Plant Pathol. J. 17(3) : 123-127 (2001)

The Plant Pathology Journal

Mini-Review

Hydroxycinnamic Acid Amides and Their Possible Utilization for Enhancing Agronomic Traits

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(Received on May 14, 2001)

Several thousands of phenolic compounds known to date are from plant sources. All these plant phenolic compounds are synthesized from either phenylpropanoid pathway producing the majority of plant phenolics or phenylpropanoid-acetate pathway leading to the synthesis of plant quinones and flavonoids. From the most simple phenolic (C_6 aromatic monomer molecules) to the most condensed tannins ($(C_6\text{-}C_3\text{-}C_6)_n$ polymer), phenolics are comprised of an extraordinarily diverse array of chemical compounds in the plant kingdom. In accordance with structural complexity, phenolics as the secondary metabolites play significant roles in the process of plant growth and development. The best known role of phenolics is providing structural support as an integral part of the cell wall component. In addition to physical mechanical support, phenolics give rise to defense barriers against pathogen attack. Another significant contribution of phenolics includes allelopathy, fruit colors, UV light absorbent, repellent against herbivores, and phytoalexins (Strack, 1997).

Of these, this review focuses on hydroxycinnamic acid derivatives, one of the most commonly found phenolics due to their structure, biosynthesis and functional importance in plants.

Biosynthesis of Hydroxycinnamic Acid Derivative Phenolics

Phenylpropanoid pathway starts with the synthesis of 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) resulting from the condensation of phosphoenolpyruvate (a glycolytic intermediate) and erythrose 4-phosphate (a pentose phosphate pathway intermediate) by the action of DAHP synthase (Fig. 1). The DAHP leads to the synthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan by the sequential reaction of 9 enzymes. This pathway is also called the shikimate pathway because shikimate is produced as an intermediate of phenylpropanoid biosynthetic pathway. One intriguing enzymatic step is the conversion of shikimate 3-phosphate into the enolether 5-enolpyruvylshikimate 3-phosphate (EPSP) by EPSP synthase which is known to be inhibited by the herbicide glyphosate, a non-selective, broad spectrum postemergence herbicide. The glyphosate is employed not only for weed removal by the depletion of the aromatic amino acids but for the experimental tool to understand the regulation of the penylpropanoid derived compounds in plants. Hydroxycinnamic acid phenolics are derived from cinnamic acid, the first synthesized phenolic molecule diverging from the primary aromatic amino acid metabolite to the secondary phenolic metabolites. This step is catalyzed by phenylalanine ammonia-lyase (PAL) which converts the amino acid phenylalanine into cinnamic acid by removing amine group of phenylalanine.

It was well characterized that PAL activity is closely cor-

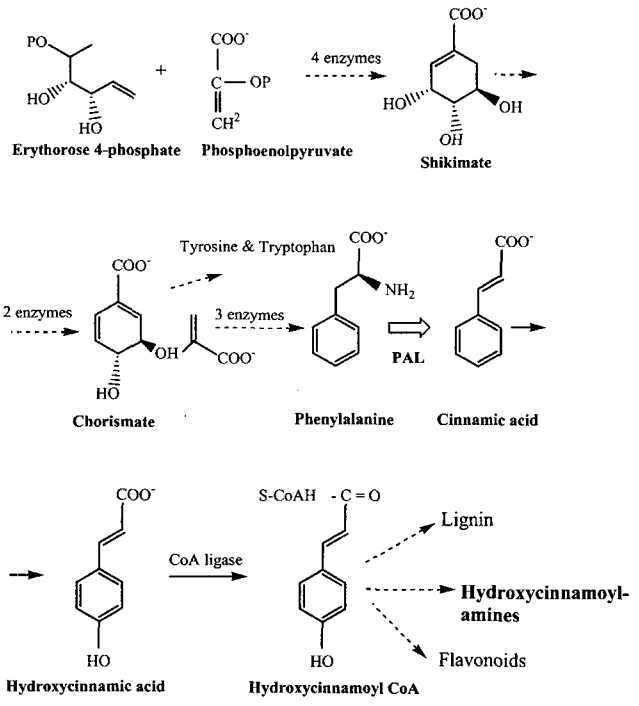

Fig. 1. Overview of phenylpropanoid pathway leading to synthesis of a series of phenylpropanoid metabolites such as lignin, flavonoids and hydroxycinnamic acid amines.

