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Detection of Wound-inducible *Trans*-Cinnamic Acid-4-Hydroxylase in Avocado, *Persea americana*, Roots

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Abstract

***Trans*-cinnamic acid-4-hydroxylase(*t*C4H) is the first cytochrome P450-dependent monooxygenase of the phenylpropanoid pathway. The roots of avocado seedlings were wounded and examined to determine whether the *t*C4H would be activated in response to wounding and/or whether *t*C4H activity be modulated by the application of exogenous *p*-coumarate. At the specified length of times, the wounded and treated roots were either frozen in liquid nitrogen or used immediately to extract microsomal proteins. The microsomal proteins were subjected to immunoblot analysis using polyclonal antibodies against CYP73 of *t*C4H gene. In this study, *t*C4H was induced in wounded roots sealed in bags within 6 hours, and in low level(10^{-8} M) of *p*-coumarate solution within 24 hours, whereas the solution without *p*-coumarate and high levels of *p*-coumarate solution repressed *t*C4H induction in wounded roots. These results indicate that *t*C4H is induced by wounding in the root of avocado, and is inhibited by the application of exogenous *p*-coumarate.**

Key words: *trans*-cinnamic acid-4-hydroxylase, *para*-coumaric acid, phenylpropanoid pathway, immunoblot

INTRODUCTION

Trans-cinnamic acid-4-hydroxylase(*t*C4H) is one of the most abundant cytochrome P450s in higher plants and catalyzes the first hydroxylation reaction of *trans*-cinnamic acid to produce *p*-coumaric acid. In the general phenylpropanoid pathway, *t*C4H is involved in the second step of the core reactions(Fig. 1) started from phenylalanine, together with phenylalanine ammonia lyase(PAL) and 4-coumaroyl CoA ligase(4CL)(1-3). The phenylpropanoid pathway provides a variety of secondary metabolites including important phenolic compounds, such as lignins, tannins, suberines, flavonoids, isoflavonoids, coumarins, anthocyanins, and several types of phytoalexins that are involved in the differentiation of plant organs and the protection of plant tissues against environmental stresses (2).

Involvement of a cytochrome P450 in the enzymatic hydroxylation of *trans*-cinnamic acid into *trans*-4-coumaric acid was first suggested by Russell(1). This cytochrome P450, essential in the plant kingdom, constitutes a super family of membrane-bound hemoproteins involved in a variety of oxidative, peroxidative, and reductive reactions of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines. Many of these enzymes also metabolize wide range of foreign chemicals including drugs, environmental pollutants, natural plant products, and alcohols. The metabolism of foreign chemicals can frequently produce toxic metabolites, of which some have been implicated as agents that might be responsible for the initiation,

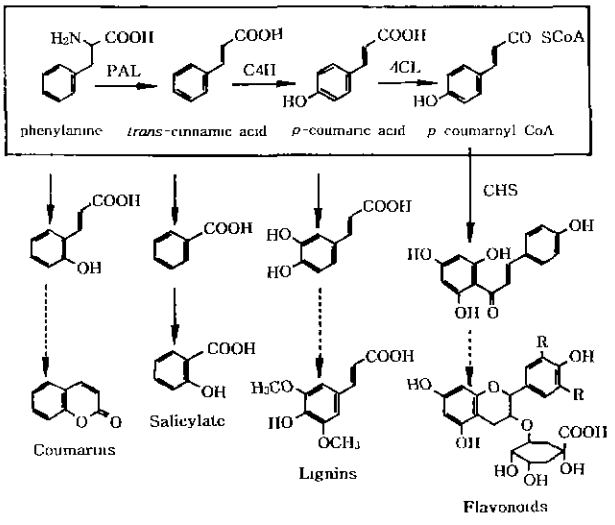

Fig. 1. The core reactions of the general phenylpropanoid pathway and related metabolites.
The core reactions are boxed.
PAL: phenylalanine ammonia lyase
*t*C4H: *trans*-cinnamic acid-4-hydroxylase
4CL: 4-coumaroyl CoA ligase

promotion, and progression of tumor(4-6).

