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Review

Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation, and metabolic engineering applications

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Abstract

A comprehensive survey of the extensive literature relevant to the evolution, physiology, biochemistry, regulation, and genetic engineering applications of plant aromatic L-amino acid decarboxylases (AADCs) is presented. AADCs catalyze the pyridoxal–5′–phosphate (PLP)-dependent decarboxylation of select aromatic L-amino acids in plants, mammals, and insects. Two plant AADCs, L-tryptophan decarboxylase (TDC) and L-tyrosine decarboxylase (TYDC), have attracted considerable attention because of their role in the biosynthesis of pharmaceutically important monoterpenoid indole alkaloids and benzylisoquinoline alkaloids, respectively. Although plant and animal AADCs share extensive amino acid homology, the enzymes display striking differences in their substrate specificities. AADCs from mammals and insects accept a broad range of aromatic L-amino acids, whereas TDC and TYDC from plants exhibit exclusive substrate specificity for L-amino acids with either indole or phenol side chains, but not both. Recent biochemical and kinetic studies on animal AADCs support basic features of the classic AADC reaction mechanism. The catalytic mechanism involves the formation of a Schiff base between PLP and an invariable lysine residue, followed by a transaldimination reaction with an aromatic L-amino acid substrate. Both TDC and TYDC are primarily regulated at the transcriptional level by developmental and environmental factors. However, the putative post-translational regulation of TDC via the ubiquitin pathway, by an ATP-dependent proteolytic process, has also been suggested. Isolated *TDC* and *TYDC* genes have been used to genetically alter the regulation of secondary metabolic pathways derived from aromatic amino acids in several plant species. The metabolic modifications include increased serotonin levels, reduced indole glucosinolate levels, redirected shikimate metabolism, increased indole alkaloid levels, and increased cell wall-bound tyramine levels.

Keywords: Aromatic amino acid decarboxylases; Dopa decarboxylase; Metabolic engineering; Pyridoxal phosphate-dependent enzymes; Secondary metabolism; Tryptophan decarboxylase; Tyrosine decarboxylase

Contents

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1. Introduction

Aromatic L-amino acid decarboxylases (AADCs) are homologous pyridoxal–5′–phosphate (PLP)-dependent enzymes of ancient evolutionary origin that catalyze the decarboxylation of aromatic L-amino acids in mammals, insects, and plants. In animals, L-Dopa decarboxylase (DDC; EC 4.1.1.28) has been the focus of much research because it is involved in several key biochemical and/or physiological processes including: (1) the catalysis of a key step in the biosynthesis of the monoamine neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine in the central and peripheral nervous systems of most mammalian species (Christenson et al., 1970); (2) a temperature- and possibly hormone-regulated step in the synthesis of biogenic amines, which are necessary for proper sclerotization of the cuticle in insects (Lunan and Mitchell, 1969); and (3) various clinical disorders, such as, Parkinson's disease and hypertension, in humans. Moreover, two peripheral DDC inhibitors, dopa hydrazide and α–methyldopa, are currently used to treat Parkinson's disease and hypertension, respectively.

In plants, AADCs are involved in the biosynthesis of several types of secondary metabolites, which are defined as compounds that are not essential for normal growth and development, but are often involved in key interactions between plants and their biotic and abiotic environments. Two particular AADCs in plants, L-tryptophan decarboxylase (TDC; EC 4.1.1.27) and L-tyrosine/L-Dopa decarboxylase (TYDC; EC 4.1.1.25), have attracted considerable attention primarily due to their roles in the biosynthesis of several important groups of pharmaceutical alkaloids. TDC and TYDC operate at an interface between primary and secondary metabolism, suggesting that they possess key regulatory functions in the control of end-product biosynthesis. Plant and animal AADCs share extensive amino acid identity and display remarkable similarities in subunit structure, molecular mass, and kinetic properties. However, plant and animal AADCs also possess striking differences in substrate specificity. In contrast to DDCs from mammals and insects, which accept a broad range of aromatic L-amino acids, TDC and TYDC from plants exhibit exclusive substrate specificity for L-amino acids with either indole or phenol side chains, but not both.

In addition to their potential regulatory functions in a variety of secondary metabolic pathways, plant AADCs are intriguing for several other reasons, including the physicochemical basis for their unique substrate specificities compared to animal AADCs; their biochemical properties as PLP-dependent enzymes; their possible degradation via a ubiquitin-mediated pathway; and their evolutionary relationship with DDC in animals. Isolated *TDC* and *TYDC* genes have also been used in a number of plant genetic engineering studies that have contributed to our understanding of the regulation of aromatic amino acid and secondary metabolic pathways. Metabolic engineering strategies that target AADCs have also demonstrated the impressive biotechnological potential and the prospective difficulties of altering key pathways, such as, aromatic amino acid metabolism, in plants. In this review, we examine the biochemical properties, the evolutionary origin, and the metabolic and physiological functions of AADCs in plants. We also discuss the current literature on the developmental and inducible regulation of TDC and TYDC activities, and summarize the results of metabolic engineering experiments using heterologous AADCs in various transgenic plants.

2. Metabolic and physiological roles of aromatic amino acid decarboxylases in plants

In addition to their role as the basic structural units of proteins, aromatic L-amino acids also serve as biosynthetic precursors for several primary and secondary metabolites in plants. The enzymes responsible for the commitment of aromatic L-amino acids to secondary metabolic pathways typically have key regulatory functions. For example, phenylalanine ammonia lyase (PAL) catalyzes the deamination of Phe to *trans*-cinnamic acid, the first step in the biosynthesis of the large and diverse array of phenylpropanoids, coumarins, flavonoids, and lignin (Hahlbrock and Scheel, 1989). Secondary metabolic pathways derived from Trp and Tyr are often initiated by substrate-specific

decarboxylation reactions that result in the formation of tryptamine and tyramine, respectively.

TDC has been implicated in the biosynthesis of the plant hormone indole-3-acetic acid (IAA), although it is not the major biosynthetic route (Bartel, 1997). TDC is also involved in the formation of the monoamine serotonin (5-hydroxytryptamine) (Grosse and Klapheck, 1979), and of simple alkaloids such as the β-carbolines, which are found in several plant families (Berlin et al., 1994). However, most research has focused on the role of TDC in the biosynthesis of the monoterpenoid indole alkaloids, which comprise a structurally diverse group of over 1800 pharmacologically active compounds that include several important pharmaceuticals such as, vinblastine (an antineoplastic agent used to treat Hodgkin's disease and other lymphomas) and ajmaline (an antiarrythmic that functions by inhibiting glucose uptake by heart tissue mitochondria) from *Catharanthus roseus*; quinine (a traditional antimalarial drug) from *Cinchona officinalis*; camp-

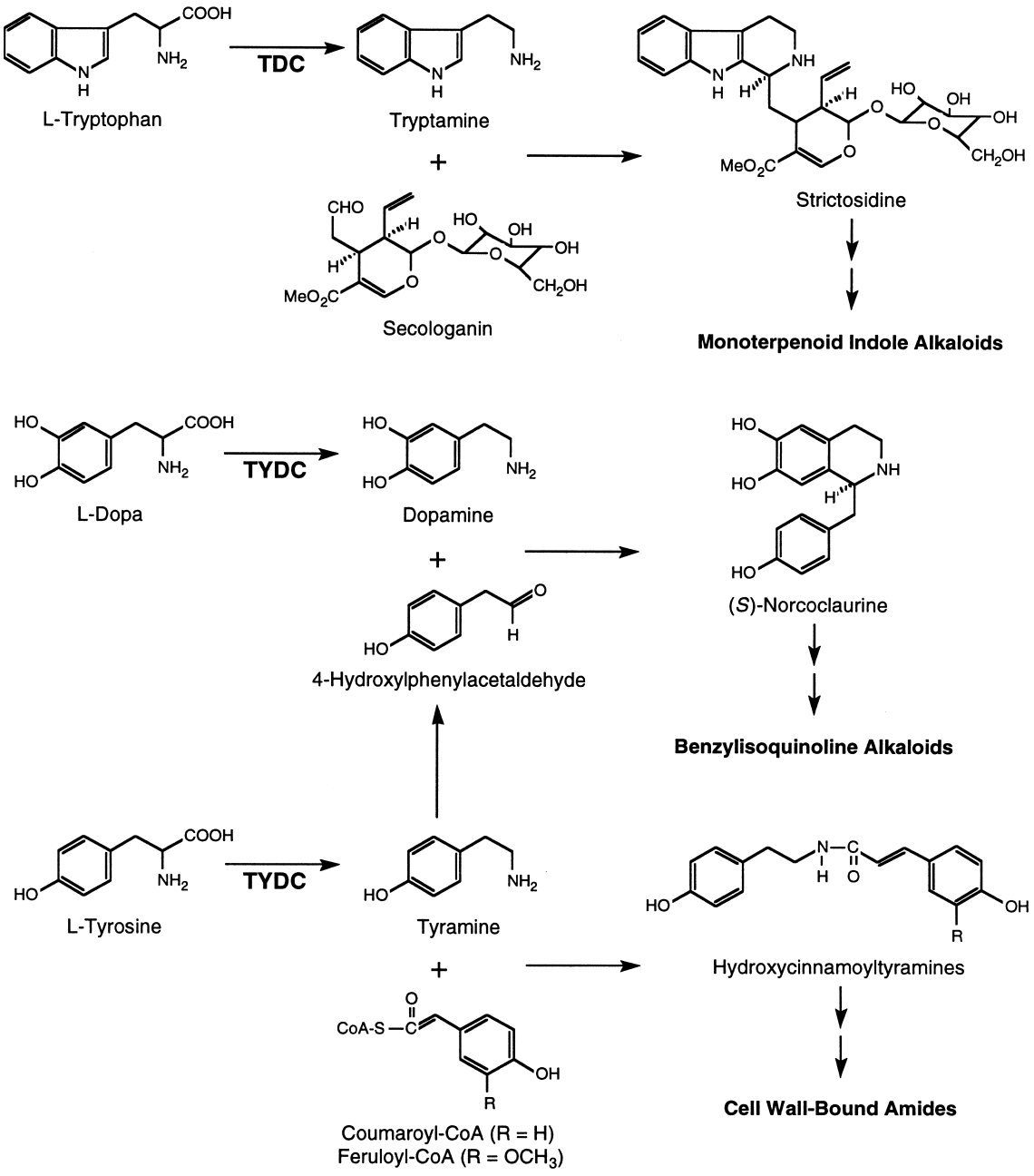

Fig. 1. Major biosynthetic functions of L-tryptophan decarboxylase (TDC) and L-tyrosine/L–Dopa decarboxylase (TYDC) in plant metabolic pathways leading to monoterpenoid indole alkaloids, benzylisoquinoline alkaloids, and cell wall-bound hydroxycinnamic acid amides.