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related with the total synthesis of phenolic compounds in plants (Maher et al., 1994). Beginning with cinnamic aicd, a series of hydroxylation and methylation reactions lead to the sequential synthesis of the common hydroxycinnamates such as p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. These hydroxycinnamates are finally activated as hydroxycinnamate-CoA forms in which hydroxycinnamate CoA ligases are responsible for these catalyses. The activation of phenolic compounds as hydroxycinnamte-CoAs serves as a building block molecule for further downstream modification, which includes condensation and conjugation resulting in flavonoids, lignin and hydroxycinnamic acid amines.

Biosynthesis of Hydroxycinnamic Acid Amines. Hydroxycinnamic acid amines are synthesized by the enzyme hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl)-transferase (THT) which catalyzes the transfer of hydroxycinnamic acids from the respective CoA esters to tyramine and various amines (Fig. 2). The enzymatic products of THT are comprised of feruloyltyramine, feruloyloctopamine, coumaroyltyramine and coumaroyloctopamine as representative phenolic amines. The synthesis of these hydroxycinnamic acid amines is closely related to a defense mechanism that protects plants from pathogen attacks. It is proposed that the hydroxycinnamic acid amines synthesized in the cytosol transport into the cell wall fraction followed by their subsequent peroxidative polymerization in which these compounds appear to be involved in cell wall

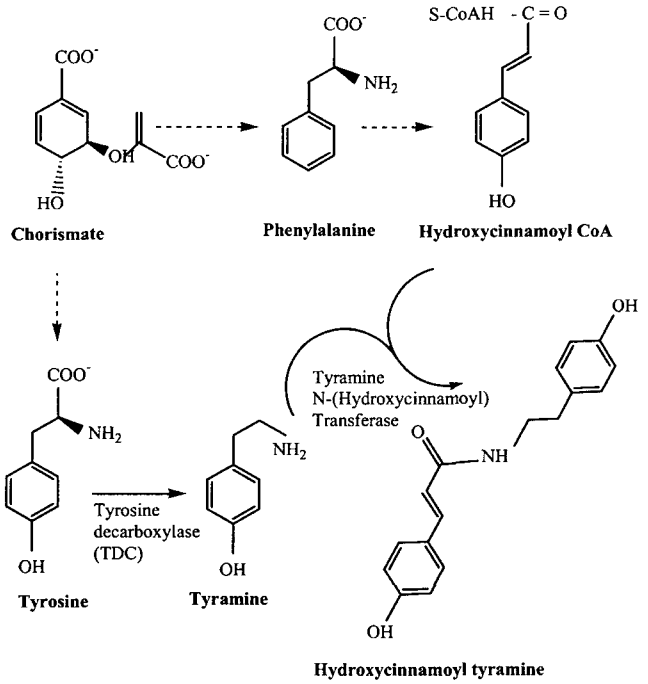

Fig. 2. Reaction scheme of tyramine N-(hydroxycinnamoyl)-transferase (THT).

