RESEARCH PAPER

Functional analysis of the essential bifunctional tobacco enzyme 3-dehydroquinate dehydratase/shikimate dehydrogenase in transgenic tobacco plants

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Abstract

In plants, the shikimate pathway occurs in the plastid and leads to the biosynthesis of aromatic amino acids. The bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHD/SHD) catalyses the conversion of dehydroquinate into shikimate. Expression of NtDHD/SHD was suppressed by RNAi in transgenic tobacco plants. Transgenic lines with <40% of wild-type activity displayed severe growth retardation and reduced content of aromatic amino acids and downstream products such as chlorogenic acid and lignin. Dehydroquinate, the substrate of the enzyme, accumulated. However, unexpectedly, so did the product, shikimate. To exclude that this finding is due to developmental differences between wild-type and transgenic plants, the RNAi approach was additionally carried out using a chemically inducible promoter. This approach revealed that the accumulation of shikimate was a direct effect of the reduced activity of NtDHD/SHD with a gradual accumulation of both dehydroquinate and shikimate following induction of gene silencing. As an explanation for these findings the existence of a parallel extra-plastidic shikimate pathway into which dehydroquinate is diverted is proposed. Consistent with this notion was the identification of a second DHD/SHD gene in tobacco (NtDHD/SHD-2) that lacked a plastidic targeting sequence. Expression of an NtDHD/SHD-2–GFP fusion revealed that the NtDHD/SHD-2 protein is exclusively cytosolic and is capable of shikimate biosynthesis. However, given the fact that this cytosolic shikimate synthesis cannot complement loss of the plastidial pathway it appears likely that the role of the cytosolic DHD/SHD in vivo is different from that of the plastidial enzyme. These data are discussed in the context of current models of plant intermediary metabolism.

Key words: 3-Dehydroquinate dehydratase/shikimate dehydrogenase, Nicotiana tabacum, RNA interference, secondary metabolism, shikimate pathway.

Introduction

The shikimate pathway is central for the biosynthesis of aromatic amino acids, folic acids, vitamins, and a number of aromatic compounds and secondary metabolites in bacteria, plants, fungi, and apicomplexa parasites but is absent from humans and other higher animals (Bentley, 1990; Herrmann, 1995; Herrmann and Weaver, 1999). In plants, aromatic amino acids are used not only as protein building blocks, but also as precursors for a large number of secondary metabolites (i.e. pigments, flavonoids, auxins,
phytoalexins, lignin, and tannins) (Herrmann, 1995). In addition, pathway intermediates may serve as substrates for a number of other metabolic pathways including biosynthesis of quinate and derived products such as chlorogenate (Herrmann and Weaver, 1999). It has been estimated that, under normal growth conditions, 20% of the carbon fixed by plants can be directed towards the shikimate pathway (Haslam, 1993).

The shikimate pathway consists of seven metabolic steps beginning with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate to form 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) and ending with the synthesis of chorismate from 5-enolpyruvylshikimate 3-phosphate (EPSP). The organization of the seven enzymes is markedly different between the three kingdoms. While the seven enzymatic steps in most prokaryotes are catalysed by monofunctional enzymes; in fungi, reactions 2–6 are catalysed by the multifunctional AROM complex.

In plants, the shikimate pathway is believed to be confined to plastids (Herrmann, 1995; Schmid and Amrhein, 1995; Mustafa and Verpoorte, 2005; Weber et al., 2005). This is supported by biochemical and molecular data. Most shikimate pathway enzymes are synthesized as precursors with an N-terminal extension, allowing the post-translational uptake of the protein into chloroplasts (Della-Cioppa et al., 1986; Schmid et al., 1992). Thus, the shikimate pathway appears to be firmly established in plastids. However, it has been proposed that the chloroplast-localized biosynthetic activity does not account for 100% of the aromatic amino acid biosynthesis observed. Hence, a spatially separated pathway in the cytosol has been proposed (Buchholz et al., 1979). Evidence for a cytosolic shikimate pathway comes from the isolation of DAHP synthase (Ganson et al., 1986), EPSP synthase (Mousdale and Coggins, 1985), and chorismate mutase (d’Amato, 1984) isoforms lacking N-terminal transit peptides. Moreover, since plastid-targeted shikimate kinase (Schmid et al., 1992) and EPSP synthase (Della-Cioppa et al., 1986) are active when they retain their transit peptides, these enzymes could also potentially be constituents of a cytosolic shikimate pathway. However, to date, operation of a complete extra-plastidial pathway has not been established.

In plants, steps three and four of the shikimate pathway are catalysed by the bifunctional enzyme 3-dehydroquinate dehydratase (EC 4.2.1.10)/shikimate dehydrogenase (EC 1.1.1.25) (DHD/SHD), which catalyses the dehydration of dehydroquinate to dehydroshikimate and the reversible reduction of dehydroshikimate to shikimate, respectively (Fig. 1A). Three different classes of SHDs have been identified across kingdoms on the basis of phylogenetic and biochemical analysis: shikimate dehydrogenase (AroE), shikimate/quinate dehydrogenase (YdiB), and shikimate dehydrogenase-like (SHD-L) (Michel et al., 2003; Singh et al., 2005). All three classes of SHDs can catalyse the oxidation of shikimate in the presence of nicotinamide adenine dinucleotide phosphate (NADP*) in vitro, albeit with varying efficiencies. The natural substrate of the SHD-L, however, has not yet been determined (Singh et al., 2005). The plant SHD has been shown to be the AroE type (Singh and Christendat, 2006).
The ratio of SHD to DHD activity has been reported to be 9:1; thus dehydroquinate is readily converted to shikimate without accumulation of dehydroshikimate (Fiedler and Schultz, 1985). Bifunctional enzymes play important roles in regulating the partitioning of metabolites at branch points in metabolic pathways to ensure sufficient production of a desired compound. DHD/SHD is found at the branch point for quinate synthesis. Dehydroquinate and dehydroshikimate, the substrates for DHD and SHD, respectively, can be partitioned towards quinate metabolism (Bentley, 1990). Substrate channelling from the DHD to the SHD site will ensure a constant flux through the shikimate pathway for chorismate biosynthesis.

Partial or full-length cDNA clones encoding DHD/SHD of pea (Deka et al., 1994), tobacco (Bonner and Jensen, 1994), and tomato (Bischoff et al., 2001) have been isolated. In 1994, Bonner and Jensen isolated a partial cDNA encoding tobacco DHD/SHD by screening a Nicotiana tabacum cDNA expression library with a DHD/SHD-specific polyclonal antibody. This cDNA comprised the entire coding region of the mature enzyme and an additional 69 upstream nucleotides, postulated to encode part of the transit peptide for plastidic targeting. The mature sequence translates into a protein with both enzymatic activities, the dehydratase at the amino-terminal and the dehydrogenase at the carboxyl-terminal part. The putative transit peptide is rich in hydroxylated amino acid residues but has a net negative charge, a feature not seen for other chloroplast transit peptides (Gavel and von Heijne, 1990). In 2001, Bischoff and co-workers cloned a full-length cDNA encoding tomato DHD/SHD (Bischoff et al., 2001). The amino acid sequence deduced revealed that DHD/SHD is most probably synthesized as a precursor with a very short (13 amino acids) plastid-specific transit peptide, which was highly similar to the tobacco counterpart.