tothecin (a potential anticancer agent) from *Camptotheca accuminata*; and strychnine (a violent tetanic poison used in homeopathy) from *Strychnos nux-vomica*. All monoterpenoid indole alkaloids are derived from the common intermediate strictosidine (Meijer et al., 1993), which is produced by a condensation reaction between tryptamine and the monoterpenoid secologanin and catalyzed by the enzyme strictosidine synthase (STR) (Fig. 1).

The decarboxylation of Tyr appears to be a ubiquitous enzymatic function in plants that plays a role in several different metabolic pathways. For example, TYDC supports the biosynthesis of the hydroxyphenylethanol glycoside, verbascoside, in *Syringa vulgaris* (Ellis, 1983), and the simple alkaloid phytoalexin, hordinine, in *Hordeum vulgare* (Leete and Marion, 1953). Recently, tyramine has also been detected as a jasmonic acid conjugate in petunia pollen (Miersch et al., 1998). TYDC is also involved in the biosynthesis of complex alkaloids, primarily of the benzylisoquinoline type, which comprises more than 2500 known compounds found mainly in five plant families. Benzylisoquinoline alkaloids are also pharmacologically active and include several widely prescribed pharmaceuticals, such as, morphine, codeine (analgesic and antitussive drugs), and papaverine (a muscle relaxant), from *Papaver somniferum*; colchicine (a microtubule disrupter and gout suppressant) from *Colchicum autumnale*; sanguinarine (an antibiotic used in oral hygeine products) from *Sanguinaria canadensis*; and (+)-tubocurarine (a nondepolarizing muscle relaxant, used as an adjuvant to anesthesia, that produces temporary paralysis) from *Chondodendron tomentosum*. Benzylisoquinoline alkaloid biosynthesis begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA) to form the first committed intermediate, (*S*)-norcoclaurine (Stadler et al., 1987) (Fig. 1). Both dopamine and 4-HPAA are derived from Tyr, but their synthesis has not been unequivocally characterized. Dopamine synthesis could result from the decarboxylation of Dopa or the hydroxylation of tyramine (Rueffer and Zenk, 1987). The capacity of TYDC to decarboxylate both Tyr and Dopa (Facchini and De Luca, 1994; Facchini and De Luca, 1995a) suggests that dopamine might be synthesized by both routes. Similarly, 4-HPAA synthesis could occur via the decarboxylation of 4-hydroxyphenylpyruvate or the oxidation of tyramine (Rueffer and Zenk, 1987); thus, TYDC is probably involved in the formation of both dopamine and 4-HPAA.

In addition to its role as an alkaloid precursor, tyramine has also been implicated as a limiting factor for sexual reproduction in tobacco (Martin-Tanguy et al., 1996), a constitutive cell wall component in xylem (Negrel and Lherminier, 1987), a cell wall constituent of wound-induced periderm (Borg-Olivier and Monties, 1993), and both as a free amine and as a hydroxycinnamic acid-conjugated amide deposited in the cell wall in response to viral infection (Negrel and Jeandet, 1987). The role of tyramine in the general physiology of plants, and as an integral component of the plant defense response, is also suggested by reports on the isolation of *TYDC* genes from species such as, parsley (Kawalleck et al., 1993) and Arabidopsis (Trezzini et al., 1993) that do not produce benzylisoquinoline alkaloids. *TYDC* appears to be an important and ubiquitous plant defense response gene which is induced by pathogens and participates in cell wall-bound hydroxycinnamic acid amide biosynthesis (Facchini et al., 1999). The deposition of hydroxycinnamic acid amides, and other phenolics, in the cell wall is believed to create a barrier against pathogens by reducing cell wall digestibility and/or by directly inhibiting the growth of fungal hyphae. Amides are formed by the transfer of hydroxycinnamic acids from hydroxycinnamoyl-CoA esters to hydroxyphenethylamines, such as, tyramine. Hydroxycinnamoyl-CoA: tyramine *N*-(hydroxycinnamoyl)transferase (THT) has been purified from several plants, including a benzylisoquinoline alkaloid-producing species (Yu and Facchini, 1999), and catalyzes the condensation of various hydroxycinnamoyl-CoA esters, especially coumaroyl- and feruloyl-CoA, with tyramine (Fig. 1).

In plants, tyramine is also one of several structurally related substituted phenethylamines, such as, dopamine, norepinephrine, adrenaline, octopamine, and *N*-methyltyramine, that exhibit extreme toxicity toward wild-type callus tissue cultures of many species, including tobacco, soybean, corn and sunflower (Christou and Barton, 1989). In contrast, crown gall tissues, generated by genetic transformation with modified strains of *Agrobacterium tumefaciens*, are resistant to the toxicity of substituted phenethylamines, including tyramine. The resistance of crown gall tissues was shown to be related to the expression of isopentyl transferase, a key enzyme in cytokinin biosynthesis (Christou and Barton, 1989). Tyramine toxicity in plant tissues occurs rapidly, but can be completely overcome by pretreatment of the tissue with exogenous cytokinin. The biochemical basis for the resistance of tobacco crown gall and callus cultures grown in the presence of cytokinins was investigated by Negrel et al. (1993). The rapid formation of black oxidation products from tyramine in sensitive tissues is caused by the activity of polyphenol oxidase (PPO), which is induced by auxin. The toxicity of tyramine was found to be due to the formation of indole quinolines after oxidation by PPOs. Growth of cultures on media that included the cytokinin benzyladenine caused a decrease in PPO activity, and an increase in THT and peroxidase activities (Negrel et al., 1993); thus, tyramine produced in cytokinin-treated or crown gall cultures is rapidly inte-

grated into cell walls. The sequestration of monoamines, such as, tyramine, to the cell wall as insoluble amides appears to be a mechanism to control their toxicity as soluble metabolites in plants. Based on its abundant overproduction in several transgenic species (Ibrahim et al., 1994), tryptamine does not exhibit the same cytotoxicity as tyramine. The differential cytotoxicity of aromatic amines has important implications for the metabolic engineering of AADCs.

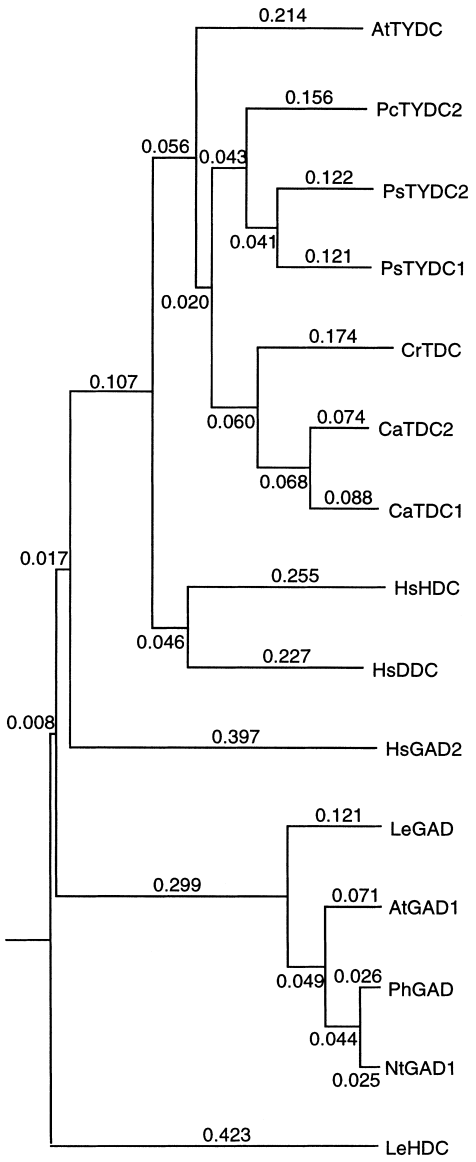

Fig. 2. Phylogenetic relationship among L-tryptophan decarboxylase (TDC), L-tyrosine/L–Dopa decarboxylase (TYDC), L-glutamate decarboxylases (GAD), and L-histidine decarboxylase (HDC) from various plants. L-Dopa decarboxylase (DDC) and other homologous human enzymes are shown as representatives of the highly conserved group II amino acid decarboxylases in animals. The length of the horizontal lines connecting the different proteins is proportional to the estimated genetic distance between the corresponding amino acid sequences.