fortification (Negrel and Martin, 1984; Negrel and Jeandet, 1987; Negrel and Lherminier, 1987; Negrel et al., 1993). The accumulation of phenolic amines in the cell wall is supposed to build up a resilient barrier against pathogens by reducing cell wall digestibility and/or by directly inhibiting the growth of fungal hyphae (Gransmaison et al., 1993). Accordingly, the synthesis of hydroxycinnamic acid amines is induced in response to various stresses including physical injury, pathogen infection and elicitor treatment from both Solanaceae plants such as potato, tobacco, tomato and grass plant such as wheat (Atsushi et al., 2000; Crarke et al., 1982; Miyagawa et al., 1998; Negrel et al., 1984; Pearce et al., 1998). In addition to Solanaceae and Gramineae, the accumulation of hydroxycinnamic acid amines upon stress responses has been reported in Papaveraceae and Liliaceae showing that these phenolic amines are ubiquitous in the plant kingdom (Facchini, 1998; McLusky et al., 1999; Yu and Facchini, 1999). In potato cell culture, coumaroyltyramine, feruloyltyramine and feruloyloctopamine accumulate both as cell wall-bound amines and as compounds secreted in the culture medium after elicitor application in which coumaroyltyramine synthesis peaks at 60 h or at 30 h, respectively (Schmidt et al., 1999). Induction of N-hydroxycinnamoyltyramine synthesis was also reported in wounded maize leaves (Ishihara et al., 2000). The level of N-hydroxycinnamoyltyramines started to increase 3 h after wounding and reached their highest level after 12 h followed by 70% of the maximum level until 72 h. Mechanical wounding in tomato leaves synthesized feruloyltyramine and coumaroyltyramine by 10 and 25 folds in response to oligosaccharide elicitor chitosan. Furthermore, it had been suggested that feruloyltyramine synthesis was not associated with octadecanoid signaling pathway, which is responsible for the synthesis of jasmonic acid (Pearce et al., 1998). As for *Phytophthora infestans* infected leaves of potato, cell wall-bound coumaroyltyramine and feruloyltyramine accumulated rapidly after spore inoculation (Keller et al., 1996). Thus, it has been considered that not only does incorporation of hydroxycinnamic acid amines in cell wall play a role as a physical barrier against pathogens, but also that their secretions into medium upon elicitor treatment in cell suspension culture imply that the hydroxycinnamic acid amines seem to be closely associated with antimicrobial compounds like phytoalexin.

Characteristics and Kinetics of Hydroxycinamoyl-CoA:Tyramine N-Hydroxycinnamoyltransferase (THT) Enzyme

THT catalyzes the synthesis of hydroxycinnamic acid amines using two different types of chemical backbones as substrates, one of which is hydroxycinnamoyl-CoA thio-

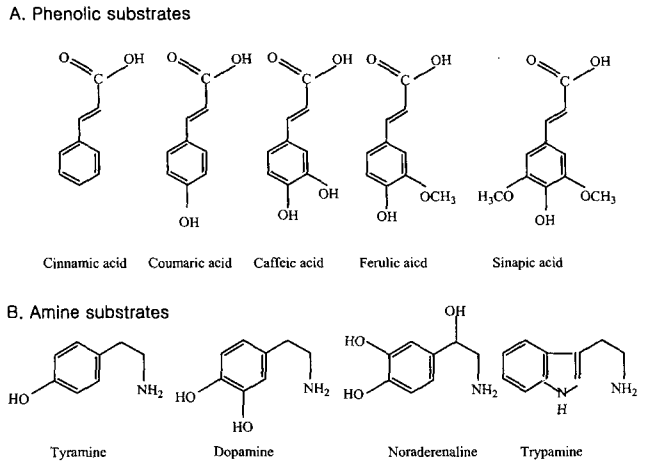

Fig. 3. Various forms of phenolic and amine type substrates utilized by THT enzyme in plants.

ester and the other is various hydroxyphenylethylamines (Fig. 3). The hydroxycinnamoyl-CoA thioesters come from subsequent modification of cinnamic acid by a series of enzymes including cinnamate-4-hydroxylase, coumarate-3-hydroxylase, caffeic acid O-methyltransferase, ferulate-5-hydroxylase and hydroxycinnamate:CoA ligase. Through these reactions, cinnamic acid is subsequently modified into hydroxycinnamoyl-CoA thioesters such as CoA thioesters of cinnamic acid, coumaric acid, caffeic acid, ferulic acid and sinapic acid. Hydroxyphenylethylamines include tyramine, octopamine, dopamine, noradrenaline and typtamine. Among these, tyramine and tryptamine are derived from decarboxylation of aromatic amino acids tyrosine and tryptophan, respectively. So far, kinetics of THT enzyme were investigated in detail from four different sources of plants such as potato, tobacco, poppy and maize (Hohlfeld et al., 1995; Negrel and Javelle, 1997; Yu and Facchini, 1999; Ishihara et al., 2000). The first experiment showing the existence of THT enzyme went back Negrel and Martin's report (1984). When tobacco leaves were infected by tobacco mosaic virus, THT enzyme activity increased after infection. It was also detected in *Nicotiana glutinosa* and *Eschscholtzia californica* cell-suspension cultures, following elicitation by chitisan (Villegas and Brodelius, 1990), and in *Solanum tuberosum* tuber discs induced by wounding (Negrel et al., 1993). Recently, the THT activity increased during development of opium poppy seedlings, occurred at a high level in roots and stems of mature plants, and was induced in cell-suspension cultures after treatment with a pathogen-derived elicitor (Yu and Facchini, 1999). In maize, the accumulation of hydroxycinnamoyltyramines was accompanied by an increase in the THT enzyme activity. This increase was initially detected 3 h after wounding and reached a maximum at 36 h, the level of activity being 40 times that in the leaves before wounding (Ishihara et al., 2000).