To elucidate the physiological role of DHD/SHD in plants, its activity was decreased by RNAi in transgenic tobacco plants. Resulting plants were thoroughly investigated for growth and biochemical and molecular parameters. Silencing of DHD/SHD resulted in a dramatic reduction of aromatic compounds and a strongly reduced growth of transgenic plants. Unexpectedly, DHD/SHD-deficient plants accumulated shikimate, the product of DHD/SHD activity. Stimulated by this rather unexpected result a second DHD/SHD isoform, capable of synthesizing shikimate within the cytosol, was isolated and biochemically characterized. This newly isolated cytosolic DHD/SHD isoform was designated NtDHD/SHD-2 and seems likely to be involved in the shikimate synthesis observed in the cytosol.

Materials and methods

Plant material and growth conditions

Tobacco plants (Nicotiana tabacum cv. Samsun NN) were grown in tissue culture under a 16 h light/8 h dark regime (150 µM quanta m$^{-2}$ s$^{-1}$ light, 21°C) at 50% humidity on Murashige–Skoog medium (Sigma, St Louis, MO, USA) containing 2% (w/v) Suc. For basic molecular and biochemical characterization, kanamycin-selected plants of the T1 generation were cultivated in soil in a greenhouse with 16 h supplemental light (~250 µM m$^{-2}$ s$^{-1}$) followed by 8 h darkness. The temperature regime followed the day/night cycle with 25°C/18°C. Detailed investigation of growth parameters was performed with plants grown on sand under greenhouse conditions and watered daily with a nutrient solution as described (Geiger et al., 1999).

Cloning of full-length NtDHD/SHD-1 from tobacco and expression in Escherichia coli

To obtain a full-length DHD/SHD clone from Nicotiana tabacum, the lacking 5' end of a published DHD/SHD sequence (Bonner and Jensen, 1994; GenBank accession no. L32794) was amplified by RACE using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and a gene-specific primer (5'-TTTACATAC-CAGCATACTGACCCCTTCCC-3'). The resulting PCR product (~500 bp) was ligated into the pCR-blunt vector (Invitrogen, Carlsbad, CA, USA) and sequenced. Sequence information allowed assembly of a putative full-length cDNA using the InforMax VectorNTI software (InforMax, Inc., USA). Finally, the entire cDNA clone, designated NtDHD/SHD-1, was amplified by RT-PCR from tobacco leaves using M-MLV[Hi+] reverse transcriptase (Promega, Madison, WI, USA) and oligod(T)30 primer for reverse transcription, as well as Pfu polymerase (Stratagene) and gene-specific primers (5'-GGGACTTTTTCAGCTGGCTGCT-3' and 5'-TTGATACAAATGCTACATAAATGTTG-3') for PCR amplification. Identity of the sequence was verified and submitted to GenBank/DDBJ/EMBL (accession no. AY578144).

For expression in E. coli, the coding region of mature NtDHD/SHD-1 protein lacking the apparent transit peptide [nucleotides (nts) 118–1785] was PCR amplified with gene-specific primers 5'-GGATCCGGGGAGGCAATGACGAGGAAC-3' and 5'-CTGCCAGTTATCTCTCCGAGCACAATGG-3'. After subcloning into pCR-blunt (Promega), the PCR fragment was ligated into the BamHI and PstI sites of pQE9 and transferred into E. coli M15(pREP4) cells for protein expression (Qiagen, Hilden, Germany). Recombinant E. coli cells were harvested 3–4 h after induction with 0.05 mM isopropylthio-β-galactoside, and soluble 6× His-tagged NtDHD/SHD-1 protein was purified under native conditions using nickel–nitrilotriacetic acid agarose according to the manufacturer’s instruction.

RNAs plasmid construction and tobacco transformation

To generate the NtDHD/SHD-1 RNAi construct, a partial fragment of tobacco NtDHD/SHD-1 (GenBank, AY578144) comprising nts 816–1368 was PCR amplified using the primers 5'-GGATCCGGGGAGGCAATGACGAGGAAC-3' and 5'-GCGAGCTAATGCTGAGGAA-3' (BamHI and SalI restriction sites are underlined). The resulting PCR product was subcloned into a pCR-blunt vector and subsequently inserted as a BamHI/SalI fragment in sense orientation downstream of the GA20oxidase intron in the pUC-RNAi vector as described by Chen et al. (2003). The same fragment was ligated in antisense orientation into the BglII/Xhol sites of pUC-RNAi already carrying the sense fragment. Finally, the resulting RNAi fragment was excised from pUC-RNAi using the flanking PstI sites and inserted into the Shfl site of pBinAR (Höfgen and Willmitzer, 1990) between the CaMV 35S promoter and ocs terminator, yielding the construct pBin-NtDHD/SHD-1-RNAi. The construct ALC-NtDHD/SHD-1-RNAi for inducible silencing was prepared using the strategy detailed in Chen et al. (2003), employing the same gene fragment as above.
Transformation of tobacco plants by *Agrobacterium*-mediated gene transfer using *A. tumefaciens* C58.C1.pGV2260 was carried out as described by Rosahl et al. (1987).

Cloning of NtDHD/SHD-2, binary plasmid construction, and Agro-infiltration

A partial 270 bp EST clone homologous to tobacco NtDHD/SHD-1 was identified from a sequenced tobacco cDNA library and amplified by RT-PCR from tobacco leaf material using gene-specific primers (5′-TCGAGAAGGAGAGCTGAAATTT-3′ and 5′-CCCGGGATGGAGTTGGTAGTGGATTC-3′) and 5′-CCCGGGATGGAGTTGGTAGTGGATTC-3′ and 5′-GCGTCTTTCACGCCAGAGTCAGTCTTTTCTCTTA-3′). The resulting PCR fragment was ligated into pCR-blunt and sequenced to verify the identity of the NtDHD/SHD isoform. 5′ and 3′ fragments of 1.2 and 0.7 kb, respectively, were obtained by RACE using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) and gene-specific primers (5′-RACE, 5′-GCGTCTTTCACGCCAGAGTCAGTCTTTTCTCTTA-3′; 5′-RACE, 5′-TCGAGAAGGAGAGCTGAAATTT-3′) and 5′-GCGTCTTTCACGCCAGAGTCAGTCTTTTCTCTTA-3′). PCR products were cloned into pCR-blunt and sequences were assembled by the software VectorNTI (InforMax, Inc.). Finally, the full-length cDNA clone containing either TTAGGATTGTTGAA-3′ was identified from a sequenced tobacco cDNA library and amplified by RT-PCR from tobacco leaf material using gene-deduced oligonucleotides, and the entire sequence, designated NtDHD/SHD-2, was determined (GenBank accession no. AY578143).