The *t*C4H activity in various plant tissues has been reported to be induced by a number of stimuli such as light, elicitors, and wounding(5-12). A plant *t*C4H enzyme has been recently purified from microsomal fractions of Manganese-treated Jerusalem artichoke tubers(13). The levels of *t*C4H activity and P450 content in tubers have been shown to be increased by wounding and subsequent storage for three days(14). Similarly, *t*C4H activity in pea hypocotyl tissue increases in response to wounding (15). Here the level of *t*C4H itself could play an important role in the overall regulation of such pathways(16,17). The genes involved in the phenylpropanoid pathway have been isolated and characterized in several plant species (2,18,19). The cDNA coding for *t*C4H, namely CYP73, was cloned by antibody screening of expression libraries from Jerusalem artichoke(20), mung bean(3), and alfalfa (21). Although much is known about the characteristics of PAL and 4CL in phenylpropanoid pathway, little is known about *t*C4H gene expression to form *p*-coumaric acid.

This study was initiated to determine whether the *t*C4H is activated in response to wounding and subsequent aging of avocado roots, and *t*C4H activity is modulated by the application of exogenous *p*-coumarate.

MATERIALS AND METHODS

Induction of *t*C4H by wounding and chemical treatments

Avocado(*Persea americana* Mill.) roots were collected from seedlings of Hass, grown in the campus of California State Polytechnic Uinversity at Pomona, California, USA. Roots were washed with deionized water, sliced into 5 mm length, frozen immediately in liquid N_2, and stored at -70°C freezer. In order to achieve wounding-promoted induction of *t*C4H, sliced roots were placed on wet filter papers, and incubated in sealed plastic bags at room temperature for 0, 3, 6, 24, and 72 hours, respectively. After incubating during the specified length of times, slices were frozen immediately in liquid N_2, and then stored at -70°C until extracted microsomal protein. To determine the effect of *p*-coumarate on wounding-induced *t*C4H activity, wounded roots were treated with various concentrations(0 M, 10^{-8}M, 10^{-6}M, 10^{-4}M and 10^{-2}M) of *p*-coumarate dissolved in solution(0.4 M mannitol, 0.5% cellulysin, 0.5% macerase) for protoplast preparation during 24 hours. The treated root slices were rinsed three times with deionized water, squeezed liquid off using filter papers, and then frozen immediately in liquid N_2 to extract microsomal protein.

Preparation of microsomal fractions

The preparation of microsomal fractions was performed as described in O'Keefe and Leto(22). Approximately 10 gms of frozen tissue samples were placed into a falcon tube and 20ml of cold microsomal preparation buffer(0.1 M MOPS/NaOH pH 7.0, 0.3M sorbitol, 5mM EDTA, 0.1%(w/v) BSA, 1mM PMSF, 5μg/ml leupeptin, and 5μg/ml pepstatin) were added. This was polytroned on the highest setting for about one minute, or until solubilized. The homogenate was centrifuged at 20,000g for 30 minutes. The supernatant was transferred to ultracentrifuge tubes and spun at 100,000g for 60 minutes. This fraction contains all microsomal proteins including *t*C4H proteins. The pellet was resuspended in 0.5ml of resuspension buffer (50% glycerol, and 0.1M MOPS/NaOH pH 7.0) and frozen until analysis.

Analytical methods

Protein concentrations of microsomal fractions were determined by the method of Bio-Rad protein assay. The microsomal fractions were subjected to immunoblot analysis following seperation by SDS-PAGE on a 10% acrylamide gel(23). After electrophoresis, proteins were transferred to nitrocellulose membrane by electroblotting using transfer buffer(20 mM Tris-base, 150 mM glycine, 0.1% SDS, and 20% methanol) as described by Towbin et al.(24). The nitrocellulose membrane was blocked with 1% bovine serum albumin for 2 hours at room temperature, and then incubated for 2 hours with primary antiserum(rabbit antiCYP73) against *t*C4H protein. The primary antiserum was diluted to 1:2,000 in 1×TBS and 1% BSA. Excess antibody was washed away with deionized water, and then 1×TBS and 0.05% Tween 20. Following incubation for 2 hours with secondary antibody, peroxidase conjugated goat anti-rabbit immunoglobulinG (Bio-Rad), diluted 1:8,000 in 1×TBS and 1% BSA, excess antibody was washed away with deionized water, 1×TBS and 0.05% Tween 20. Color was developed with the substrate 4-chloro-1-napthol(Sigma) for 20 minutes.

RESULTS AND DISCUSSION

The microsomal proteins from roots of avocado seedlings were electrophoresised and subjected to immunoblot analysis to determine whether the *t*C4H is induced in response to wounding and/or whether *t*C4H activity is modulated by the application of exogenous *p*-coumarate.

