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Effect of Wounding and Chemical Treatments on Expression of the Gene Encoding Cinnamate-4-Hydroxylase in *Camptotheca acuminata* Leaves

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The phenylpropanoid pathway plays an important role when plants are exposed to environmental stresses, such as wounding or pathogen attack. Its activity leads to the production of lignin, flavonoids, and phytoalexins. Cinnamate 4-hydroxylase (C4H) is a cytochrome P450-dependent monooxygenase that catalyses the hydroxylation of cinnamic acid to p-coumaric acid. We isolated *C4H* cDNA from *Camptotheca acuminata* and investigated the expression pattern of the *C. acuminata C4H* (CaC4H) gene following stress treatments. A search against the BLOCKS database of conserved protein motifs indicated that CaC4H shares common features with C4Hs from other species. C4H transcripts accumulated in the leaves in response to mechanical wounding or the application of molecules involved in the stress response, i.e., ethylene, methyl jasmonate, and hydrogen peroxide. Interestingly, the application of aminoethoxyvinylglycine, salicylic acid, or diphenylene iodonium, which are biosynthetic inhibitors of ethylene, methyl jasmonate, and hydrogen peroxide, respectively, did not inhibit this wound-induced expression. Based on these results, we suggest that C4H functions in response to various stresses in *C. acuminata* leaves.

Keywords: Camptotheca acuminata, cinnamate 4-hydroxylase, stress molecules, wound response

Cytochrome P450 (P450) is a super family of oxygen- and NADPH-dependent microsomal enzymes that catalyze a variety of reactions, such as hydroxylation, deamination, oxidation, and N-dealkylation (Mitoma et al., 1956; Estabrook et al., 1963). In plants, P450s are involved in myriad biochemical pathways, including those devoted to the synthesis of phenylpropanoids, alkaloids, terpenoids, lipids, cyanogenic glycosides, and glucosinolates, as well as gibberellins, jasmonic acid, and brassinosteroids (Chapple, 1998). Most plant phenolic compounds are products of phenylpropanoid metabolism, and play important roles in injury healing or as anti-pathogens, e.g., phytoalexin (Smith et al., 1994), lignin (Hahlbrock and Scheel, 1989), suberin (Bernards and Lewis, 1992), and other wound-induced polyphenolic barriers (Dixon and Paiva, 1995).

In the phenylpropanoid pathway, three distinctive cytochrome P450-dependent enzymes have been identified, with cinnamate-4-hydroxylase (C4H) being the most intensively studied. Its greatest physiological role is to serve as an early rate-limiting step and to sequentially regulate the biosynthesis of sophisticated secondary metabolites. This pathway is the main route for lignin biosynthesis; induced lignification is a critical defense against wounding or pathogens (Hahlbrock and Scheel, 1989). Despite many research efforts, the regulation of lignin biosynthesis, particularly with respect to the stress response, is not yet clearly understood. Several defense-related genes in the early pathway, such as for phenylalanine ammonia lyase (Lois and Hahlbrock, 1992; Diallinas and Kanellis, 1994), C4H (Fahrendorf and Dixon, 1993; Mizutani et al., 1993), and chalcone synthase, are regulated at the transcriptional level (Liang et al., 1989; Joos and Hahlbrock, 1992; Teutsch et al., 1993; Frank et al., 1996; Batard et al., 1997; Bell-Lelong et al., 1997; Richard et al., 2000; Betz et al., 2001). However, their signal pathways are still controversial.

In this study, we examined the effect of wounding and several chemical effectors, known as either triggers in defense-gene activation or mediators in the defense-related signal transduction pathway, on *C4H* transcript accumulation in *Camptotheca acuminata*.