To create a binary construct for transient expression in plants, the entire coding region of NtDHD/SHD-2 was amplified by PCR using the primers 5′-GCGTCTTTCACGCCAGAGTCAGTCTTTTCTCTTA-3′ and 5′-GCGTCTTTCACGCCAGAGTCAGTCTTTTCTCTTA-3′, thereby introducing Sall restriction sites (underlined) at the 5′ end and 3′ end, respectively. After subcloning, the PCR fragment was inserted into the SalI site of pBinAR downstream of the CaMV 35S promoter to yield pBin-NtDHD/SHD-2.

For infiltration of *N. tabacum* leaves, the *A. tumefaciens* strain C58C1 was infiltrated into the abaxial air space of 4-week-old plants as described (Voinnet et al., 2003). The p19 protein of tomato bushy stunt virus was used to suppress gene silencing. Co-infiltration of *Agrobacterium* strains containing pBin-NtDHD/SHD-2 and the p19 construct was carried out at OD600 of 1.0.

Construction of fusion proteins with GFP variants and microprojectile bombardment

To obtain reporter gene fusion constructs for transient expression in leaf tissue, the coding regions of the respective NtDHD/SHD isoform were either cloned by RT-PCR from leaf tissue (NtDHD/SHD-1) or PCR amplified from the plasmid pBin-NtDHD/SHD-2, using primers 5′-CCCCGGGATGGGATGTGAGTTC-3′ and 5′-TCTAGAATTCTCCGAAGCACAATGTTGA-3′ for NtDHD/SHD-1, and primers 5′-CCCCGGGATGGGATGTGAGTTC-3′ and 5′-TCTAGAATTCTCCGAAGCACAATGTTGA-3′ for NtDHD/SHD-2, respectively. The oligonucleotides were designed to delete the stop codon and to introduce SmaI sites at the 5′ end and XbaI sites at the 3′ end, respectively. PCR fragments were subcloned and inserted into the SmaI/XbaI sites of a pPF19-based vector (Timmermans et al., 1990) containing either nGFPs, EFYF, or ECFP instead of the GUS gene (pPF19–GFP, pPF19–YFP, pPF19–CFP, kindly provided by A Wachter, Heidelberg, Germany), to yield plasmids pPF19–NtDHD/SHD-1, YFP, pPF19–NtDHD/SHD-2, GFP, or pPF19-NtDHD/SHD-2, CFP. DNA-plasmids were coated onto 1 μm gold particles, and the abaxial epidermis of tobacco was bombarded either with a single construct (pPF19–GFP vector, pPF19–NtDHD/SHD-2, GFP) or simultaneously with two constructs (pPF19–NtDHD/SHD-1, YFP and pPF19–NtDHD/SHD-2, GFP) using a Helios Gene Gun according to the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA, USA). Images were obtained with a confocal microscope LSM 510 META (Carl Zeiss, Göttingen, Germany). Excitation light of 485, 488, and 514 nm produced by an argon laser and different emission filters of 475–510, 510–525, and 520–545 nm allowed detection of CFP, GFP, or YFP, respectively. Images were superimposed with scans of chlorophyll-derived red fluorescence and transmission images by means of the Zeiss LSM Version 3.0 software.

Measurement of DHD/SHD enzyme activity

DHD/SHD enzyme activity of recombinant NtDHD/SHD-1 protein and of tobacco plants was determined with shikimate as substrate by quantifying the production of NADPH spectrophotometrically at 334 nm. An aliquot (0.5 μg) of purified 6× His-NtDHD/SHD-1 protein (see above) was incubated in 800 μL of reaction buffer (200 μM NADP in 100 mM glycine–NaOH buffer, pH 9.0) at room temperature for 3 min and the reaction was started by adding 2 μL of 100 mM shikimate. For determination of DHD/SHD activity in stable transformants or agro-infiltrated leaves, 500 mg of leaf material was homogenized in liquid nitrogen and extracted with 500 μL of protein extraction buffer [50 mM TRIS–HCl, pH 6.8, 5 mM MgCl2, 5 mM mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, 1 mM EGTA, and 0.1 mM Pefabloc protease inhibitor]. Twenty microlitres of leaf extracts were incubated in 780 μL of reaction buffer (1 mM NADP in 100 mM glycine–NaOH buffer, pH 9.0) for 5 min at room temperature, and the reaction was started in the spectrophotometer (Uvikon 922, Kontron Instruments) after reaching a stable baseline by addition of shikimate as described.

Enzymatic determination of shikimate and dehydroshikimate/dehydroquininate

Shikimate was determined enzymatically in plant extracts by measuring the turnover of NADP to NADPH following the addition of recombinant DHD/SHD protein (see above). Five hundred milligrams of leaf tissue were extracted with trichloroacetic acid (TCA) as described previously (Jelitto et al., 1992), and 50 μL of the neutralized extract [16% (v/v) TCA] were incubated in 550 μL of reaction buffer 1 (100 μM NADP in 100 mM glycine–NaOH buffer, pH 9.0). The production of NADPH was determined spectrophotometrically at 334 nm with 20× multiplications using a Sigma ZWS-II photometer (Sigma, Darmstadt, Germany), and the reaction was started by adding 0.5 μg of purified DHD/SHD enzyme. Calculation was based on calibration curves prepared with 1–20 nmol shikimate ml−1 giving rise to the linear equation y=1.0504x+0.1974 (R2=0.9993). Recovery experiments were undertaken to confirm the accuracy of the measurements. A defined portion of shikimate (1–20 nmol ml−1) was added to the leaf material before extraction and determined exactly as described. The recovery rates were between 92% and 101% of the amount added.

Since DHD/SHD catalyses a reversible reaction, dehydroquininate/dehydroshikimate was determined in the same extract when the reaction was carried out in the reverse direction. Ten microlitres of TCA extract was incubated in 590 μL of reaction buffer 2 (100 μM NADP in 100 mM HEPES–KOH buffer, pH 7.2) and the turnover of NADPH to NADP was followed at 334 nm with 10 times multiplication after addition of recombinant DHD/SHD protein as described.

Carbohydrate and amino acid determination

Soluble sugars and starch were determined in leaf samples extracted with 80% (v/v) ethanol/20 mM HEPES–KOH pH 7.5 as described (Stitt et al., 1989). Amino acid contents were measured from the same extracts with HPLC as described (Rolletschek et al., 2002).
Single amino acids were identified by co-chromatography with authentic standards and quantified by comparison with internal standards.