3. Evolutionary origins of plant aromatic amino acid decarboxylases

Sandmeier et al. (1994) compared the amino acid sequences from nine different PLP-dependent amino acid decarboxylases and grouped the enzymes into four distinct classes that appear to be evolutionarily unrelated to one another. The classes were defined as follows: group I, represented by glycine decarboxylase; group II, comprised of L-glutamate decarboxylase (GAD), L-histidine decarboxylase (HDC), DDC, TYDC, and TDC; group III, which includes prokaryotic forms of ornithine and lysine decarboxylases, and the prokaryotic biodegradative type of arginine decarboxylase; and group IV, comprised of eukaryotic forms of ornithine and lysine decarboxylases, and the prokaryotic biosynthetic type of arginine and diaminopimelate decarboxylases (Sandmeier et al., 1994). The various groups appear to have evolved along separate lineages, even in some cases, when substrate specificities are identical. The evolutionarily conserved group II enzymes, which include all known AADCs in both plants and animals, also appear to be distantly related to amino transferases and other members of the alpha family of PLP-dependent enzymes (Sandmeier et al., 1994).

Significant and extensive sequence similarities among prokaryotic and eukaryotic PLP-dependent decarboxylases have also been identified (Jackson, 1990). Quantitative methods used to calculate the relative evolutionary distances between representative pairs of group II decarboxylases strongly suggest a common and ancient evolutionary origin (Jackson, 1990). The phylogenetic relationship among all group II enzymes reported from plants, together with human DDC, GAD, and HDC, as representative examples of the homologous animal enzymes, is shown in Fig. 2. The phylogeny of group II decarboxylases is generally established along functional lines, although there are some notable exceptions. Among species, plant AADCs exhibit amino acid identities between 50 and 68%, with subgroups that demarcate the specificity of the enzymes for Trp or Tyr (Fig. 2). Likewise, GADs which have been isolated from petunia (Baum et al., 1993), tomato (Gallego et al., 1995), Arabidopsis (Turano and Fang, 1998), and tobacco (Yun and Oh, 1998), show identities between 75 and 95%, but substantially less identity is found with mammalian GAD (Fig. 2). Mammalian GADs, represented by human GAD2 (Karlsen et al., 1991) in Fig. 2, display greater sequence conservation with plant and animal AADCs, than with plant GADs. Interestingly, plant GADs share considerably more identity (44%) with GAD from *Escherichia coli* than they do with GADs from eukaryotic organisms (Baum et al., 1993). The overall identity between plant GADs and AADCs is only

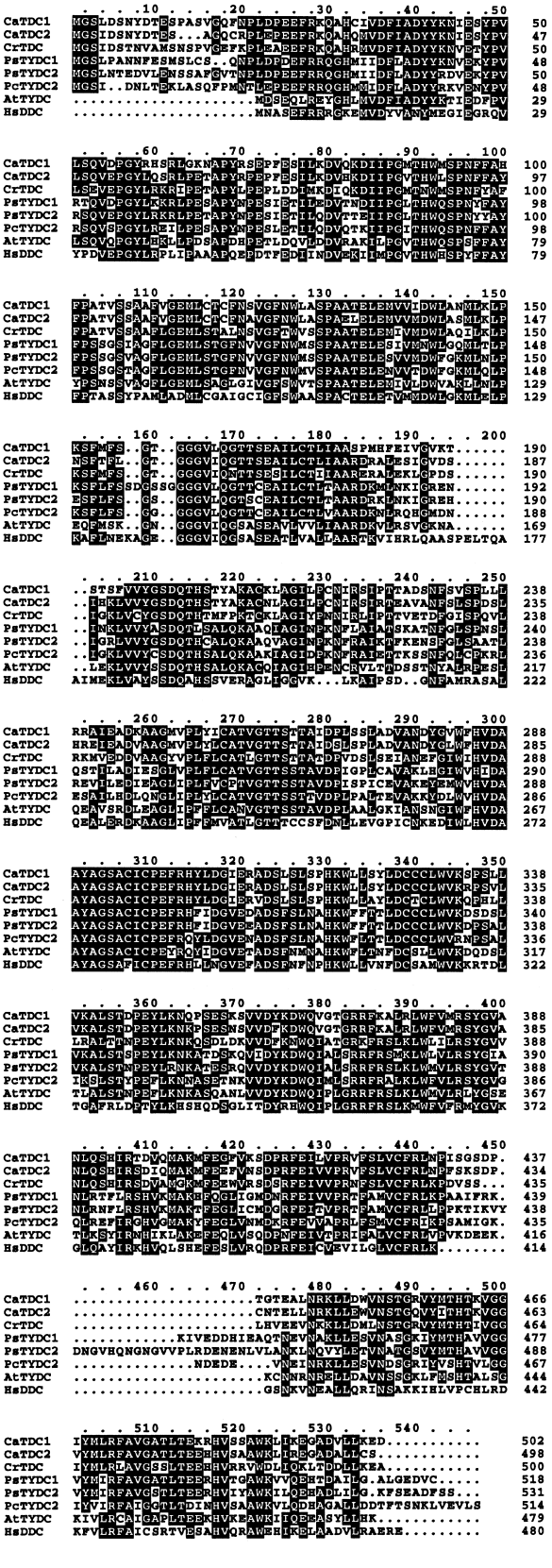

Fig. 3. (*Caption opposite*).

about 12–15%. However, the homology between plant GADs and AADCs is more apparent when the PLP binding domains are compared. One reason for the low overall homology between plant GADs and other PLP-dependent amino acid decarboxylases is the presence of a calmodulin binding domain in the plant protein, as part of a carboxyl-end extension (Baum et al., 1993); thus, the regulation of plant GAD appears to involve Ca^{2+} as a second messenger. GAD catalyzes the conversion of glutamic acid to γ-aminobutyric acid (GABA). In vertebrates and invertebrates, GABA functions as a major inhibitory neurotransmitter by modulating the function of ion channels. In plants, the role of GABA has not been established, but it accumulates in response to hypoxia, cold and heat shock, drought, and mechanical stress, and might be involved in signal transduction (Baum et al., 1993; Turano and Fang, 1998).

AADCs from plants and animals share between 27 and 37% identity with animal HDCs, which are represented by human HDC (Yamauchi et al., 1990) in Fig. 2. However, an HDC from tomato that shares strong homology with PLP-dependent HDCs from prokaryotes (Picton et al., 1993), shows only cursory homology with mammalian HDCs, and eukaryotic AADCs (Fig. 2). As with GAD, the homology between tomato HDC and plant AADCs in the putative PLP binding domain is considerably higher than the overall amino acid identity. Decarboxylation of His results in the formation of histamine, which is associated with allergic reactions and smooth muscle contraction in mammals. The roles for HDC and histamine in plants are unknown, although HDC transcripts were found to accumulate in tomato fruit during the early stages of ripening (Picton et al., 1993).

An alignment of the deduced amino acid sequences for plant and human (Ichinose et al., 1989) AADCs is shown in Fig. 3. These enzymes display extensive amino acid identity that reveals a remarkable degree

Fig. 3. Alignment of the deduced amino acid sequences for various aromatic L-amino acid decarboxylases from plants and animals: AtTYDC, *Arabidopsis thaliana* putative tyrosine decarboxylase (GenBank accession number AC006569); PsTYDC1, *Papaver somniferum* tyrosine/Dopa decarboxylase-1 (U08597); PsTYDC2, P. *somniferum* tyrosine/Dopa decarboxylase-2 (U08598); PcTYDC2, *Petroselinum crispum* tyrosine decarboxylase-2 (M96070); CrTDC, *Catharanthus roseus* tryptophan decarboxylase (M25151); CaTDC1, *Camptotheca accuminata* tryptophan decarboxylase-1 (U73656); CaTDC2, C. *accuminata* tryptophan decarboxylase-2 (U73657); HsDDC, *Homo sapiens* Dopa decarboxylase (M76180). Shaded boxes indicate residues that are identical in at least 50% of the aligned proteins. Gaps introduced into sequences to maximize alignments are represented by dots.

of sequence conservation through several evolutionary boundaries, including the divergence of plants and animals and the establishment of substrate-specific AADCs in various plant species. The alignment of several homologous, yet substrate-specific, AADCs reveals the location of both variable and invariant, or conservatively substituted residues that allows for the rational design of specific functional mutants to further investigate the catalytic properties of AADCs.

4. Biochemical and molecular properties of plant aromatic amino acid decarboxylases

The remarkable sequence fidelity among all aromatic amino acid decarboxylases, regardless of their origin or substrate specificity, is underscored by the extensive similarity of their hydropathy profiles (Fig. 4). For example, human DDC accepts a broad range of aromatic amino acid substrates and shares ~40% amino