MATERIALS AND METHODS

Plant Material and Treatments

C. acuminata plants were grown from seed under

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greenhouse conditions. Leaves of similar size and age were detached and wounded with a cork borer. These leaf discs were then incubated in water for various periods at room temperature (RT). Afterward, the samples were placed in 3-L sealed jars containing either air alone as a control for the ethylene experiments or an ethanol-dipped cotton swab as a control for the methyl jasmonate experiments. For the hydrogen peroxide treatment, samples were floated on 20 mM sodium phosphate buffers containing various concentrations of H_2O_2. For experiments that combined wounding with the effects of biosynthesis inhibitors, we tested aminoethoxyvinyl glycine (AVG; ethylene synthesis inhibitor), salicylic acid (SA; jasmonic acid synthesis inhibitor), and diphenylene iodonium (DPI; NADPH oxidase inhibitor). In those trials, detached leaves were first pre-incubated for 4 h in 20 mM sodium phosphate buffer that contained one of the inhibitors, then injured with a cork borer. All treatments were carried out at RT, and all samples were frozen in liquid nitrogen immediately after collection and stored at –80°C.

Cloning of *C. acuminata C4H* (*CaC4H*)

Single-strand cDNA was constructed with a First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, USA), according to the manufacturer's instructions. Degenerate primers (forward: 5´-ACCGG G/A/T/C AA A/G GG G/A/T/C CA A/G GA C/T ATG GT-3´; reverse: 5´-GC A/G TC A/G TG G/A/T/C A A/G A/G TTCAT A/G TG-3´ encoding the amino acids TGKGQDMV and HMNLHDA, respectively) were used for amplification. PCR fragments were cloned into pGEM T-Easy (Promega, USA) and sequenced via the dideoxy chain termination method (Sanger et al., 1977) with an ABI373 automated DNA sequencer (Perkin-Elmer, USA). To obtain full-size CaC4H cDNA, 5´ and 3´ rapid amplification of either the 5´ or 3´ cDNA ends (RACE) was performed (5´/3´ RACE kit, Roche, USA).

Northern Analysis of *CaC4H* Expression

RNA was isolated as previously described by Sambrook et al. (1989). Equal amounts of total RNA (20 µg) were electrophoresed on agarose-formamide/formaldehyde gels, blotted onto Nytran Plus positively charged nylon membranes (Schleicher and Schuell, USA), and UV-cross-linked. ^{32}P radio-labeled random priming probes for the C. *acuminata C4H* cDNA gene were prepared using the Klenow enzyme, hybridized and washed at 42°C, and exposed to phosphor-image plates. Quantification of RNA hybridization signals was evaluated with a BAS 2500 Phosphor image analyzer (Fuji, Japan).

Analysis of Soluble Hydroxycinnamates

To analyze soluble hydroxycinnamates, we extracted leaves in 50% methanol. A 20-µL sample of each extract was assessed by HPLC on a C18 column (Waters, USA) using a gradient from 1% acetic acid to 100% acetonitrile, at a flow rate of 1 mL min^{-1}. Eluted compounds were detected at 322 nm and compared with the retention times for standard soluble hydroxycinnamate compounds.

RESULTS AND DISCUSSION

Isolation and Characterization of *C. acuminata C4H* cDNA

We extracted total RNA from C. *acuminata* roots and prepared first-strand cDNA for PCR amplification because it has been reported that *C4H* transcripts are highly accumulated in the stems and roots of *Arabidopsis* (Bell-Lelong et al., 1997). Using 5´ and 3´ RACE, we obtained full-length cDNA that contained a 1518-b open reading frame (ORF). Sequencing of this C. *acuminata C4H* cDNA (Genbank accession number AY621152) revealed an ORF encoding 506 amino acids. The CaC4H protein sequence was compared by multiple alignments with other available C4H sequences (NCBI protein Blast), using global comparison methods (ClustalW v. 1.8). CaC4H was found to most resemble C4H from *Populus* (81% identity), *Gossypium arboretum* (80%), *Glycine max* (79%), *Capsicum annuum* (78%), and *Arabidopsis thaliana* (77%).