**Chlorophyll determination**

Chlorophyll was measured in ethanol extracts and concentrations were determined as described in Lichtenenthaler (1987).

**Chlorogenic acid determination**

Leaf samples (100 mg) were homogenized in liquid nitrogen and successively extracted with 500, 300, and 200 µl of methanol (gradient grade; Sigma-Aldrich, Germany). Supernatants of each extraction step were collected after centrifugation (16 000 g, 5 min) and the pooled samples were used for further analysis. Prior to the measurements, the methanol concentration of the sample was brought up to 20% using buffer A (9.5% acetonitrile and 1% acetic acid in purest H₂O). A Waters HPLC system (Waters, Eschborn, Germany) consisting of a gradient pump (Waters no. 600), a degassing module, an autosampler (no. 717), and a UV detector (no. 417) was used for determination of chlorogenic acid. The gradient was brought up to 100% acetonitrile. For determination of chlorogenic acid, the gradient was brought up to 100% acetonitrile. For the catabolic reaction, a reversed phase column [Luna 5µ C18(2), 250×4.6 mm; Phenomenex, Aschaffenburg, Germany] was used. The column was equilibrated with buffer A at a flow rate of 1 ml min⁻¹ and chlorogenic acid was recorded by the UV detector at 280 nm.

**Lignin determination**

Lignin content was determined spectrophotometrically (280 nm) using thioglycolic acid following the protocol as described by Campbell and Ellis (1992). Calculation was done according to a calibration curve prepared with pure lignin (5–25 mg). Lignin staining of transverse sections of petioles was performed by means of phloroglucinol–HCl reagent essentially as described by Biemelt et al. (2004). Tobacco petioles (6 weeks old) were decolorized in 70% (v/v) ethanol for 6–8 h, washed with distilled water, and sliced by scalpel. The sections were stained in 1% (w/v) phloroglucinol and electro-transferred to a nitrocellulose membrane (Porablot; Macherey-Nagel, Düren, Germany). Development of the immunoblots was performed with the SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Rockford, IL, USA). After incubation with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce).

**Results**

**Biochemical characterization of recombinant NtDHD/SHD-1**

A cDNA fragment encoding the predicted mature NtDHD/SHD-1 was PCR amplified from tobacco cDNA and heterologously expressed in E. coli. The recombinant protein was purified to apparent homogeneity using metal-affinity purification (data not shown), and this enzyme preparation was used to determine a number of biochemical parameters. Enzyme pH preference was determined in both the forward and reverse reaction in different buffer systems. The pH optimum for the anabolic reaction (from dehydroquinate to shikimate) was between 6.8 and 7.2. For the catabolic reaction, a higher pH optimum was determined. The reaction velocity reached a maximum at pH 9.0 and pH 9.4. The effect of temperature on NtDHD/SHD-1 activity was determined for both the forward and reverse reaction. The velocity of the NtDHD/SHD-1 catalysed reaction rose linearly from 5°C to 30°C, reaching a maximum value at 30°C, which remained constant until 45°C. Thereafter, the reaction velocity dropped rapidly as a result of the deactivation of the protein (data not shown). Thus the optimum temperature for NtDHD/SHD-1 was determined to be between 30°C and 45°C. DHD/SHD catalyses two neighbouring reversible reactions. Limited by the detection of dehydroshikimate, only the shikimate–NADP and dehydroquinate–NADPH pairs could be employed to determine the kinetic properties of NtDHD/SHD-1. The SHD domain, which catalysed the oxidation of shikimate to dehydroshikimate, exhibited typical Michaelis–Menten kinetics with $K_M$ of 31±7 µM and $K_M$ of 130±15 µM (data not shown).
RNA interference of NtDHD/SHD-1 strongly inhibits DHD/SHD activity in transgenic tobacco plants

To analyse functionally the in planta role of NtDHD/SHD-1, the corresponding transcript was targeted through an RNA interference (RNAi) strategy. To this end, a fragment of the tobacco NtDHD/SHD-1, ranging from nt 816 to nt 1368 of the respective cDNA clone (GenBank, AY578144), was amplified by PCR and inserted first in antisense orientation downstream of the CaMV 35S promoter, followed by a short intron, and by the same NtDHD/SHD fragment, but in sense orientation. This resulting plasmid pBin-DHD/SHD-RNAi was transformed into tobacco plants by Agrobacterium-mediated gene transfer, and 80 kanamycin-resistant primary transformants were screened by RNA blot hybridization after transfer into the greenhouse. Fourteen transgenic plants showed decreased transcript levels (data not shown), and depletion of DHD/SHD was further confirmed by enzyme activity measurement. Six independent transgenic lines with 16–87% remaining DHD/SHD activities were finally selected for detailed analysis in the T1 generation. Based on enzyme activities, DHD/SHD RNAi plants from the T1 generation were classified into four groups: high (H, DHD/SHD activities 85–60%); middle (M, DHD/SHD activities 60–40%); low (L, DHD/SHD activities 40–20%); and very low (VL, DHD/SHD activities 20–5%) (Fig. 1B).

Silencing of NtDHD/SHD-1 severely inhibits growth of transgenic tobacco plants

Silencing of NtDHD/SHD-1 led to dramatic phenotypical changes of the transgenic tobacco plants. A 40–60% reduction of DHD/SHD activity (group M plants) led to chlorotic leaves. Bleached areas developed in intercostal regions of mature leaves. A further reduction (60–80%) resulted in a dwarfen phenotype. When DHD/SHD activity dropped below 20% of wild type, transgenic plants stopped growing and frequently died in 1–2 weeks after transfer from tissue culture to the greenhouse (Fig. 1C).

To correlate the reduction in DHD/SHD activity with biomass production, NtDHD/SHD-1-RNAi plants (T1 generation) were germinated and cultivated in sand culture as described in Materials and methods. Growth parameters were acquired by measuring fresh weight (FW), dry weight (DW), and plant height (Table 1). An up to 40% reduction of DHD/SHD did not result in growth retardation. Whereas 40% to 60% reduction of enzyme activity led to a 33% reduction in fresh weight, a 42% reduction in dry weight, and a 37% reduction in plant height, respectively. In plants with 20% or lower DHD/SHD activity, biomass production severely decreased to 7% (fresh weight and dry weight) and 36% (plant height) as compared with wild-type plants. These results indicate that the loss of DHD/SHD activity leads to a concomitant reduction in biomass production of transgenic plants.