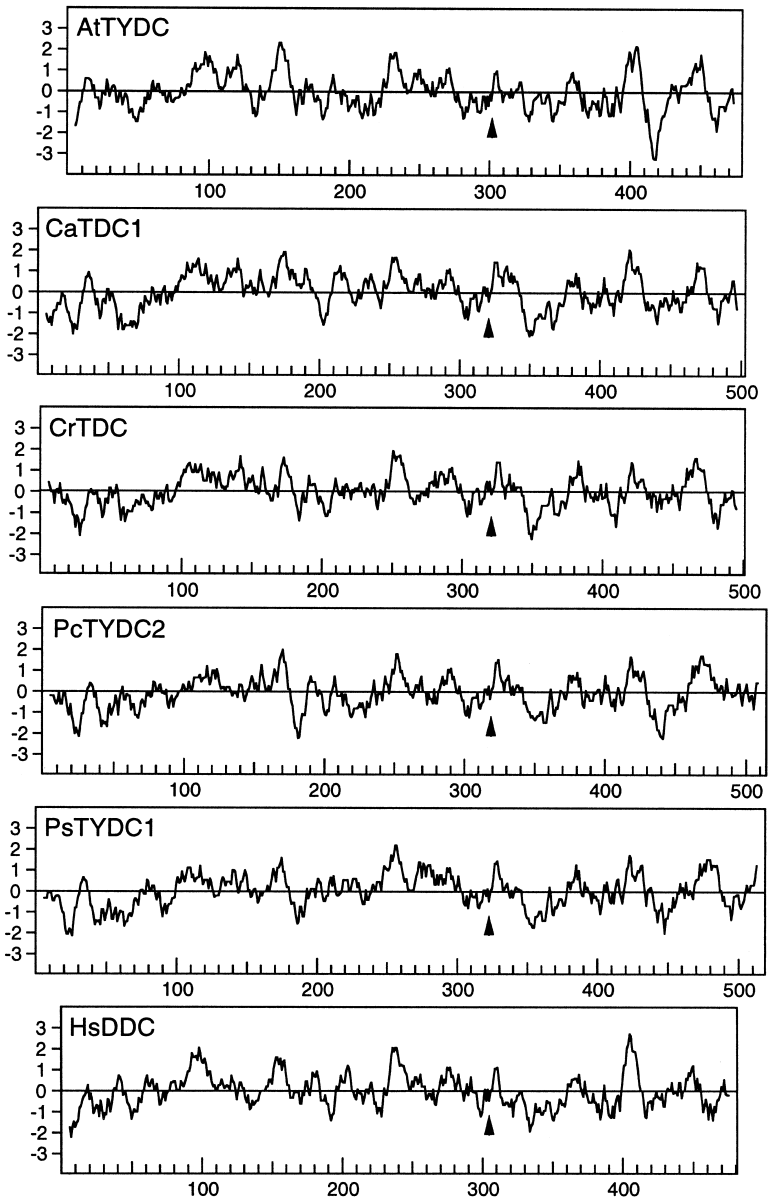

Fig. 4. Hydropathy profiles for various aromatic L-amino acid decarboxylases from plants and animals: AtTYDC, *Arabidopsis thaliana* putative tyrosine decarboxylase; PsTYDC1, *Papaver somniferum* tyrosine/Dopa decarboxylase-1; PcTYDC2, *Petroselinum crispum* tyrosine decarboxylase-2; CrTDC, *Catharanthus roseus* tryptophan decarboxylase; CaTDC1, *Camptotheca accuminata* tryptophan decarboxylase-1; HsDDC, *Homo sapiens* Dopa decarboxylase. The average hydrophobic index of nine consecutive amino acids was assigned to the middle residue for each amino acid in the protein. Positive and negative values indicate hydrophobic and hydrophilic domains, respectively. The approximate locations of putative pyridoxal-5′-phosphate binding sites are indicated by arrowheads.

acid identity with the highly substrate-specific AADCs from plants. Few distinct hydropathic domains can be found in the human DDC when it is compared with plant TYDCs and TDCs. Moreover, the extensive similarity in the hydropathy plots for TDC and TYDC is in marked contrast to their absolute substrate specificity for either indole or phenol side chains, respectively. It can be concluded that the substrate specificity of various AADCs is probably the result of relatively minor amino acid substitutions.

Recent kinetic (Black and Smarrelli, 1986; Bertoldi et al., 1996; Bertoldi et al., 1998; Hayashi et al., 1993) and site-specific mutagenesis (Ishii et al., 1996) studies support the classic catalytic mechanism

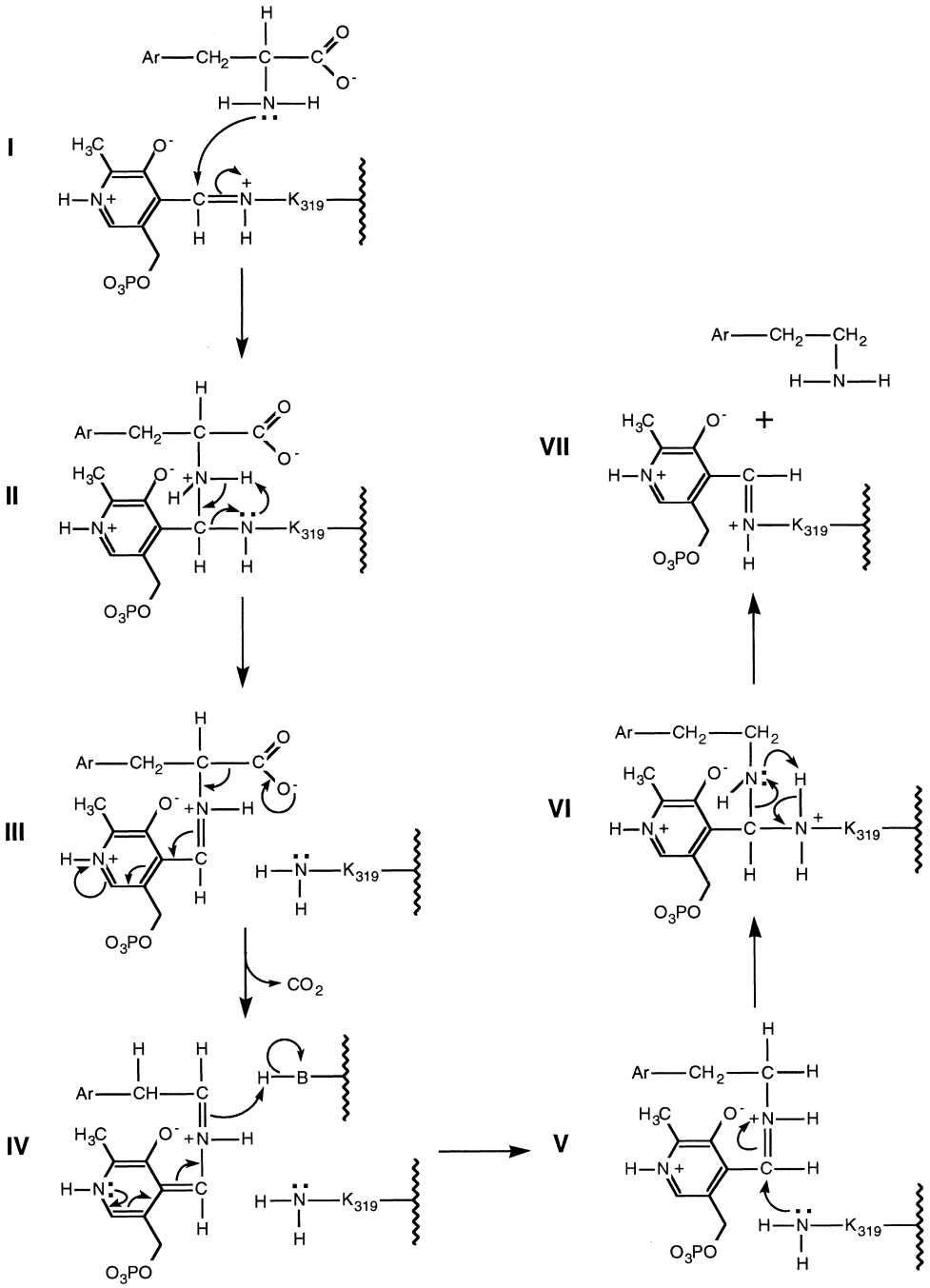

Fig. 5. Putative reaction mechanism for the PLP-dependent decarboxylation of aromatic L-amino acids by AADCs. Ar refers to the aromatic side chain of Trp, Tyr, or Dopa. In the scheme illustrated, PLP forms a Schiff base with the ϵ-amino group of K_{319} in the PLP binding domain of *Catharanthus roseus* TDC or *Papaver somniferum* TYDC2. Interconversion of the Lys-PLP Schiff base with the substrate-PLP Schiff base proceeds without release or incorporation of a water molecule (transaldimination).

for PLP-dependent enzymes shown in Fig. 5 (Metzler et al., 1954; Boeker and Snell, 1972), which should be directly applicable to plant TDC- and TYDC-catalyzed reactions. Although no direct work has been done to verify the reaction mechanism for TDC or TYDC from plants, the extensive sequence and structural homology between AADCs from diverse eukaryotic sources (Fig. 3) implies that the catalytic mechanism is conserved among AADCs from both plants and animals.

In their substrate-free form, AADCs are associated with the coenzyme PLP via a Schiff base linkage with the ϵ-amino group of a specific Lys residue (Lys_{303} in animal DDC and Lys_{319} in *C. roseus* TDC and *P. somniferum* TYDC2) (Bossa et al., 1977; Ishii et al., 1996). When the substrate enters the active site of the enzyme, the α-amino group of the substrate initiates a nucleophilic attack on the C-4′ of PLP (**I** in Fig. 5). This results in a transaldimination reaction (**I**, **II**, **III**), in which the enzyme-PLP Schiff base linkage is broken and a new Schiff base is formed between the substrate and PLP, thus liberating the ϵ-amino group of the PLP-binding Lys (Ishii et al., 1996). The substrate is then decarboxylated (**III**), producing a carbanion which is stabilized by the electron-accepting protonated pyridine ring of PLP (Nishino et al., 1997). The quinonoid intermediate that forms (**IV**) is then protonated at the α-carbon of the substrate to restore the aromatic character of the pyridine ring of PLP. The proton donor for this step is probably a His residue; an essential His residue has been identified in DDCs through chemical modification studies (Dominici et al., 1985) and site-directed mutagenesis (Ishii et al., 1996). Chemical modification of His residues in pig kidney DDC using diethylpyrocarbonate (DEPC) resulted in complete inactivation of the enzyme, but did not interfere with most of its ability to bind substrate (Dominici et al., 1985). Moreover, the DEPC-mediated inactivation of pig kidney DDC is completely reversible with hydroxylamine. These data support a crucial role for a His residue in catalysis, but not in substrate binding. In a second study using site-directed mutagenesis to identify key residues, it was shown that the mutation of His_{192} to Ala in rat liver DDC resulted in a 10^4-fold decrease in the activity of the enzyme (Ishii et al., 1996). Sequence alignments reveal that His_{192} in animal DDCs is almost fully conserved in all AADCs suggesting that it plays an important catalytic role (Fig. 3).