A search against the BLOCKS database of conserved protein motifs indicated that CaC4H has features in common with C4Hs from other species. All the conserved helix regions typically obeyed the consensus (P/I) PGPx (G/P) xP (i.e., the H1 regions in Fig. 1) identified in other eukaryotic P450s. This region is thought to act as a 'hinge' that is required for optimal orientation of the enzyme with regard to the membrane (Yamazaki et al., 1993). Likewise, the conserved heme-binding domain contained the signature sequence FGxGRRxCxG (H2 regions in Fig. 1) in the C-terminal region, where the conserved cysteine serves as a fifth ligand for the heme iron, with the combined occurrence of a putative ER membrane

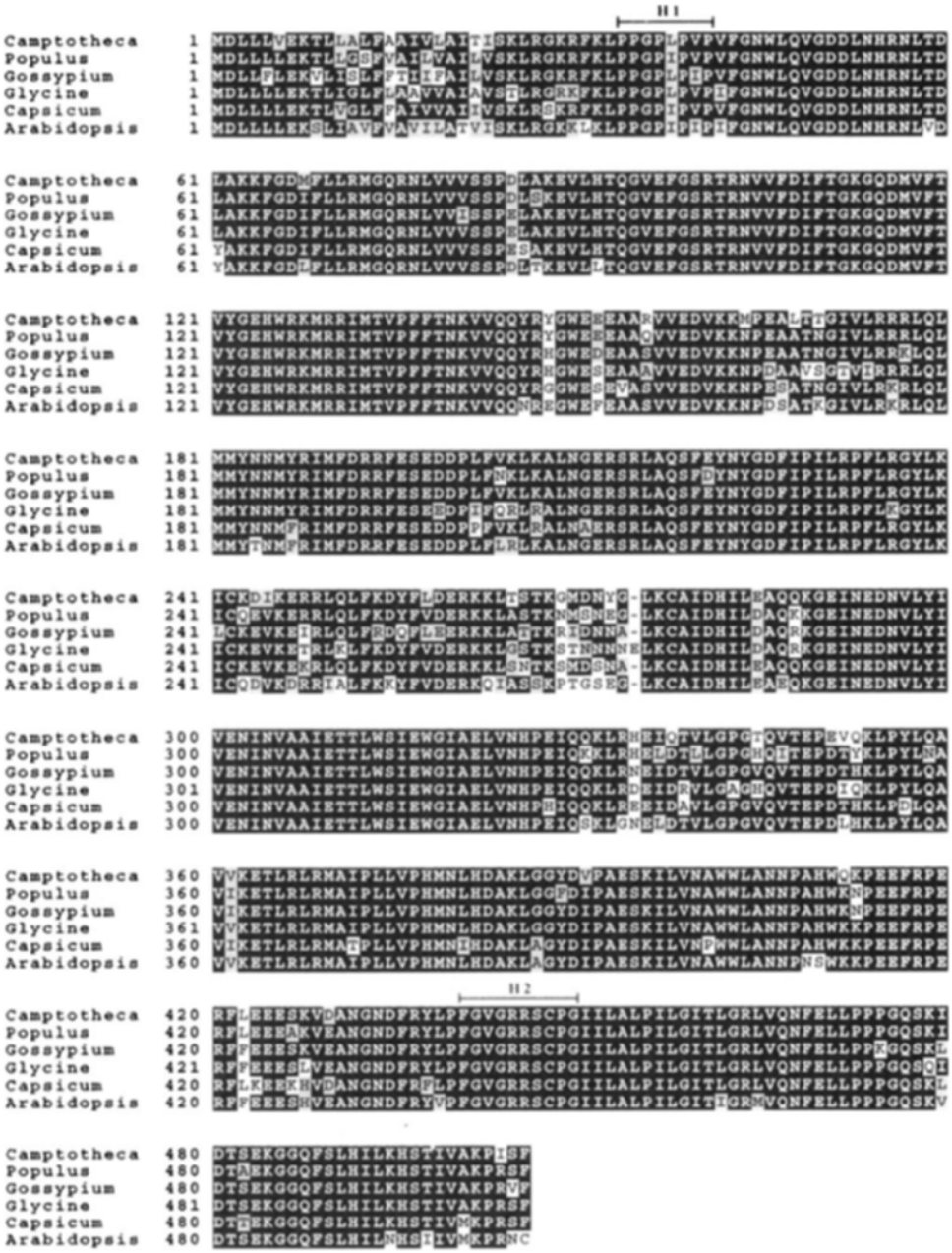

Figure 1. Deduced amino acid sequences for C4H from *C. acuminata* aligned with those of C4H proteins from *Populus* (AF302495), *G. arboretum* (AF286648), *G. max* (X92437), *C. annuum* (AF212318), and *A. thaliana* (U71080). Identical amino acids are marked in black; putative helical (H1) and heme-binding domains (H2) are indicated.

anchor at the N-terminus (Ortiz de Montellano, 1995). These data provide evidence that our *CaC4H* gene encodes a cinnamate-4-hydroxylase.

The Effects of Wounding on *CaC4H* Gene Expression