To evaluate the impact of wounding on *t*C4H expression, sliced roots were incubated for 0, 3, 6, 24, and 72 hours on wet papers sealed in plastic bags to prevent desication prior to protein extraction. The microsomal fraction from ripe avocado mesocarp containing detectable P450 antigen was loaded in lane 3 and resolved by SDS-PAGE(Fig. 2, lane 3) to compare with *t*C4H activity of avocado roots. No detectable *t*C4H antigen was observed in the microsomal fraction extracted from roots which were incubated less then 3 hours after wounding treatment (lane 4, 5). However, a *t*C4H antigen comigrating with purified native avocado *t*C4H starts to accumulate from 6 hours incubation and gradually increased throughout the 72 hours of wounding in sealed bags as shown in lane 6(6 hours), 7(24 hours) and 8(72 hours) of Fig. 2.

The *t*C4H is a plant-specific cytochrome P450 which is encoded by the gene CYP73 and catalyzes the second step of the multibranched phenylpropanoid pathway(10). In plant, P450s perform many oxygenation reactions in secondary metabolism, in sterols and fatty acid derivative synthesis, and in the detoxification of xenobiotics and foreign chemicals including drugs, environmental pollutants, natural plant products, and alcohols(5,6,10). Because of their importance in xenobiotic and drug metabolism, these enzymes have been throughly studied in mammalian liver (25-27) and insect(28-30). Cytochrome P450s are also heavily involved in the responses of plants to stress. These stimuli include wounding, pathogens, elicitors, xenobiotics and chemicals. However, ripe avocado contains one of the highest levels of the P450 protein. Therefore, it has been used to prepare P450 protein for analysis. and also provided the first P450 gene to be cloned and sequenced from a plant source(31,32). Polyclonal antibodies were generated against the CYP71A1 protein, and used in a western blot analysis of Hass fruit at various stages of ripening. An antigen of approximately 48,000 daltons was absent in unripe fruit, but observed in ripening tissue (33,34).

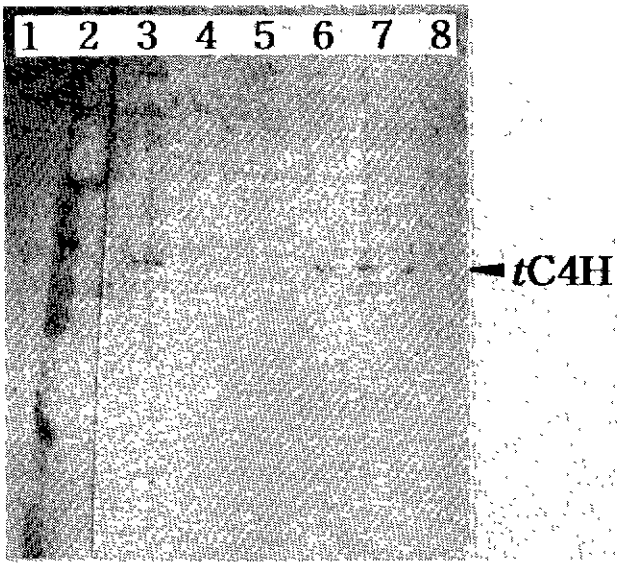

Fig. 2. Immunoblot analysis of wounding-promoted induction of *trans*-cinnamic acid-4-hydroxylase (*t*C4H) enzyme in avocado, *Persea americana*, root tissues.
1: Protein molecular weight marker, 2: Dye, 3: Microsomal fraction from the mesocarp of ripe avocado, 4: Microsomal fraction from the root tissues of 0 hr. after wounding, 5: 3hr. root tissues. 6: 6hr root tissues, 7. 24hr. root tissues, 8: 72hr. root tissues.

More than 16 P450-catalyzed reactions have been reported in the pathway leading to the biosynthesis of phenylpropanoids(10). The phenylpropanoid pathway gives rise to a wide array of metabolites. These compounds participate in many plant-defense responses(8). Induction of the phenylpropanoid pathway is also required during vascular differentiation to provide the precursors of lignin. The first enzyme of the pathway, PAL shows complex expression but is probably coordinately expressed with the P450, *t*C4H and other genes of the lignin pathway although expression of some of the later steps may be delayed. The *t*C4H has been immunolocalized to vascular tissue in French bean. In poplar stems, *t*C4H showed a different tissue distribution in activity from ferulate 5-hydroxylase, it being found in the xylem and sclerenchyma (6). Plant exposure to divalent cations like Mn^{2-} or Cd^{2+} has been shown to increase the P450 specific content and related activities in Jerusalem artichoke and sweet potato. Induction of P450 by organic xenobiotics(alcohol, drugs, pesticides) is now very well documented in several plant materials. The regulation of Jerusalem artichoke P450 levels through exposure to barbiturates, clofibrate, ethanol and several herbicides is most well studied system (6). The *t*C4H has been shown to be rapidly induced at the transcriptional level in *Helianthus* in response to wounding(20) and in alfalfa in response to elicitor(21).