The species produced from protonation of the quinonoid intermediate (**V**) is then attacked by the nucleophilic ϵ-amino group of Lys_{319} at C-4′ of PLP. This attack represents the first step in another transaldimination reaction (**V**, **VI**, **VII**) which ultimately results in release of the amine product from the active site. Site-directed mutagenesis studies have shown that the PLP-binding Lys is essential for product release, but not for the decarboxylation step. When Lys_{303} in rat liver DDC was replaced with Ala, the mutagenized enzyme (K303A) retained the ability to bind PLP and decarboxylate L-Dopa, but the product was not released. Instead, an adduct was formed between the enzyme and the product. Also, the rate constant of K303A for the formation of the enzyme–substrate Schiff base was negligible compared with the native enzyme, suggesting that the PLP-binding Lys is essential in catalyzing Schiff base formation. This is also supported by the fact that Schiff bases are more susceptible to nucleophilic attack than free aldehydes (Nishino et al., 1997).

Ishii et al. (1996) have suggested possible roles for other residues in animal AADCs, based on sequence alignments and site-directed mutagenesis. For example, Arg_{355} (Arg_{361} in *C. roseus* TDC and *P. somniferum* TYDC2) is invariant among AADCs, and might be important for recognition of the α-carboxylate group of the substrate through electrostatic interactions. When this residue was replaced with Ala, the enzyme activity decreased 10^4-fold. However, replacement with Lys showed almost no effect on enzyme activity, indicating that the presence of a positively charged residue at this position is necessary and sufficient for catalysis. The negatively charged carboxylate group of Asp_{271} (in animal AADCs) is believed to be involved in stabilization of the protonated form of the pyridine nitrogen of PLP (Ishii et al., 1996). This residue is absolutely conserved in all AADC sequences, at a position 32–33 amino acids before the PLP-binding Lys. This residue corresponds to PLP-stabilizing Asp residues in other group II decarboxylases. When this residue was replaced with Ala, activity decreased by 10^4-fold, indicating that a negative charge in this position is essential (Ishii et al., 1996). Further mutagenic and structural studies must be performed on plant AADCs to evaluate the role of select residues on substrate binding and catalysis.

TDC has been isolated from several plant species (Grosse and Klapheck, 1979; Baxter and Slaytor, 1972; Gibson et al., 1972) and purified to apparent homogeneity from various *C. roseus* tissues (Noé et al., 1984; Fernandez et al., 1989a; Pennings et al., 1989a). The enzyme has been characterized as a soluble cytosolic protein (Stevens et al., 1993), with an isoelectric point of approximately 5.9 and a native M_r of 115,000, consisting of two identical subunits ($M_r = 54{,}000$). TDC has been shown to be a pyridoxoquinoprotein that contains one molecule of pyridoxal phosphate and one molecule of pyrroloquinoline quinone per subunit (Pennings et al., 1989b). The enzyme displays Michaelis–Menten kinetics and a high substrate specificity, since L-Tyr, L-Dopa, and L-Phe were not accepted as substrates. Only L-Trp and a variety of

Trp analogues were decarboxylated, including the naturally occurring amino acid L-5-hydroxytryptophan and the toxic analogues 4-fluorotryptophan, 5-fluorotryptophan, and 4-methyltryptophan (Sasse et al., 1983). D-Trp was found to be a non-competitive inhibitor, whereas tryptamine was a competitive inhibitor ($K_i = 0.31$ mM). The substrate specificities and reported K_m values for TDC from tomato, *C. roseus*, and *C. accuminata* are given in Table 1. All reported plant TDCs accept Trp as a substrate, but are inactive toward amino acids with phenol side chains, such as, Tyr and Dopa.

TYDC has also been isolated from a variety of plants (Gallon and Butt, 1971; Tocher and Tocher, 1972; Hosoi et al., 1970; Hosoi, 1974; Roberts and Antoun, 1978; Chapple, 1984; Skinner et al., 1987), and was purified to apparent homogeneity from California poppy *(Eschscholzia californica)* and meadow rue *(Thalictrum rugosum)* (Marques and Brodelius, 1988a). Native TYDC was found to be a homodimeric enzyme with a $M_r = 112{,}000$. The isoelectric point was estimated to be between pH 5.2 and 5.4 which is close to, but slightly lower than that determined for TDC (Noé et al., 1984). The enzyme decarboxylated L-Tyr and L-Dopa, but was inactive toward L-Phe and L-Trp. The higher K_m of TYDC for Tyr, relative to that of TDC for Trp (Table 1), might reflect the larger intracellular pool of soluble Tyr and/or the more rapid flux of Tyr biosynthesis, as compared to Trp. All reported plant TYDCs accept Tyr and Dopa as substrates, but are not active towards Trp (Table 1). The relative activity toward Tyr and Dopa is species-dependent; TYDC from parsley shows the strongest preference for Tyr (3:1 over Dopa), whereas TYDC from *Sanguinaria* has the greatest affinity for Dopa (30:1 over Tyr).

Several potent inhibitors of animal AADCs have been reported, including the α-fluoromethyl analogues, α-fluoromethyl(3,4-dihydroxyphenyl)alanine (α-FMD) and α-fluoromethyltyrosine (α-FMT) (Maycock et al., 1980). These suicide inhibitors are effective against some, but not all, plant AADCs (Chapple et al., 1986). For example, TYDC from *Hordeum vulgare* is strongly inhibited by α-FMD, but TYDCs from *Nicotiana tabacum* and *Sanguinaria canadensis* are relatively insensitive to α-fluoromethyl analogues. The variability might be linked to the proposed catalytic mechanism (Fig. 5), since the compounds are believed to enter the active site of AADCs and form a PLP-conjugate which becomes covalently linked to a specific functional group during the course of the decarboxylation reaction (Maycock et al., 1980). The benzylisoquinoline alkaloids, sanguinarine and chelerythrine, whose biosynthesis involves TYDC, also caused strong and irreversible inhibition of AADC activity (Drsata et al., 1996). Dithiothreitol prevented the inhibitory effect of sanguinarine and chelerythrine indicating that the mechanism of inhibition involves an interaction with thiol groups essential for AADC activity.

The Phe analogue L-α-aminoxy-β-phenylpropionate (AOPP), which is a potent in vivo inhibitor of PAL (Amrhein et al., 1983), was also found to inhibit TYDC preparations from a variety of plants, although TYDCs from *Syringa vulgaris* and *H. vulgare* were the most sensitive. Marques and Brodelius (1988b) also reported that *Eschscholzia* TYDC was strongly inhibited by AOPP, but largely unaffected by α-FMD and

Table 1
Substrate specificities of AADCs isolated from plants and animals

Organism	Enzyme	Relative enzyme activity (%)			Apparent K_m (mM)			Reference
		Tyr	Dopa	Trp	Tyr	Dopa	Trp	
Lycopersicon esculentum	TDC	0	nd[a]	100	–	nd	3.0	Gibson et al. (1972)
Catharanthus roseus	TDC	0	0	100	–	–	0.075 0.013[b]	Noé et al. (1984)
Camptotheca	TDC1	0	0	100	–	–	nd	López-Meyer and Nessler (1997)
Camptotheca accuminata	TDC2	0	0	100	–	–	nd	López-Meyer and Nessler (1997)
Papaver somniferum	TYDC1	90	100	0	1.0	1.0	–	Facchini and De Luca. (1995a)
Papaver soniferum	TYDC2	65	100	0	1.0	1.0	–	Facchini and De Luca (1995a)
Pertoselinum crispum	TYDC2	100	28	0	nd	nd	–	Kawalleck et al. (1993)
Thalictrum rugosum	TYDC	100	74	0	0.27	0.25	–	Marques and Brodelius (1988b)
Eschscholzia californica	TYDC	100	nd	0	1.0	1.1	–	Marques and Brodelius (1988b)
Syringa vulgaris	TYDC	100	63	0	0.33	nd	–	Chapple (1984)
Sanguinaria canadensis	TYDC	3	100	0	nd	nd	–	Chapple (1984)
Hordeum vulgare	TYDC	33	100	0	nd	nd	–	Chapple (1984)
Cytisus scoparius	TYDC	0	100	0	nd	nd	–	Tocher and Tocher (1972)
Sus scrofa (pig)	DDC	< 1	100	3 10[b]	8.4	0.19	1.0	Christenson et al. (1970)
Drosophila melanogaster	DDC	3	100	nd	3.3	0.08	nd	Clark et al. (1978)

[a] Not determined.
[b] Using 5-hydroxytryptophan.

α-FMT. No reports on the effectiveness of AOPP as an inhibitor of TDC have appeared, although it might be assumed that the mechanism of inhibition is conserved since aminoxy compounds, such as, AOPP, are generally inhibitors of carbonyl enzymes (Chapple et al., 1986). TYDC, TDC, and PAL all contain analogous carbonyl functional groups at their active sites; PLP in the case of TYDC and TDC, and dehydroalanyl in the case of PAL.