C4H can be induced by various stresses, such as wounding, fungal attack, and chemical effectors (Batard et al., 1997; Bell-Lelong et al., 1997; Ro et al., 2001). Its expression is easily detected in many plant tissues, where it catalyzes a reaction important for defense and lignification (Chapple, 1998). Wounding activates phenylpropanoid compounds, including chlorogenic acid, alkyl ferulate esters, and cell wall-bound phenolic esters (Chapple,

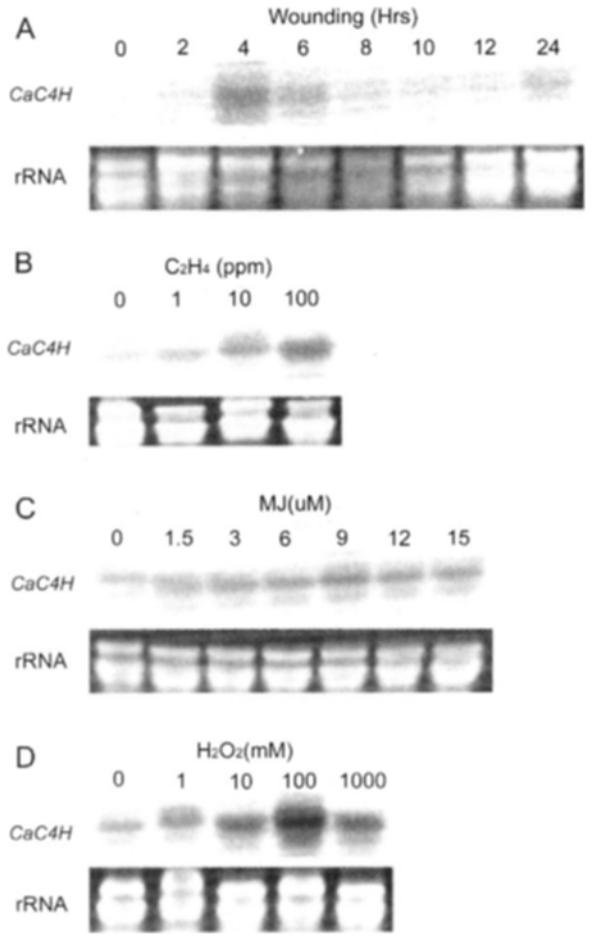

Figure 2. Effect of wounding and chemicals related to wound-signaling on gene expression. **(A)** *CaC4H* expression at indicated time after wounding. **(B-D)** Detached leaves were treated with ethylene, methyl jasmonate (MJ), or hydrogen peroxide (H_2O_2), respectively, at concentrations indicated.

1998). These compounds may serve as precursors for lignin biosynthesis. To identify the wound-induced gene expression of *C4H*, which is known to play an important role in the PAL pathway, we injured detached leaves and monitored changes in the transcription of *CaC4H* over time. Levels started to increase after 2 h and persisted for up to 24 h after wounding (Fig. 2A), a result very similar to that reported with *C4Hs* from other plant systems (Mizutani et al., 1993; Batard et al., 1997). For leaves that were chemically treated for 24 h, *CaC4H* expression was highly induced by 100 ppm ethylene or 100 mM hydrogen peroxide. However, methyl jasmonate hardly affected its expression (Fig. 2B-D).