First attempts to purify THT from tobacco leaves infected by tobacco-mosaic virus had failed due to both the low abundance of the THT and the occurrence of several forms of the enzyme, possibly isoenzymes, in the cell free extracts (Fleurence and Negrel, 1989). In 1995, Dr. Stracks group in Germany had success for partial purification of THT with a 380-fold enrichment from cell-suspension culture of potato (Hohlfeld et al., 1995). It had been shown that the partially purified THT produced formation of feruloyltyramine with divalent cation Ca^{2+} or Mg^{2+} enhancement and pH 6.8 as optimum activity. As summarized in Table 1, the partially purified potato THT had a high affinity toward feruloyl-CoA and 4-coumaroyl-CoA. The potato THT was also able to convert caffeoryl-CoA to caffeoryltyramine, however, it did not accept this CoA ester in the THT from tobacco leaves (Negrel and Martin, 1984). There was a preferential specificity for tyramine as an acceptor in the presence of feruloyl-CoA, followed by octopamine. Two years later, Dr. Javelles group reported the purification of THT from tobacco cell-suspension cultures treated with a commercial preparation of pronase (Negrel and Javelle, 1997). The best substrates were feruloyl-CoA, followed by sinapoyl-CoA, cinnamoyl-CoA and p-coumaroyl-CoA whereas no caffeoryltyramine could be produced using caffeoryl-CoA as acyl donor. In the presence of feruloyl-CoA, tyramine was the best substrate as acyl acceptor, followed by octopamine and dopamine. THT enzyme purified from opium poppy also exhibited multiple substrate specificity for cinnamoyl-CoA derivatives and hydroxyphenethylamines. The best substrates were feruloyl-CoA for cinnamoyl-CoA substrate and tyramine for amine substrate, respectively (Yu and Fac-

Table 1. Substrate affinity of the tyramine N-(hydroxycinnamoyl)-transferase enzymes from several plant species

Substrates	Potato	Tobacco	Poppy	Maize	Pepper
Hydroxycinnamoyl-coAs					
cinnamoyl-CoA	+++[a]	++	++	–	+++
feruloyl-CoA	+++	+++	+++	+++	++
caffeoyl-CoA	++	–	–	+	++
4-coumaroyl-CoA	++	+	+	++	+
sinapoyl-CoA	++	+++	++	+++	++
Amines					
tyramine	+++	+++	+++	+++	+++
octopamine	+++	++	nd	nd	++
dopamine	++	+	+	++	nd
noradrenaline	+	–	nd	nd	nd
tryptamine	nd	nd	nd	+++	nd

[a] +++: highest affinity, ++: relatively high affinity, +: low, –: no affinity, nd: not determined

chini, 1999). The recombinant pepper THT enzyme was purified using a bacterial overexpression system (Back et al., 2001). The purified enzyme has a broad substrate specificity including acyl donors such as cinnamoyl-, sinapoyl-, feruloyl-, caffeoyl-, and 4-coumaroyl-CoA and acceptors such as tyramine and octopamine. Long after identification of amines of hydroxycinnamic acid with tyramine and tryptamine in maize (Martin-Tanguy et al., 1982; Ehmann, 1974), THT activity was first measured in wounded leaf segments of maize (Ishihara et al., 2000). The kinetics of maize THT purified showed different profiles of substrate specificity exhibiting broader ranges of amines utilized as substrates such as tyramine, tryptamine, dopamine and phenethylamine in preferential substrate order. As for hydroxycinnamic acid derivatives as substrates, the best substrates were feruloyl-CoA, followed by sinapoyl-CoA, p-coumaroyl-CoA and caffeoryl-CoA.