More recent studies have documented increases in *t*C4H mRNA abundance in response to a number of stimuli such as light, pathogens, elicitors, and wounding(8,10,21). The cDNA of CYP73 coding for *t*C4H was cloned by antibody screening of expression libraries from Jerusalem artichoke (20), mung bean(3), and alfalfa(21). In this experiment, I have found that wounding of avocado roots leads to an increase in *t*C4H accumulation.

To determine whether *t*C4H activity is modulated by the application of exogenous *p*-coumarate, wounded root tissues were incubated for 24 hours in the protoplast preparation solution containing various concentrations of *p*-coumarate, or without *p*-coumarate(0 M of *p*-coumarate) as a control. The microsomal proteins extracted from tissues treated with *p*-coumarate were also electrophoresised with the microsomal fraction of ripe avocado mesocarp, and subjected to immunoblot analysis(Fig. 3). The *t*C4H was activated in response to only 10^{-8}M *p*-coumarate solution within 24 hours, but was not induced in response to high levels of *p*-coumarate as shown in Fig. 3. However, wounded avocado roots in sealed bags produced *t*C4H

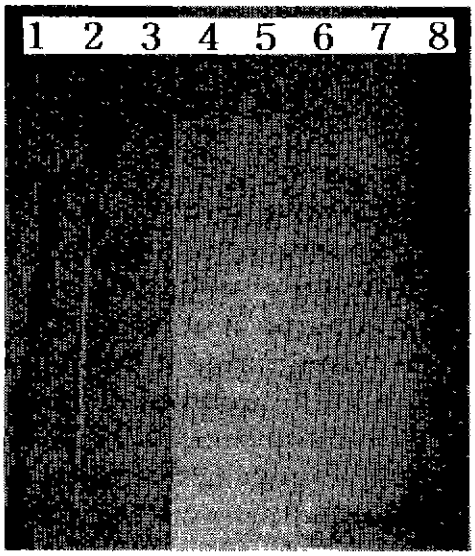

Fig. 3. Immunoblot analysis of *t*C4H enzyme derived from the root tissues of *Persea americana* in response to wounding and subsequent treating with various concentrations of *p*-coumaric acid (*p*-CA).
1: Protein molecular weight marker, 2: Dye, 3: Microsomal fraction from the mesocarp of ripe avocado, 4: Microsomal fraction from wounded root tissues treated with the solution without *p*-CA, 5: Microsomal fraction from wounded root tissues treated with 10^{-2} M *p*-CA solution, 6: 10^{-4}M *p*-CA root tissues, 7: 10^{-6} M *p*-CA root tissues, 8: 10^{-8}M *p*-CA root tissues.

within 6 hour incubation after cutting, as described above, whereas in the solution without *p*-coumarate didn't produce *t*C4H even after 24 hours.

Enzymes involved in the general phenylpropanoid pathway are usually expressed in a coordinate manner, but the regulatory mechanisms underlying coordinated expression are unclear. Both the substrate, cinnamic acid and the product, *p*-coumaric acid, of *t*C4H have been implicated in the regulation of upstream and downstream enzymes of the phenylpropanoid pathway(10,18).

In addition, *t*C4H activity is modulated in response to the application of exogenous *p*-coumarate as described by Lamb and Rubery(16), suggesting that the endogenous pools of phenylpropanoid intermediates may play an important role in the regulation of *t*C4H gene expression, as has been documented for other genes in this pathway (17). In the experiment of Jerusalem artichoke, *Helianthus tuberosus*, *t*C4H activity which was just detectable in dormant tissues was increased 14-folds after 48 hours of aging in aerated water, and greater induction of activity was observed in the presence of 0.25% DMSO(approximately 18-fold). Addition of some chemicals such as clofibrate, diethylhexyphthalate and 8-methoxypsoralen to the incubation medium resulted in a decrease in *t*C4H activity. Other chemicals, such as Mn^{2-}, phenobarbital, aminopyrine, lindane, biphenyl, and flavone, increased *t*C4H activity(10).

In this report, the results showed that *t*C4H activity is induced by wounding and subsequent aging of avocado root tissues, that *t*C4H is detected in response to the aging in the low concentration(10^{-8}M) of *p*-coumarate, but is inhibited in response to the solution without *p*-coumarate and the application of higher concentrations of exogenous *p*-coumarate.

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