Involvement of Signal Molecules in *CaC4H* Expression Induced by Physical Wounding

Ethylene, methyl jasmonate, and hydrogen peroxide are involved in wound-induced expression of the proteinase inhibitor gene in tomato and potato (Pena-Cortes et al., 1995; O'Donnell et al., 1996; Titarenko et al., 1997; Orozco-Cardenas et al., 2001). To gain further insight into the molecular signals involved in such expression of the *C4H* gene in *C. acuminata*, we investigated transcript levels in wounded leaves concomitant with chemical treatment as a function of time. When injured leaves were treated with 100 ppm ethylene or 15 μM methyl jasmonate, expression was more rapid, i.e., by 1 h, there than in wounded leaves that received no chemical (Fig. 3A and B). These results support the idea that ethylene and methyl jasmonate mediate wound-induced transcriptional stimulation of the *C4H* gene in *C. acuminata*. In contrast, the response from treatment with 100 mM H_2O_2 did not differ from that of the control (Fig. 3C).

To determine whether biosynthesis of these signal molecules is necessary for wound-induced expression of *CaC4H*, we performed time-course experiments using biosynthesis inhibitors that contained

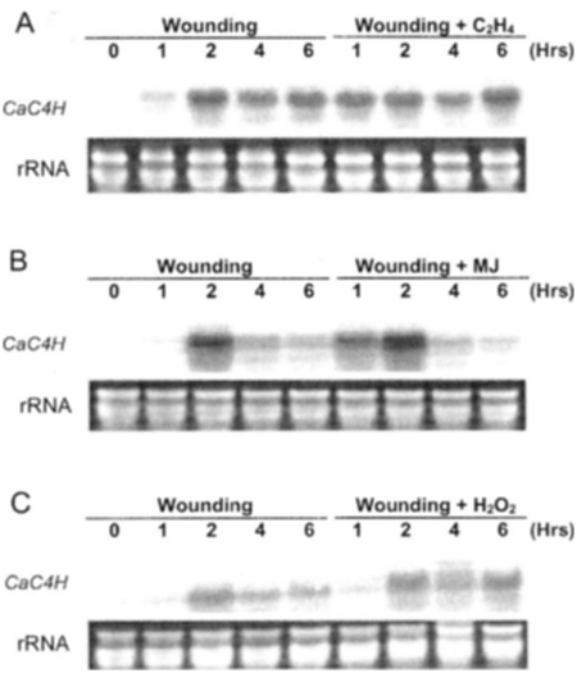

Figure 3. Expression of *CaC4H* transcripts in wounded leaves treated with different chemicals related to wound-signaling. Leaves were wounded with cork borer, then incubated with: **(A)** 100 ppm ethylene, **(B)** 15 μM methyl jasmonate, or **(C)** 100 mM hydrogen peroxide for periods indicated.