TYDC is encoded by a single-copy gene in *Arabidopsis* (Trezzini et al., 1993), and a small family of about four genes in parsley (Kawalleck et al., 1993). In contrast, the *TYDC* gene family in opium poppy is comprised of ~15 members that can be divided into two subgroups, based on sequence identity, represented by *TYDC1* and *TYDC2* (Facchini and De Luca, 1994). At least some *TYDC* genes in the opium poppy genome are clustered, since *TYDC8* and *TYDC9* were located only 3.2 kb apart on the same genomic clone with their transcription units oriented in opposite directions (Facchini et al., 1998). TDC is encoded by a single-copy gene in *C. roseus* (De Luca et al., 1989; Goddijn et al., 1994), but by two autonomously regulated genes, *TDC1* and *TDC2*, in *C. accuminata* (López-Meyer and Nessler, 1997). All plant genes encoding AADCs lack introns, except for the *TYDC* gene from *Arabidopsis* which contains 12 introns (GenBank accession number AC006569; chromosome II BAC F11A3 genomic sequence). The lack of introns in most AADC genes from plants suggests that the introns in the *Arabidopsis TYDC* gene are the result of relatively recent genomic insertions.

5. Regulation of aromatic amino acid decarboxylases in plants

Much of the research on AADCs in plants has focused on the regulation of gene expression in alkaloid-producing species. *TDC* and *TYDC* both exhibit developmental, tissue-specific, and inducible expression. TDC activity and mRNA levels are transiently induced in developing seedlings of *C. roseus* (De Luca et al., 1988), *Cinchona ledgeriana* (Aerts et al., 1990), and *C. accuminata* (López-Meyer and Nessler, 1997), three indole alkaloid-producing plants. Maximum TDC activity or mRNA levels were detected approximately 5–10 days after imbibition in each species, and were coordinated with the appearance of other enzymes involved in alkaloid biosynthesis. Only one (*TDC1*) of the two genes encoding TDC in *C. accuminata* was expressed during seedling development, or in the mature plant. TYDC mRNAs are also transiently expressed during opium poppy seedling development, with maximum levels occurring during the emergence of the radicle, between 3 and 6 days after imbibition (Facchini et al., 1998; Maldonado-Mendoza et al., 1996). *TYDC2*-like genes appeared to be expressed at much higher levels than *TYDC1*-like genes during opium poppy seedling development (Facchini et al., 1998).

In mature *C. roseus* plants, *TDC* and *STR* gene expression was shown to be coordinately regulated (Pasquali et al., 1992). For both genes, the highest mRNA levels were found in roots, with much lower levels in leaves, stems and floral organs. The relative abundance of TDC mRNAs was correlated with the relative accumulation of total indole alkaloids produced in various *C. roseus* organs. St-Pierre et al. (1999) used in situ hybridization and immunocytochemistry to establish the cellular distribution of TDC, STR, and two late steps in indole alkaloid biosynthesis, desacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline 4-*O*-acetyltransferase (DAT). TDC and STR mRNAs and proteins were found specifically in the epidermis of stems, leaves, and flower buds, whereas they were most abundant in the protoderm and cortical cells around the root apical meristem. In contrast, D4H and DAT were specifically associated with laticifers and idioblasts in leaves, stems, and flower buds; thus, a pathway intermediate derived from tryptamine requires intercellular translocation to complete the biosynthesis of indole alkaloids in *C. roseus*.

In *C. accuminata*, TDC1 mRNAs accumulate most abundantly in the shoot apex and stems, to a lesser extent in leaves, fruits, and seeds, but only to low levels in roots (López-Meyer and Nessler, 1997). The relative abundance of TDC1 mRNAs was correlated with the relative accumulation of the indole alkaloid camptothecin. Expression of *TDC2* in *C. accuminata* only occurred after treatment of plant tissues with elicitors, such as, yeast extract or methyl jasmonate. The autonomous regulation of these genes suggests that *TDC1* participates in a developmentally regulated chemical defense system in *C. accuminata*, whereas *TDC2* is part of a defense mechanism induced during pathogen challenge (López-Meyer and Nessler, 1997).

TYDC is encoded by a large gene family in opium poppy; thus, differential expression of TYDC genes is not unexpected (Facchini and De Luca, 1994, 1995b; Facchini et al., 1998). Members of the *TYDC1*-like subgroup are abundantly expressed only in roots, whereas members of the *TYDC2*-like subgroup are mostly expressed in roots and stems. However, individual *TYDC* genes within each subgroup exhibit further differential regulation (Facchini et al., 1998). The association of TYDC enzyme activity with the alkaloid-containing latex in opium poppy is well-established (Roberts and Antoun, 1978; Roberts et al., 1983). In situ hybridization using antisense TYDC1 and TYDC2 mRNAs as probes supports the expression of *TYDC*

genes in laticifer-rich regions of the phloem in stems and roots (Facchini and De Luca, 1995b).

TYDC gene expression was further studied by introducing six different opium poppy *TYDC* promoter-β-glucuronidase (*GUS*) fusions into transgenic tobacco. GUS activity was measured, and histochemically localized, in various organs and tissues of the transgenic plants (Facchini et al., 1998; Maldonado-Mendoza et al., 1996). The organ- and tissue-specific expression pattern of *TYDC* promoter-*GUS* fusions in transgenic tobacco paralleled that of corresponding *TYDC* genes in opium poppy. In mature plants, *GUS* expression was most abundant in the internal phloem of shoot organs, and the stele in roots. Select *TYDC* promoter-*GUS* fusions were also wound-induced in transgenic tobacco, suggesting that the basic mechanisms of developmental and inducible *TYDC* expression are conserved across species. A conserved mechanism of transcriptional regulation for *TYDC* genes in plants is consistent with the ubiquitous role of TYDC in hydroxycinnamic acid amide biosynthesis.

The regulation of AADCs has been studied most extensively in plant cell suspension cultures because of the relative simplicity of manipulating the metabolism of cells grown in vitro, compared to intact plants. For example, when cell suspension cultures of *C. roseus* were transferred from growth media to 'alkaloid production media', which is characterized by a high concentration of sucrose, but low levels of growth regulators, phosphate, and nitrogen-containing compounds (Knobloch et al., 1981), increased indole alkaloid accumulation is generally associated with induced TDC activity. Although such results suggest that TDC represents a rate-limiting step in indole alkaloid biosynthesis, TDC induction alone is not sufficient for an increase in alkaloid biosynthesis (Knobloch and Berlin, 1983; Mérillon et al., 1986). The induction of TDC activity in response to altered media composition occurs at the level of transcription of the *TDC* gene in *C. roseus* cultures. Other factors, including exposure of the cells to fungal elicitors (Pasquali et al., 1992; Noé and Berlin, 1985; Eilert et al., 1987; Roewer et al., 1992) and treatment with auxin (Aerts et al., 1992; Goddijn et al., 1992) also induce accumulation of TDC mRNAs.

Application of auxin, either indolebuteric acid (IBA) or 2,4-dichlorophenoxyacetic acid (2,4-D) also enhanced and prolonged the increase in TDC activity in developing *C. roseus* seedlings (Aerts et al., 1992). Although TDC activity and immunoreactive anti-TDC protein levels were similar in the aerial parts of auxin-treated and control seedlings, TDC activity and polypeptide levels were found in radicles of auxin-treated, but not in control, seedlings. In contrast, the addition of 1-naphthaleneacetic acid (NAA), IAA, or 2,4-D to *C. roseus* cell suspension cultures grown in auxin-free media resulted in the transcriptional down-regulation of *TDC* gene expression (Goddijn et al., 1992). Hairy roots transformed with *Agrobacterium rhizogenes* also showed a reduction of TDC mRNAs after NAA treatment. The apparent inconsistency of the effects of auxins on TDC activity in seedlings and cell cultures indicates that generalizations cannot be made when comparing two physiologically different systems, such as, seedlings and cell cultures.

Treatment of *T. rugosum* (Gügler et al., 1988) and opium poppy (Facchini et al., 1996) cell cultures with fungal elicitors also caused a rapid and transient induction in TYDC activity and mRNA levels. In opium poppy cell cultures, TYDC1-like mRNA levels were induced rapidly but declined to near baseline levels within 5 h. In contrast, TYDC2-like transcript levels increased more slowly, but were sustained for an extended period. Overall, induction of TYDC mRNAs preceded that of PAL mRNAs. The inducible expression of TYDC genes in parsley was also demonstrated by elicitor treatment of cell suspension cultures (Kawalleck et al., 1993) by in situ hybridization of leaf tissue infected with *Phytophthora megasperma* (Schmelzer et al., 1989). The transient and local accumulation of TYDC mRNAs around infection sites demonstrates that the elicitor induced expression of TYDC genes is cell suspension cultures in physiologically relevant.

A few basic components of the signal transduction mechanisms involved in the inducible expression of AADC genes have been identified. The induction of *TDC* by fungal elicitors in *C. roseus* cultures was found to involve the jasmonate biosynthetic pathway and protein phosphorylation in the signal transduction pathway (Menke et al., 1999). Exogenous methyl jasmonate or the jasmonate precursor α-linolenic acid induced expression of *TDC* and *STR* (Menke et al., 1999). Exposure to methyl jasmonate vapour also resulted in an increase in TDC activity in *C. roseus* seedlings (Aerts et al., 1994; Vazquez-Flota and De Luca, 1998). In contrast, diethyldithiocarbamic acid, a potent inhibitor of jasmonate biosynthesis, or the protein phosphatase inhibitor K-252a, blocked the elicitor-induced production of jasmonate and expression of *TDC* and *STR* genes in *C. roseus* cultures (Menke et al., 1999). The induction of *TYDC* by fungal elicitors in opium poppy cell cultures was also shown to involve protein phosphorylation/dephosphorylation as part of the signal transduction pathway. Staurosporine, a protein kinase inhibitor, and okadaic acid, an inhibitor of protein phosphatases 1 and 2A, blocked the induction of TYDC1-like, but not TYDC2-like or PAL, mRNAs. These results suggest that separate processes are involved in the induction of *TYDC* and other defense response genes in opium poppy. Moreover, methyl jasmonate-induced expression of *TYDC* genes has also been demonstrated in cell cultures of

opium poppy (Facchini et al., 1996) and parsley (Ellard-Ivey and Douglas, 1996). Although the expression levels of some *TYDC* genes in opium poppy increased after exposure to methyl jasmonate, sanguinarine accumulation was not induced by methyl jasmonate treatment (Facchini et al., 1996). It would appear that jasmonate might be involved as a signal in the developmental and elicitor-induced activation of AADC genes in plants, but the regulation of these steps is ncoupled from other genes involved in the biosynthesis of some secondary metabolites derived from Trp and Tyr.