Cloning of THT and Its Molecular Comparisons to Related Acyltransferase

Dr. Strack's group first reported molecular cloning and bacterial expression of a potato THT cDNA from a cDNA library constructed from mRNA of elicitor-treated potato cells. The potato THT encodes a protein with a calculated molecular mass of 28.4 kDa. Recombinant THT purified from *Escherichia coli* exhibited a broad substrate specificity, similar to that of native potato THT, accepting cinnamoyl-CoA, 4-coumaroyl-CoA, caffeoryl-CoA, feruloyl-CoA and sinapoyl-CoA as acyl donors and tyramine, octopamine and noradrenaline as acceptors tested. THT transcript was induced and peaked 5 h after elicitor treatment in accordance with THT enzyme activity. Northern blot had shown that THT mRNA was detected predominantly in roots of soil-grown potato plants. In addition, THT is encoded by a small gene family when judged by southern blot. Secondly, THT gene was identified and sequenced from tobacco by immunoblot screening of a cDNA expression library constructed from mRNA purified from tobacco leaves infiltrated with an incompatible strain of *Ralstonia solanacearum* (Farmer et al., 1999). The cloned cDNA encodes protein of 226 amino acids with calculated molecular masses of 26 kDa. THT transcript was only found to be expressed in tobacco root tissues. Thirdly, a cDNA encoding THT was cloned from the leaves of UV-C treated *Capsicum annuum* (hot pepper) using a differential screening strategy (Back et al., 2001). The predicted protein encoded by the THT cDNA is 250 amino acids in length and has a relative molecular mass of 28,221. The protein sequence derived from the cDNA shares 76% and 67% identity with the potato and tobacco THT protein sequences, respectively. In UV-C treated plants, the THT mRNA was strongly induced in leaves, and the elevated level of expression was stable for up to 36 h. THT mRNA also increased in leaves that were detached from the plant but not treated with UV-C. THT expression was measured in different plant tissues, and was constitutive at a similar level in leaf, root, stem, flower and fruit. Induction of THT mRNA was correlated with an increase in THT protein.

THT belongs to the class of acyltransferase showing the closest homology with other enzymes of this class, namely spermidine/spermine N-acetyltransferase (SSATs) and diamine acetyltransferase (DAT). Two conserved domains have been identified in N-acetyltransferase from microbes and animals that are also present in plant THTs. One of the conserved domains contains a stretch of seven amino acid residues (RGFGIGS), that was shown to be required for activity and binding of acetyl-CoA (Lu et al., 1996). As determined in human SSAT in which dimerization is necessary to form active enzymes, plant THT enzyme also seems to be active as a dimmer.

Metabolic Engineering for Improving Agricultural Traits Through the Genetic Manipulation of THT. As mentioned earlier in this review, THT enzyme is located in the middle of modification branch pathway of phenylpropanoid metabolites that include lignin and flavonoid. In addition to various genes encoding key enzymes of phenylpropanoid branch pathway such as tryptophan decarboxylase (TDC), caffeic acid O-methyltransferase (COMT) and coumarate coenzyme A ligase (4CL), THT also opens up the possibility of engineering important plants including alfalfa, rice and tree. The recent finding of THT enzyme inhibition by the addition of OH-PAS which inhibited lignification (Farmer et al., 1999), gives rise to the possibility of lowering lignin content by way of downregulation of THT gene. Actually, lignin is an undesirable component of raw pulp used in paper making, and it requires elimination by chemical treatment. As for forage such as alfalfa, modification of lignin composition and/or content can lead to improvement of forage digestibility. THT may play an important role for engineering alfalfa having better digestibility. The overexpression of THT in host plants can accumulate hydroxycinnamic acid amines that endow cell wall fortification and seem to exhibit antimicrobial effect as well. Those plants will be expected to have more resistance toward pathogen attack.

Acknowledgements

This work was supported by grant No. (2001-2-22100-002-3) from the Basic Research Program of the Korea Science & Engineering Foundation. This is a peer-reviewed paper that was presented at the 3rd symposium on "Molecular Plant-Microbe Interactions" held on December 22, 2000 in

Korea University, Seoul.

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