Table 1. Growth parameters of tobacco plants with decreased DHD/SHD activity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group</th>
<th>Plant Height (cm)</th>
<th>Shoot FW (g)</th>
<th>Shoot DW (g)</th>
<th>Shoot FW/DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>–</td>
<td>13.5±2.6</td>
<td>7.13±1.24</td>
<td>0.41±0.18</td>
<td>17.39</td>
</tr>
<tr>
<td>DHD/SHD-1</td>
<td>H</td>
<td>12.9±2.3</td>
<td>6.32±1.46</td>
<td>0.34±0.10</td>
<td>18.59</td>
</tr>
<tr>
<td>DHD/SHD-63</td>
<td>M</td>
<td>8.6±2.1</td>
<td>4.83±0.67</td>
<td>0.24±0.07</td>
<td>20.13</td>
</tr>
<tr>
<td>DHD/SHD-63</td>
<td>L</td>
<td>7.2±1.7</td>
<td>1.79±0.19</td>
<td>0.10±0.05</td>
<td>17.9</td>
</tr>
<tr>
<td>DHD/SHD-9</td>
<td>VL</td>
<td>4.9±1.2</td>
<td>0.50±0.083</td>
<td>0.03±0.01</td>
<td>16.67</td>
</tr>
</tbody>
</table>

The shikimate pathway links primary and secondary metabolism. Under normal growth conditions, it was estimated that about 20% of fixed carbon is directed towards the shikimate pathway (Haslam, 1993). To determine whether reduced flux towards the shikimate pathway would influence primary metabolism, carbohydrate contents in DHD/SHD-silenced plants were determined. Similar amounts of carbohydrates were detected in all DHD/SHD RNAi plants when compared with wild type (data not shown). This result suggests that the inhibition of DHD/SHD had no effect on primary metabolism.

Silencing of NtDHD/SHD-1 leads to accumulation of dehydroquinate and shikimate

Inhibition of DHD/SHD activity was expected to lead to an accumulation of the substrate (dehydroquinate) and a depletion of the product (shikimate) of the enzyme reaction. Therefore, dehydroquinate and shikimate levels were determined in leaf extracts of wild-type and transgenic plants using the enzymatic assay mentioned above. In wild-type plants and NtDHD/SHD-1 RNAi plants with >60% DHD/SHD activity, no dehydroquinate could be detected (Fig. 2A), whereas in plants with <60% residual DHD/SHD activity, 1.7–9.3 μmol of dehydroquinate were measured per gram leaf material (fresh weight). Accumulation of dehydroquinate in these plants correlated with the reduction in DHD/SHD activity (see Fig. 1B). Unexpectedly, shikimate, the product of DHD/SHD, also accumulated in DHD/SHD RNAi plants; 3.5- to 8-fold higher levels of shikimate were detected in plants with <60% residual DHD/SHD activity as compared with wild-type controls (Fig. 2B), whereas in plants with >60% DHD/SHD activity, no shikimate accumulation was observed.

Inhibition of DHD/SHD leads to reduced chlorogenate and lignin content

The shikimate pathway converts erythrose 4-phosphate and PEP into aromatic amino acids that could be
metabolized further into a large number of different phenolic compounds. To determine the effect of DHD/SHD silencing on secondary metabolism, chlorogenate and lignin were determined in transgenic plants. In plants with >60% DHD/SHD activity (DHD/SHD 1-H), a slight decrease of chlorogenate and lignin was measured with values of 96% and 93% of wild-type plants, respectively. A 40–60% reduction of DHD/SHD activity led to a remarkable reduction in aromatic compounds, in which lignin and chlorogenate dropped to 63% and 67% of wild-type values, respectively. In plants with <20% DHD/SHD residual activity, only 43% of chlorogenate and 37% of lignin accumulated (Fig. 3).

To visualize lignin histochemically in transgenic plants, leaf material was stained by the phloroglucinol–HCl method. As demonstrated in Fig. 3C, vascular tissues in petioles of wild-type plants were stained brilliant red, indicating high concentrations of phenolic compounds and lignin, whereas in DHD/SHD-silenced plants, vascular tissue in petioles was only slightly stained pale red, suggesting that less phenolic compounds and lignin accumulated in the transgenic plants (Fig. 3C).

Molecular analysis of shikimate synthesis in tobacco

DHD/SHD silencing does not lead to down-regulation of shikimate pathway enzymes at the transcript level

To investigate whether silencing of DHD/SHD would influence the expression of other shikimate pathway genes, the transcripts of DAHPS, dehydroquinate synthase, shikimate kinase, EPSP synthase, and chorismate synthase were probed by northern blot hybridization in both transgenic and control plants. As demonstrated in Fig. 4, silencing of DHD/SHD did not affect expression of downstream genes and the adjacent upstream gene, dehydroquinate synthase. DAHPS was the only gene to be regulated at the transcript level. Accumulation of DAHPS transcripts increased up to four times in DHD/SHD-VL plants as compared with wild-type controls.

Shikimate feeding of leaf discs reveals normal metabolism in DHD/SHD-RNAi plants

As described above, shikimate accumulated in DHD/SHS-silenced plants. This unexpected observation might be explained by an impaired downstream processing of shikimate. To test this hypothesis, wild-type and transgenic leaf discs were floated on 50 mM HEPES solution (pH 7.0) containing 20 mM shikimate at room temperature for 16 h. As a control, leaf discs were floated on 50 mM HEPES solution without shikimate under the same conditions. Afterwards, leaf discs were washed with distilled water, dried on filter paper, and subjected to amino acid determination. In the absence of shikimate or prior to the experiment, the content of phenylalanine and tyrosine was lower in transgenic plants (substantially so in the case of phenylalanine but only slightly so in the instance of tyrosine) as compared with wild-type controls. Following feeding on shikimate these differences disappeared. Similar amounts of phenylalanine (79–118% of wild-type plants) and tyrosine (84–122% of wild-type plants) accumulated in leaf discs of transgenic plants after feeding 20 mM shikimate (Supplementary Fig. S1 available at JXB online). This result indicates that silencing DHD/SHD did not impair downstream enzymes of the shikimate pathway in transgenic plants.