The expression characteristics of the *C. roseus* TDC promoter, linked to the *GUS* reporter gene, were examined in transgenic tobacco plants, and in transiently transfected tobacco protoplasts (Goddijn et al., 1994). No differences in *GUS* activities between leaf, stem, and root were detected in tobacco plants transformed with a 2-kb *TDC* promoter-*GUS* construct. The lack of specificity of the *TDC* promoter in transgenic tobacco is in contrast to the organ-specific accumulation of TDC mRNA in *C. roseus* plants (Goddijn et al., 1992). Successive deletions of the 2-kb *TDC* promoter to −398 resulted in reduced levels of GUS activity, but did not affect the expression pattern of the reporter gene in transgenic tobacco. Further deletion to −232 resulted in complete elimination of GUS activity in the transgenic plants, although this promoter remained functional in transiently transfected tobacco protoplasts (Goddijn et al., 1994). Promoter regions from *TDC* were further studied in transgenic tobacco, by a loss-of-function assay, to identify *cis-elements* involved in basal expression or elicitor induction (Ouwerkerk and Memelink, 1999). Three functional regions in the *TDC* promoter from −160 to −37, relative to the start of transcription, have been identified. The region from −160 to −99 was shown to act as the main transcriptional enhancer, whereas two separate elicitor-responsive elements were found between −99 and −87 and between −87 and −37.

Deletion analysis of the *TYDC7* gene from opium poppy revealed the location of putative regulatory domains necessary for the expression of the *GUS* reporter gene in a transient assay system based on microprojectile bombardment of cultured opium poppy cells (Park et al., 1999). A region between −393 and −287 was found to be essential for promoter activity. A time-course for the induction of TYDC7 mRNAs in wounded cells was nearly identical to that for GUS activity in cells bombarded with promoter-*GUS* constructs when the −393 to −287 region of *TYDC7* was present. These results suggests that the wound signal caused by the entry of DNA-coated microcarriers into opium poppy cells was sufficient to induce *TYDC7* promoter activity, and that wound-responsive regulatory elements are located within the −393 to −287 region. Although *TDC* and *TYDC* promoter analyses have begun to reveal the location of *cis*-elements involved in gene activation, much work remains to be done on the molecular mechanisms that regulate AADC gene expression in plants.

Immunoblot analysis has shown that TDC activity follows the relative abundance of the TDC protein (Fernandez et al., 1989a), supporting the role of transcription as the primary point of regulation. However, the decrease in TDC activity during seedling development is also accompanied by the appearance of several minor immunoreactive polypeptides (Fernandez and De Luca, 1994). Two of these minor proteins of M_r 63,000 and 68,000 were detectable with both anti-TDC and anti-ubiquitin antibodies. Addition of ATP to crude extracts caused a rapid loss of TDC activity and the appearance of proteolytically processed forms that react with anti-TDC antibodies (Fernandez et al., 1989b; Fernandez and De Luca, 1994). The appearance of these processed forms of TDC could be prevented by treatment with hemin, an inhibitor of ubiquitin-mediated proteolysis. Cell free studies suggest that TDC might exist in equilibrium between a dimeric active form and a monomeric inactive form that is susceptible to irreversible degradation (Fernandez et al., 1989b). When the equilibrium shifts to monomer production, TDC is rapidly ubiquitinated (predominantly once or twice), before it is degraded by an ATP-dependent proteolytic process; thus, while TDC activity is clearly regulated at the transciptional level, the ubiquitin pathway appears to play an important post-translational role in regulating the half-life of the enzyme. Although there is no direct evidence, the extensive sequence identity between TDC and TYDC suggests that the latter might also be ubiquitinated and post-translationally regulated in a manner similar to that of TDC.

6. Metabolic modifications involving aromatic amino acid decarboxylases in transgenic plants

Metabolic engineering is broadly defined as the improvement of cellular activities by the manipulation of enzymatic, transport, and regulatory functions using recombinant DNA technology (Bailey, 1991). Isolated *TDC* and, more recently, *TYDC* genes have been used to genetically alter the regulation of secondary metabolic pathways derived from aromatic amino acids in several plant species (Table 2). The biotechnological objectives of these modifications include: (1) increasing the accumulation of valuable pharmaceuticals in medicinal plants, or introducing novel phytochemical pathways into plants of agronomic importance; (2) reducing the accumulation of undesirable compounds in plant-derived products; and (3) improving the resist-

ance of crop species against pests and disease-causing pathogens. However, these efforts to alter plant metabolic pathways using *TDC* and *TYDC* genes have often produced unpredictable results, primarily due to our limited understanding of the network architecture of metabolic pathways. Most current models of metabolic regulation in plants are still based on individual reactions, and do not consider the integration of several pathways sharing common branch points. The use of transgenic plants expressing a heterologous *TDC* or *TYDC* gene has proven to be a powerful technique to study metabolic regulation, and is improving our understanding of the physiological roles for specific secondary metabolic pathways.

Chimeric *TDC* and *TYDC* gene constructs, consisting of the cauliflower mosaic virus (CaMV) *35S* promoter, followed by the *TDC* or *TYDC* coding region and the nopaline synthase (NOS) transcription terminator, have been introduced into various plant species (Table 2). For example, introduction of *TDC* from *C. roseus* into transgenic tobacco plants resulted in the accumulation of approximately 1% (dry weight) tryptamine (Songstad et al., 1990; Goddijn et al., 1995; Poulsen et al., 1994). Generally, tryptamine accumulation was shown to be directly proportional to the level of TDC activity in each plant. Normal growth and development of these plants was not affected despite the creation of a large sink for Trp, and a large cellular pool of soluble tryptamine. Moreover, no significant difference was detected in the activity of key aromatic amino acid biosynthetic enzymes (Poulsen et al., 1994). IAA levels were identical in high-tryptamine and control plants (Songstad et al., 1990) suggesting that tryptamine pool size might not have a significant influence on IAA synthesis, or that the tryptamine produced was quickly sequestered to the vacuole where it was unavailable for IAA synthesis. Transformed tobacco plants expressing heterologous *TDC* were also reported to accumulate up to 30-fold more tyramine, in addition to elevated levels of tryptamine, compared to wild type plants (Songstad et al., 1991). An alteration in the specificity for Trp of the heterologous TDC enzyme in transgenic plants has not been reported; thus, the most plausible explanation for these data is that the increased demand for Trp synthesis might have up-regulated shikimate and/or aromatic amino acid metabolism, resulting in increased Tyr synthesis and, consequently, higher tyramine accumulation due to an endogenous TYDC activity. Tobacco plants expressing *C. roseus TDC* also showed a 97% reduction in the reproduction rate of sweet potato whitefly (*Bemisia tabaci*) larvae, relative to wild type tobacco, suggesting that tryptamine production in transgenic crops might be a useful strategy to confer protection against insect damage without the application of agrochemicals (Thomas et al., 1995). The mechanism by which tryptamine production in transgenic tobacco affects insect reproduction is not known. However, it has been suggested that tryptamine disrupts whitefly larval and pupal development, and the process of adult leaf selection for feeding and oviposition, by blocking neuromuscular and Glu transmission (Thomas et al., 1995).

Berlin et al. (1993, 1994)) introduced heterologous *TDC* into callus and root cultures of *Peganum harmala*, which were derived from seedlings transformed with *A. tumefaciens* carrying the chimeric gene in either the pTi or pRi background. *P. harmala* tissue cultures accumulate two simple and interrelated secondary products derived from tryptamine: the harmane-type β-carboline alkaloids and serotonin. Callus and root cultures of *P. harmala* transformed with *35S-TDC* contained elevated levels of TDC activity relative to control cultures. Serotonin levels in transgenic sus-

Table 2
Metabolic modifications using AADC genes in transgenic plants

Metabolic modification	Engineered enzyme	Tranformed species	Source of gene	Reference
Increased tryptamine levels	TDC	Tobacco	*C. roseus*	Songstad et al. (1990)
Increased tyramine levels	TDC	Tobacco	*C. roseus*	Songstad et al. (1991)
Increased serotonin levels	TDC	*Peganum harmala*	*C. roseus*	Berlin et al. (1993, 1994)
Reduced indole glucosinolate levels	TDC	Canola	*C. roseus*	Chavadej et al. (1994)
Redirection of shikimate metabolism; reduced phenylalanine levels; increased disease susceptibility	TDC	Potato	*C. roseus*	Yao et al., 1995
Increased tryptamine levels in hairy root cultures	TDC	*C. roseus*	*C. roseus*	Islas et al. (1994)
Increased tryptamine levels in crown gall tissue	TDC	*C. roseus*	*C. roseus*	Goddijn et al. (1995)
Increased indole alkaloid levels in cell cultures	TDC and STR	*C. roseus*	*C. roseus*	Canel et al. (1998)
Strictosidine production when supplied with exogenous secologanin	TDC and STR	Tobacco	*C. roseus*	Hallard et al. (1998)
Increased cell wall-bound tyramine levels; decreased cell wall digestibility	TYDC	Canola	*P. somniferum*	Facchini et al. (1999)

pension cultures with elevated TDC activity were 10- to 20-fold higher than in control cultures. Similarly, serotonin accumulation in transgenic root cultures with elevated TDC activity was two- to three-fold higher than in control cultures. In contrast, β-carboline alkaloid levels were not affected by the overexpression of *TDC* in *P. harmala* cultures (Berlin et al., 1993). This result was not unexpected since tryptamine supply was previously shown to be limiting for serotonin, but not for β-carboline alkaloid, biosynthesis (Berlin et al., 1994). The mechanism for the metabolic channeling of Trp into serotonin and β-carboline alkaloids in *P. harmala* is not known. One possibility is that two TDCs are present in separate subcellular locations, and the additional tryptamine produced in transgenic cultures is available to only one isoform.

