels. Again, ALS transcripts were unaffected by these treatments (data not shown).

**DISCUSSION**

The predicted protein sequences from the _Arabidopsis_ DAHP synthase cDNAs are markedly different from their microbial counterparts. Nevertheless, the _Arabidopsis_ DHS1 cDNA complements mutations in _yeast_ strains _aro3_ and _aro4_ genes. As complementation was observed only in segregants and not in the primary transformants, it is unlikely that these

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**Table 1. Plant and microbial DAHP synthase amino acid sequence similarity**

<table>
<thead>
<tr>
<th><em>Arabidopsis</em></th>
<th><em>Potato</em></th>
<th><em>E. coli</em></th>
<th><em>Yeast</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>DHS1</td>
<td>ABD2</td>
<td>AroF</td>
<td>AroH</td>
</tr>
<tr>
<td>85 (76)</td>
<td>82 (73)</td>
<td>35 (13)</td>
<td>31 (11)</td>
</tr>
<tr>
<td>DHS2</td>
<td>86 (79)</td>
<td>35 (12)</td>
<td>35 (13)</td>
</tr>
<tr>
<td>ABD21</td>
<td>37 (14)</td>
<td>32 (11)</td>
<td>30 (12)</td>
</tr>
<tr>
<td>AroF</td>
<td>71 (53)</td>
<td>67 (46)</td>
<td>71 (49)</td>
</tr>
<tr>
<td>AroG</td>
<td></td>
<td>72 (56)</td>
<td>72 (55)</td>
</tr>
<tr>
<td>AroH</td>
<td></td>
<td>63 (43)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses represent percent similarity; numbers in parentheses represent percent identity (see Materials and Methods).

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**Fig. 2.** (A) Southern blot analysis of _Arabidopsis_ genomic DNA hybridized to _DHS1_ and _DHS2_ cDNA probes. Size markers are in base pairs. (B) Schematic representation of the _Arabidopsis_ fourth chromosome, showing the positions of _DHS1_ and _DHS2_ in relation to other genetic markers. Symbols to the left of the vertical line represent mapped restriction fragment length polymorphisms and visible gene markers, numbers to the right represent map distances in centimorgans (21).

**Fig. 3.** Complementation of yeast strain _YBK7_ (MATa, _aro3-2, aro1-1, ura3-1, GAL*). (A) Growth of _YBK7_ transformed with plasmids on synthetic complete medium lacking phenylalanine, tryptophan, tyrosine, and uracil, supplemented with galactose (Gal, – _Aro_). The expression plasmid pDAD2 was created by introducing a polylinker and the _PHO5_ terminator downstream of the _GAL1_ promoter in pCGS109 (a gift of J. Scham and J. Mao, Collaborative Research) (D. Miller, D. Pellman, and G.R.F., unpublished data). _GAL-DHS1_ and _GAL-DHS1_ constructs were created by cloning the 1.9-kilobase-pair _EcoRI_ fragment containing the entire _DHS1_ cDNA in the forward and reverse orientation, respectively, downstream of the _GAL1_ promoter. _Aro*_ segregants of _YBK7_ carrying the GAL-DHS1 construct were isolated as described in the text and three independent clones were restreaked on the plates shown here. As a positive control, a 1.7-kilobase-pair _HindIII–XbaI_ fragment containing the _aro3_ gene (23), under the control of its own promoter, was cloned into plasmid YEP352 (24) and transformed into _YBK7_. (B) Growth of the same transformed _YBK7_ strains on selective medium supplemented with glucose (Glc, – _Aro_) instead of galactose.
cDNAs would have been identified by direct complementation from a transformed cDNA library. The poor complementing activity of the GAL-DHS1 construct may be due to inhibitory effects of the chloroplast transit-peptide sequence on expression of particular plant cDNAs in microbial backgrounds (28).

The different primary structures of plant and microbial DAHP synthase proteins may reflect differences in metabolic regulation. DAHP synthase isozymes from E. coli and Salmonella typhimurium (29), Neurospora crassa (30), and S. cerevisiae (31) are each specifically feedback-inhibited by phenylalanine, tryptophan, or tyrosine. In contrast, DAHP synthases from plant species do not appear to be feedback-inhibited by these amino acids (32, 33). Activity of plant DAHP synthase enzymes may be regulated instead by intermediates in pathways leading from the free aromatic amino acid to auxins, phenolics, and other secondary metabolites.

Defense responses to wounding and pathogenic attack involve the induction of PAL and other enzymes specific to lignin synthesis and secondary metabolism (34). Consequently, coordinate induction of aromatic amino acid biosynthesis may be required to supply aromatic precursors for the synthesis of defensive secondary metabolites. The relatively early induction of DHS1 and PAL expression (19) by avirulent Pseudomonas strains may be critical for the timely production of compounds necessary to contain bacterial proliferation. In Arabidopsis and other Brassicaceae, these compounds may include sulfur-containing aromatic glucosinolates believed to function as part of a general defense system (35, 36). The belated increase in DHS1 RNA levels in response to infiltration with the virulent Psm ES4326 strain may reflect a delayed recognition process or stress caused by disease progression. Induction of only one of the two Arabidopsis DAHP synthase genes by either wounding or bacterial infection indicates a clear difference in the regulation of the two genes and implies DHS1 as the major isozyme responding to changes in secondary metabolite biosynthesis.

Genes encoding four Arabidopsis aromatic amino acid biosynthetic enzymes have now been cloned: DAHP synthase, tryptophan synthase B (3), 3-enol-pyruvylshikimate-5-phosphate synthase (4), and anthranilate synthase (K. Niyogi and G.R.F., unpublished work); each is duplicated. Duplication of the entire Arabidopsis aromatic pathway at the genetic and enzymatic level may allow independent regulation of aromatic amino acid synthesis in response to distinct physiological requirements such as secondary metabolism and protein synthesis. Previous suggestions of duplicated aromatic pathways in plants (37) were based on biochemical separation of two pathway enzyme activities, DAHP synthase and chorismate mutase, into chloroplastic and cytosolic isofoms displaying different regulatory properties (33, 38, 39). While plastids appear to contain all the enzymes required for aromatic amino acid biosynthesis (37), the absence of cytosolic activities corresponding to other pathway enzymes argues against the existence of a complete aromatic pathway in the cytosol (40, 41). In parsley, chloroplas-associated DAHP synthase activity is induced when cells are treated with fungal elicitors, while the cytosol-associated activity remains unchanged (42). By analogy, our expression data predict that DHS1 corresponds to the chloroplastic form and DHS2 to the cytosolic. However, the putative chloroplast transit peptides on both DHS1 and DHS2 suggest that if Arabidopsis has a cytosolic DAHP synthase isozyme, it must either be encoded by a distinct gene or result from an alteration of the DHS1 or DHS2 protein. We have not detected additional DHS genes in low-stringency hybridization experiments.

Our data suggest that duplicate, independently regulated aromatic amino acid biosynthetic pathways may reside within the chloroplast. This idea is supported by the fact that all cloned Arabidopsis aromatic amino acid biosynthetic genes encode putative chloroplast transit peptides (refs. 3 and 4; K. Niyogi and G.R.F., unpublished work). Independent regulation may also involve different cell-type-specific expression patterns for each member of a gene pair, as observed for pea glutamine synthetase genes in transgenic tobacco (43). Transgenic plants containing DHS promoter sequences fused to reporter genes can address this issue.

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