Inducible RNA interference of NtDHD/SHD-1

Given that the surprising result of elevated shikimate levels in the transformants constitutively silenced in NtDHD/SHD-1 was observed in plants also displaying a strong growth phenotype, it was decided to create plants which could be silenced by chemical induction. This approach was carried out to allow evaluation of this phenomenon in plants exhibiting developmental equivalence to the wild type and thus to evaluate whether it is a direct consequence of the genetic manipulation or merely a pleiotropic one. For this purpose, the alcR system, which had previously been used in the dissection of complex phenotypes, was chosen for the present study.
Following Agrobacterium-mediated gene transfer of the alcR-driven RNAi construct, 80 kanamycin-resistant plants were transferred to the greenhouse. To screen for primary transformants 42-d-old plants were induced with 1% (v/v) ethanol via root drenching (Chen et al., 2003; Schaarschmidt et al., 2004). Young leaves, ~10 cm in length at the point of induction, were followed over the course of the experiment. Samples for enzyme analysis were taken directly prior to induction and following induction over a range of periods. Before induction similar levels of DHD/SHD activity were detected (data not shown) suggesting that the transcription of the dsRNA is under strict control of the alcR cassette. DHD/SHD activity decreased dramatically following 3 d of induction, with five lines, displaying residual activities ranging from 15 to 40% of wild type, selected for detailed analysis in the T1 generation. Notably, in contrast to the situation observed in the plants constitutively silenced in the expression of NtDHD/SHD-1, the inducible lines displayed only weak phenotypic changes following induction such as mild leaf wrinkling and were not compromised in their growth in the time periods of induction described here. To analyse the T1 of Alc-DHD/SHD-RNAi plants they were subjected to induction following exactly the same procedure outlined above. The dynamic alteration of DHD/SHD transcripts was analysed by northern hybridization, revealing that the DHD/SHD mRNA started to decrease as early as 12 h after incubation and continued to decrease until 60 h after induction (Fig. 5A). In contrast, the reduction of DHD/SHD activity lagged behind, with transgenic lines maintaining wild-type activity until 48 h after induction, with significant reductions in activity (down to 20% of wild-type level) only detected 60–72 h after induction (Fig. 5B).

**Kinetic analysis of shikimate pathway intermediates**

Since the alcR gene switch allows real-time analysis of the consequences of silencing the DHD/SHD enzyme, 20 T1
plants each of the alc-NtDHD/SHD-RNAi lines 3 and 4 were induced and analysed. On the basis of their DHD/SHD enzyme activity, eight plants (five from line 3 and three from line 4) were selected for the determination of shikimate pathway intermediates. Dehydroquinate could not be detected in wild type or either transformant before ethanol induction; however, this metabolite started to accumulate in the transformants 2–3 d after ethanol induction in a manner that correlated with the silencing of DHD/SHD (Fig. 5C). In contrast shikimate started to accumulate 1–2 d later than dehydroquinate. These data suggest that whilst the accumulation of dehydroquinate is a direct consequence of the inhibition of DHD/SHD that of shikimate is most probably a secondary effect (Fig. 5D).

Identification and cloning of a cDNA encoding a cytosolic DHD/SHD isofom from N. tabacum

A cDNA encoding a DHD/SHD isozyme was cloned from N. tabacum, and designated as NtDHD/SHD-2 (Materials and methods). Upstream of the first ATG, a stop codon (27 nts upstream) was located in-frame with the coding sequence, indicating that the cDNA contained the complete coding region. The cDNA encodes a peptide of 518 amino acids, the sequence of which is 48% identical to that of NtDHD/SHD-1. Compared with the amino acid sequence of NtDHD/SHD-1, NtDHD/SHD-2 lacks the plastid-specific transit peptide (13 amino acids) at the N-terminus (Bischoff et al., 2001), and was predicted to be cytosolic by the software ChloroP (Emanuelsson et al., 1999). Conserved domain database (CDD, NCBI) search recognized both DHD and SHD domains from the deduced amino acid sequence of NtDHD/SHD-2. The DHD domain is located at the N-terminus, from amino acids 13 to 230, whilst the SHD domain is located at the C-terminus of the protein ranging from amino acids 235 to 513. A database BLAST search (Altschul et al., 1990) with the NtDHD/SHD-2 sequence revealed the presence of related sequences in a number of other plant species such as tomato, potato, poplar, and rice (Fig. 6) all apparently lacking a transit peptide but comprising both conserved domains. A closer inspection of the two genes from rice revealed that they are arranged in tandem on the same BAC clone (GenBank acc. no. AP003204) and expression of both putative DHD/SHD isoforms is supported by corresponding ESTs (data not shown).

The crystal structure of Arabidopsis DHD/SHD and the amino acid residues critical for substrate binding and catalysis have been determined recently (Singh and Christendat, 2006). A comparison of the active site alignments of the Arabidopsis DHD/SHD with the tobacco DHD/SHD-1 reveals that the key active site residues are conserved across both domains in the two enzymes. In the Arabidopsis DHD active site, Arg279 functions as a key binding group and
Fig. 6. Alignment of plant DHD/SHD protein sequences. Deduced DHD/SHD sequences with an apparent chloroplastic transit peptide from *N. tabacum* (NdDHD/SHD-1, GenBank accession no. AY578144), tomato (LeDHD/SHD-1, AF033194), rice (OsDHD/SHD-1, BAD61389), and *Arabidopsis* (AtDHD/SHD-1, AAF08579) were compared with putative cytosolic isoforms from *N. tabacum* (NdDHD/SHD-2, AW578143) and rice (OsDHD/SHD-2, NP918759), as well as with translated ESTs from tomato (LeDHD/SHD-EST, BF096277), potato (StDHD/SHD-EST, CK245886), and *Populus trichocarpa* (PtDHD/SHD-EST, CV244968) using the ClustalW program. Identical residues are shaded in black, and similar residues are shaded in grey.

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*Fig. 6.* Alignment of plant DHD/SHD protein sequences. Deduced DHD/SHD sequences with an apparent chloroplastic transit peptide from *N. tabacum* (NdDHD/SHD-1, GenBank accession no. AY578144), tomato (LeDHD/SHD-1, AF033194), rice (OsDHD/SHD-1, BAD61389), and *Arabidopsis* (AtDHD/SHD-1, AAF08579) were compared with putative cytosolic isoforms from *N. tabacum* (NdDHD/SHD-2, AW578143) and rice (OsDHD/SHD-2, NP918759), as well as with translated ESTs from tomato (LeDHD/SHD-EST, BF096277), potato (StDHD/SHD-EST, CK245886), and *Populus trichocarpa* (PtDHD/SHD-EST, CV244968) using the ClustalW program. Identical residues are shaded in black, and similar residues are shaded in grey.
Lys241 and His214 are catalytic groups (Singh and Christendat, 2006). These residues correspond to Arg207, Lys169, and His142, respectively, in NtDHD/SHD1 (Fig. 6). In the NtSHD/DHD-2 polypeptide the Lys residue (Lys160), which is involved in the formation of a Schiff base intermediate (Singh and Christendat, 2006), is also conserved; however, the His residue is substituted by Phe (Phe134) and the Arg is replaced by Gln (Gln198). In contrast, Lys385 and Asp423 (numbered with respect to the Arabidopsis protein), located in the SDH active site are conserved in all three DHD/SHD enzymes and have been proposed to be involved in proton transfer during catalysis (Singh and Christendat, 2006). The crystal structure of the Arabidopsis SDH domain indicated that Ser336, Ser338, and Tyr550 are important for binding shikimate (Singh and Christendat, 2006). All three residues are conserved in NtDHD/SHD-1 (Ser264, Ser266, and Tyr473, respectively). In NtDHD/SHD-2 only the Ser336 (Ser254 in NtSHD/DHD-2) and the Tyr550 (Tyr467) are conserved, with the Ser338 substituted by Gly (Gly256).