Introduction of *TDC* gene into *Brassica napus* (canola) resulted in the redirection of Trp into tryptamine, rather than indole glucosinolates, in all parts of the plant (Chavadej et al., 1994). The indole glucosinolate content of mature seeds from transgenic plants was only 3% of that found in wild type seeds. In oilseed crops, such as, canola, the presence of indole glucosinolates in seeds decreases the meal palatability and, consequently, its value as animal feed. This study is an elegant example of how the introduction of a heterologous AADC can be used to create an artificial sink to divert metabolic flow and reduce the levels of undesirable aromatic amino acid-derived products. Surprisingly, expression of *35S-TDC* in transgenic potato resulted in a drastic alteration in the balance of key substrate and product pools involved in the shikimate and phenylpropanoid pathways Yao et al. (1995). In potato, the redirection of Trp to tryptamine caused a decreased in the levels of Trp, Phe, and Phe-derived phenolic compounds in transgenic tubers, compared with wild type controls. The wound- and pathogen-induced accumulation of chlorogenic acid, the major soluble phenolic ester in potato tubers, and lignin were reduced due to the limited availability of phenolic monomers. The transgenic tubers were also more susceptible to pathogen infection, which was attributed to a modification in the cell wall composition of the plants. This study shows that the creation of artificial metabolic sinks using heterologous AADCs in transgenic plants can alter the availability of substrates even if the foreign gene operates outside the pathway involved in substrate supply. In metabolic engineering applications involving AADCs, it is crucial to consider the flux of branch point substrates involved in the general biosynthesis and metabolism of aromatic amino acids.

The transformation of different plant species with heterologous *TDC* or *TYDC* genes has also shown that the extent to which a specific AADC activity can be increased is species-dependent. For example, TDC activity and tryptamine accumulation in transgenic plants expressing *35S-TDC* were 3- and 12-fold higher, respectively, in tobacco compared to potato, and 10- and 50-fold higher, respectively, in tobacco compared to canola (Ibrahim et al., 1994). Moreover, in canola transformed with *35S-TYDC*, all T_0 plants showed no expression of the transgene and suppressed levels of TYDC activity relative to wild type plants; thus, the broad application of plant genetic engineering strategies involving AADCs requires an improved understanding of the mechanisms affecting transgene expression and heterologous enzyme activity. Insight into this problem can be gleaned from the work of Leech et al. (1998), in which the integration frequencies and expression levels of *35S-TDC* and *35S-STR* transgenes in transgenic tobacco were studied. Both transgenes originated from *C. roseus* and were assembled, together with a selectable marker gene, on a single transforming plasmid. Analysis of 150 independent transformants showed a 100% co-integration frequency of the two unselected genes. However, both transgenes were expressed in only 33% of the plants; in 26% of the plants both *TDC* and *STR* transgenes were silenced, whereas a preferential silencing of either the *TDC* or the *STR* transgene occurred in 41% in the plants. No correlation between the number of integration events and the levels of transcripts was apparent, suggesting that the variations in *TDC* and *STR* expression can be attributed to effects other than differences in copy number.

Extensive phenotypic diversity was also observed for alkaloid production in *C. roseus* cell suspension cultures overexpressing *TDC* and *STR*, either together or separately (Canel et al., 1998). Cultures transformed only with the *STR* transgene showed 10-fold higher STR activity, compared to wild type cultures, and accumulated over 200 mg l^{-1} of strictosidine and several other monoterpenoid indole alkaloids, but maintained wild type levels of TDC activity. In contrast, high TDC activity encoded by the *TDC* transgene, introduced either alone or in combination with the *STR* transgene, did not affect alkaloid accumulation. This result is consistent with previous indications that the production of tryptamine is not a limitation for alkaloid formation in *C. roseus* cell cultures (Knobloch and Berlin, 1983; Mérillon et al., 1983; Facchini and DiCosmo, 1991). In contrast, high STR activity was necessary, but apparently not sufficient, to sustain a high rate of alkaloid biosynthesis (Canel et al., 1998).

Studies on the effect of *TYDC* overexpression in transgenic plants have also begun. Canola (*B. napus*) was transformed with *35S-TYDC* genes encoding TYDC isoforms from opium poppy (Facchini et al., 1999). All primary transformants displayed a suppressed level of wild type TYDC activity, and transgene mRNAs were not detected. Surprisingly, silencing

of *TYDC1* was overcome in the T_1 progeny of self-pollinated T_0 plants, since high levels of TYDC1 mRNAs were detected and TYDC activity was 4-fold higher than in wild type plants. However, TYDC1 mRNA and enzyme activity declined to wild type levels in subsequent generations. In contrast, silencing of *TYDC2* was maintained through four consecutive generations. Plants expressing high levels of *TYDC1* showed a 30% decrease in cellular Tyr pools and a two-fold increase in cell wall-bound tyramine compared to wild type plants. An increase in cell wall-bound aromatic compounds was detected in these plants by UV autofluorescence microscopy, and the relative digestibility of cell walls, measured by protoplast release efficiency, was significantly reduced. This study supports the involvement of *TYDC* and tyramine in cell wall development via the synthesis of hydroxycinnamic acid amides. The engineering of cell wall-bound amide metabolism might provide an effective biotechnological strategy to reduce crop susceptibility to a broad spectrum of pathogens by decreasing cell wall digestibility.

An innovative application of plant AADC genes, with implications on genetic and metabolic engineering technologies, was shown in the development of a novel selection system for plant transformation. *TDC* can be used as a useful selectable marker because of its ability to convert the toxic Trp analogue 4-methyltryptophan into the non-toxic compound 4-methyltryptamine (Goddijn et al., 1993). Expression of a *35S-TDC* in plants with no endogenous TDC activity allowed for selection of transformants on media containing 4-methyltryptophan. It might be possible to develop a similar strategy with TYDC as a selectable marker using a toxic Tyr analogue, such as, 2-fluorotyrosine, which can be decarboxylated to form a non-toxic amine.

7. Conclusions and future directions

We have presented a comprehensive survey of the extensive literature on, and relevant to, the evolution, physiology, biochemistry, regulation, and genetic engineering applications of plant AADCs. We have shown that plant AADCs display a high degree of sequence identity with AADCs from mammals and insects, and significant homology with PLP-dependent decarboxylases of Glu and His in both plants and animals. However, despite the extensive conservation of sequence, plant and animal AADCs differ largely in their substrate specificities: TDC accepts only Trp, TYDC accepts only Tyr and Dopa, whereas animal DDC accepts a broad range of aromatic amino acids. The precise substrate specificity of plant AADCs allows the enzymes to operate at the interface between primary (i.e., shikimate and aromatic amino acid) metabolism and specific secondary metabolic pathways. In particular, TDC represents an initial step in monoterpenoid indole alkaloid biosynthesis, whereas TYDC serves as an entry point to the benzylisoquinoline alkaloid and hydroxycinnamic acid amide biosynthetic pathways.

All AADCs in nature are PLP-dependent homodimeric enzymes that likely share a common catalytic mechanism involving the formation of a Schiff base between PLP and an invariable Lys residue, followed by a transaldimination reaction with an L-aromatic amino acid substrate. TDC and TYDC activities in plants are clearly regulated at the level of transcription; however, the post-translational processing of TDC by a ubiquitin-mediated degradation pathway, has also been proposed. Although *TDC* and *TYDC* genes are developmentally controlled, they are also induced by environmental factors, such as, pathogen-derived elicitors and wounding, via a jasmonate-mediated mechanism. Several impressive metabolic engineering studies have been conducted in a variety of transgenic plants using isolated AADC genes. The metabolic modifications include increased serotonin levels, reduced indole glucosinolate levels, redirected shikimate metabolism, increased indole alkaloid levels, and increased cell wall-bound tyramine levels.

Despite the impressive amount of research performed on plant AADCs, much remains to be learned about this interesting and important group of enzymes. The primary targets for future research on plant AADCs include: (1) a detailed physicochemical characterization of the enzymes with particular emphasis on solving their three-dimensional structure using X-ray crystallography; (2) the further analysis of ubiquitin-mediated inactivation and degradation processes; (3) the isolation of *cis*-elements and transcription factors involved in the molecular regulation of *TDC* and *TYDC* gene expression; and (4) the continued development of metabolic engineering applications involving, in particular, the heterologous expression of *TYDC* in benzylisoquinoline alkaloid-producing plants and crop species to better understand the network architecture of alkaloid and hydroxycinnamic acid amide metabolism. Detailed knowledge of the biochemistry and molecular regulation of aromatic amine biosynthesis in plants has the potential to create new agricultural biotechnology opportunities for pharmaceutical production and pest/disease resistance.

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