**Enzymatic activity of NtDHD/SHD-2 and in vivo localization of NtDHD/SHD-1 and 2**

Initial attempts to express the NtDHD/SHD protein in *E. coli* failed as the recombinant protein formed insoluble inclusion bodies. Therefore, it was decided to express the protein transiently *in planta* using *Agrobacterium*-infiltration (Voinnet et al., 2003). To this end, two constructs comprising the full-length cDNA of NtDHD/SHD-1 and 2, respectively, under the control of the CaMV 35S promoter were transiently expressed in tobacco leaves alongside a control plasmid expressing the GUS reporter gene. Three days after infiltration, total DHD/SHD activity was determined in the infiltrated leaf material. As expected, total DHD/SHD activity increased ~3-fold in leaves infiltrated with NtDHD/SHD-1 as compared with the leaf infiltrated with the control plasmid (Fig. 7F). Total DHD/SHD activity also increased after infiltration of NtDHD/SHD-2, clearly confirming the enzymatic activity of this isoform.

The subcellular localization of NtDHD/SHD-2 was determined in epidermal cells of tobacco (*Nicotiana tabacum*) source leaves using the green fluorescent protein (GFP) fused to the C-terminus of the full-length NtDHD/SHD-2 protein. After biolistic transfer into tobacco leaves, the subcellular localization of the fusion protein was determined using confocal laser scanning microscopy. Free GFP served as a control for cytosolic localization. Comparison of the fluorescence pattern of NtDHD/SHD-2,GFP with that of free GFP clearly indicated co-localization of the two proteins in the cytosol (Fig. 7A, B).

To confirm further the differential localization of the two SHD/DHD isoforms from tobacco, NtSHD/DHD-1 and -2 were C-terminally tagged with yellow (YFP) and cyan fluorescent protein (CFP), respectively, and localization of the two fusion proteins was investigated after co-bombardment of the constructs into tobacco leaf cells. The two fusion proteins were found to have distinct localization
patterns (Fig. 7C, E). As observed before for the GFP fusion protein, the NtDHD/SHD-2,CFP signal clearly localized to the cytosol, whereas the NtDHD/SHD-1,YFP fluorescence was confined to the plastids. When taken together these data clearly indicate that NtDHD/SHD-2 encodes a cytosolic isoform of tobacco DHD/SHD.

Discussion

It has been demonstrated previously that compromising plastidial shikimate biosynthesis, by the antisense inhibition of DAHP synthase, leads to potato plants exhibiting a stunted growth phenotype characterized by reduced stem length, width, and lignin content (Jones et al., 1995). Early studies also documented the importance of this pathway in the synthesis of plant secondary compounds including indole acetic acid, pigments, such as anthocyanins, and antimicrobial compounds such as phytoalexins and lignin which have been demonstrated to play vital roles in plant defence (Vance et al., 1980; Moerschbacher et al., 1990; Nicholson and Hammerschmidt, 1992), wound healing (Bostock and Stermer, 1989), and maintenance of structural integrity and water transport capacity (Lewis and Yakamoto, 1990). More recently, the mutant cue1 was identified in a genetic screen for chlorophyll ab/b-binding protein underexpressors (Li et al., 1995); the mutation mapped to the PEP/phosphate translocator of the plastid inner envelope membrane which has been proposed to import PEP into the stroma, where it serves as one of the substrates of the shikimate pathway (Fischer et al., 1997). Phenotypically cue1 is quite distinctive; it exhibits a reticulate leaf phenotype with dark green paravinal and light green interveinal regions as well as exhibiting deficiencies in chloroplast and mesophyll development (Li et al., 1995; Streatfield et al., 1999). However, a detailed genetic and biochemical analysis of this phenotype revealed that it was not simply caused by a general restriction of the shikimate pathway (Voll et al., 2003). In the present study it is demonstrated that RNAi-mediated reduction of the expression of NtDHD/SHD-1 in tobacco also leads to severe phenotypic and metabolic alterations. A 50% reduction of DHD/SHD activity (DHD/SHD-M plants) resulted in strongly bleached leaves, and a further reduction of DHD/SHD activity to 40% of the wild-type value led to strong decreases in growth parameters, highlighting the importance of DHD/SHD for plant development. Further reduction of endogenous DHD/SHD activity (DHD/SHD-VL plants) led to a lethal phenotype, in which case transgenic plants were stunted, and died 2–3 weeks after being transferred to the greenhouse. These phenotypes are largely consistent with what would be expected following a severe restriction in shikimate biosynthesis. Moreover, the approximately linear correlation between DHD/SHD residual activity and the level of phenolic compounds that the transgenic lines contain highlights the crucial role of this plastid-localized enzyme in controlling the flux of carbon towards secondary metabolism.

Given the marked reduction in the expression of the bifunctional DHD/SHD and the clear growth phenotype and lignin deficiency exhibited by the transgenic lines it was somewhat surprising that these plants displayed increased, as opposed to decreased, levels of dehydroquinate and shikimate. Expression analysis of genes encoding all seven enzymes of the shikimate pathway in the DHD/SHD RNAi plants revealed that DAHPS was the only one found to be regulated at the transcriptional level. Activation of DAHPS at the transcriptional level has been described previously following abiotic stresses such as mechanical wounding (Dyer et al., 1989) or glyphosate treatment (Pinto et al., 1988), indicating its potential importance in controlling carbon flow through the shikimate pathway. However, the fact that the up-regulation of DAHPS was not accompanied by a co-ordinate change in other shikimate pathway enzymes suggests that this is unlikely to be the major mechanism underlying the increased dehydroquinate and shikimate levels of the transformants. Another possible reason for the accumulation of dehydroquinate and shikimate is a restriction in the in vivo activity of downstream enzymes of the shikimate pathway. In keeping with this hypothesis is the fact that the transformants generated in this study displayed reduced levels of lignin and phenylpropanoids. However, leaves of transgenic and wild-type plants were able to synthesize a similar level of phenylalanine and tyrosine following incubation in shikimate, suggesting that reduction in lignin and phenylpropanoids was merely due to reduced substrate supply to downstream enzymes of chorismate synthesis rather than an active inhibition of the enzyme activities per se.

Silencing of a pathway enzyme typically disturbs the conversion of substrate to product, leading to an accumulation of the substrate and a depletion of the product (Rolleston, 1972). In Nt-DHD/SHD-RNAi plants, however, the reduction of the DHD/SHD enzyme activities resulted in a build-up of both dehydroquinate and shikimate, which are substrate and product of this enzyme, respectively. The accumulation of dehydroquinate and shikimate in transgenic plants was, furthermore, strongly negatively correlated with DHD/SHD residual activity. The accumulation of a shikimate observed in transgenic plants is unexpected for two reasons. On one hand, as the product of DHD/SHD, the silencing of DHD/SHD in transgenic plants would be anticipated to result in a reduced biosynthesis of shikimate in planta. On the other hand, the silencing of DHD/SHD did not impair the metabolism of shikimate by the downstream enzymes. Alternatively, shikimate could be synthesized from dehydroquinate via quinate, thus bypassing the DHD/SHD
reaction. In this scenario, quinate is synthesized from dehydroquinicline by an NADP-dependent quinate oxidoreductase and subsequently converted to shikimate by a quinate hydrolase (Leuschner et al., 1995). However, given the proposed plastidal localization of the quinate hydrolase, this sequence of reactions is unlikely to be responsible for the shikimate accumulation as one would expect that any shikimate produced in plastids would be available to support aromatic amino acid and lignin synthesis.

When the considerations above are taken together it is clear that shikimate content should decrease in DHD/SHD-silenced plants. To interpret the accumulation of shikimate in DHD/SHD-silenced plants a metabolite shuttle model is proposed. According to this model, the silencing of NtDHD/SHD-1 leads to a high-level accumulation of dehydroquinicline in chloroplasts. A proportion of this metabolite, which notably accumulates to very high levels in the transgenics, could be transported from chloroplasts to the cytosol and be converted to shikimate by a postulated cytosolic DHD/SHD isozyme. The extraplant shikimate produced by this enzyme is, however, most probably neither efficiently re-imported into chloroplasts, nor metabolized by downstream shikimate pathway enzymes in the cytosol, resulting in an elevated cellular shikimate level in the transformants. The cloning of a cytosolic DHD/SHD-like enzyme (NtDHD/SHD-2) gives strong support to this model, although the actual export of dehydroquinicline from the plastid into the cytosol has thus far not been experimentally demonstrated. However, the enzymatic activity of NtDHD/SHD-2 has been confirmed by Agrobacterium-infiltration, and subcellular localization using GFP-fusion proteins clearly revealed that this enzyme is a cytosolic protein.

Active site alignment between the Arabidopsis DHD/SHD and NtDHD/SHD-2 revealed that many of the amino acid residues critical for substrate binding and catalysis are conserved between the two proteins, suggesting that these enzymes support similar catalytic functions. However, there appears to be deviation in amino acid type at some other key positions. The role of the active site residues for binding and catalysis in the Arabidopsis SHD domain has been assessed by site-directed mutagenesis (Singh and Christendat, 2006). All critical residues except Ser338 (changed to Gly256 in NtDHD/SHD-2) are conserved between the Arabidopsis DHD/SHD and NtDHD/SHD-2. Mutagenesis of Ser338 to Ala in the Arabidopsis enzyme resulted in a 10-fold reduction in the turnover rate, and this residue has been implicated to be involved in binding and also in positioning of the substrate for catalysis (Singh and Christendat, 2006). Given the replacement of this critical residue by Gly in NtSHD/DHD-2 it appears likely that the cytosolic enzyme retains its ability to synthesize shikimate, albeit with a lower turnover rate. Interestingly, NtSHD/DHD-2 contains an additional Ser (Ser247) which is supposed to be positioned deeper in the shikimate binding pocket. Taken together, the conservation of some of the critical residues for catalysis is in close agreement with the DHD/SHD activity of NtDHD/SHD-2 which it is possible to demonstrate. However, on the basis of comparison to SDH-L forms from other organisms, it is tempting to speculate that the preferred substrate of the enzyme in vivo is in fact a derivative of shikimate exhibiting a larger functional group at the C1 position ring (Singh and Christendat, 2006). Thus, NtDHD/SHD-2 might involve different, albeit chemically related, substrates than NtDHD/SHD-1 and thus lead to a different product than the reaction catalysed by plastidal enzyme. Hence, it appears unlikely that the biological function of NtDHD/SHD-2 in vivo is the synthesis of shikimate in the cytosol. Circumstantial evidence in support of such a hypothesis is provided by the fact that overexpression of DHD/SHD-2 did not result in an increase in activity as that observed on overexpression of DHD/SHD-1. Furthermore, several examples in the literature indicate that genes of plant secondary metabolism have been recruited from ancestors of primary metabolism (Pichersky and Gang, 2000; Ober, 2005). New genes almost always arise by gene duplication followed by divergence. If the original gene had an essential function, as is the case for DHD/SHD, gene duplication is a prerequisite (Pichersky and Gang, 2000). The genomic organization of NtDHD/SHD-1 and 2 is unknown; however, a closer inspection of the two rice orthologues revealed their tandem location within the genome, strongly implicating that they arose through a gene duplication event. It is a common feature of enzyme evolution that the substrate of a new enzyme often closely resembles that of the enzyme from which it is derived. Thus relatively few amino acid substitutions can allow an altered enzyme to recognize a novel substrate whilst retaining the ability to utilize the original substrate albeit at lower efficiency (Pichersky and Gang, 2000). Future experiments will focus on the biochemical characterization and on a structure-function analysis of DHD/SHD-2 to elucidate its biological function further. Furthermore, non-aqueous fractionation will have to elucidate the subcellular distribution of shikimate in DHD/SHD-silenced plants.

In conclusion, in the current experiments, it was possible to provide direct evidence for the essential role of a plastidal isofrom of DHD/SHD in the synthesis of phenylalanine and its secondary metabolite derivatives including chlorogenic acid and lignin. These plants were also characterized by dramatic growth deficiencies and an elevated content of shikimate. After cloning of an additional gene for a DHD/SHD-like enzyme and confirming a cytosolic localization for its product, it is proposed that this enzyme is responsible for the shikimate accumulation observed in DHD/SHD-silenced tobacco plants.
Intriguingly, despite this capability, this pathway is not able to complement that localized in the plastid, most probably due to a spatial restriction in the availability of the shikimate to the chloroplast. The fact that the shikimate produced by DHD/SHD does not enter the aromatic amino acid or lignin biosynthesis pathways is highly interesting and suggests that the cytosolic isoform may, in vivo, catalyse an alternative reaction.

As such, the future elucidation of the functional role of cytosolic DHD/SHD is likely to provide important insight into the evolution of both shikimate and intermediary metabolism in plants.

Supplementary material

Supplementary material can be found at JXB online.

Supplementary figure 1. Tyrosine (Tyr) and phenylalanine (Phe) content in leaf discs of DHD/SHD-RNAi plants prior to and following exogenous application of shikimate in comparison to the wild type controls.

References


dissection of the complex metabolic phenotype obtained following its constitutive expression. *Plant Molecular Biology* **56**, 91–110.