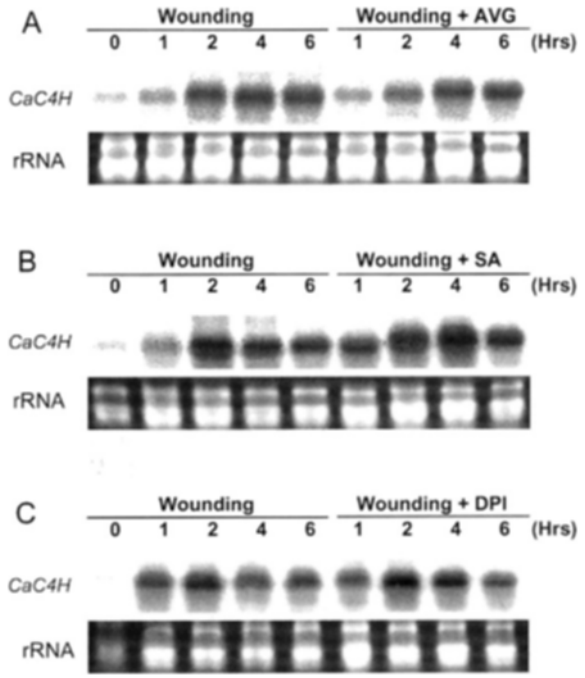

Figure 4. Effect of inhibitors involved in biosynthesis of wound-signaling molecules on wound-induced *CaC4H* expression. Detached leaves were pretreated with each inhibitor for 4 h and injured with cork borer following incubation in phosphate buffer containing each inhibitor for time indicated. AVG, ethylene biosynthesis inhibitor; SA (salicylic acid), methyl jasmonate biosynthesis inhibitor; DPI, H_2O_2-involved inhibitor.

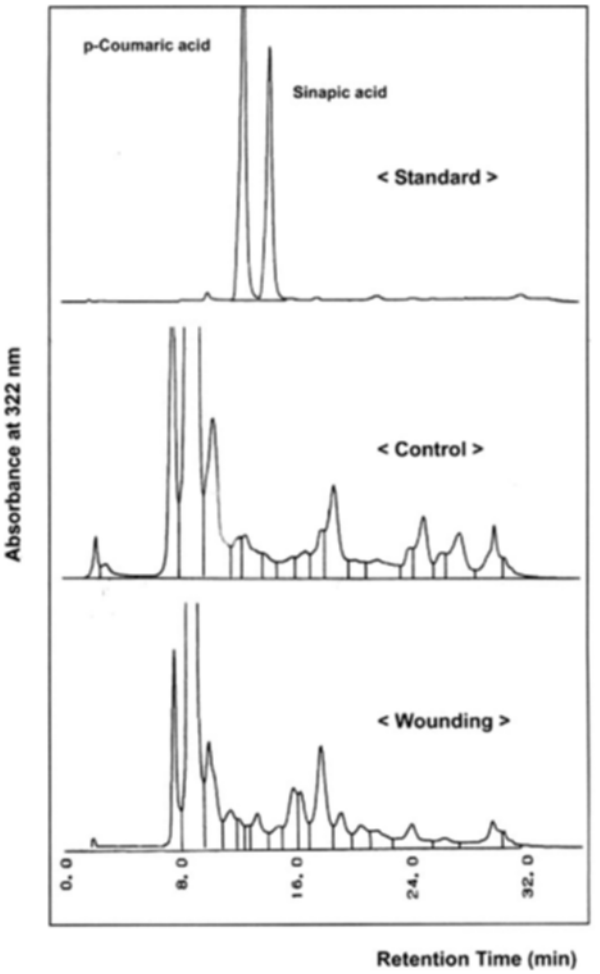

Figure 5. HPLC profiles of hydroxycinnamates extracted from control and injured leaves. Detached leaves were wounded and incubated for 4 h to determine hydroxycinnamates.

aminoethoxyvinyl glycine (AVG; ethylene synthesis inhibitor), salicylic acid (SA; jasmonic acid synthesis inhibitor), and diphenylene iodonium (DPI; NADPH oxidase inhibitor). When injured leaves were treated with SA, AVG, or DPI, the degree of wound-induced expression did not differ from that seen when wounding alone was monitored (Fig. 4A-C). Therefore, these data imply that biosynthesis of ethylene, jasmonate, and hydrogen peroxide is not involved in wound-induced regulation of the *C4H* gene in *C. acuminata*, but these chemicals may act as chemical stressors.

Effects of Wounding on Soluble Hydroxycinnamate Accumulation

To correlate the results of our *C4H* expression analysis with early phenylpropanoid metabolism, we examined soluble hydroxycinnamate accumulation in wounded leaves. There, the levels of free hydroxycinnamates that eluted from HPLC increased at retention times between 10 and 20 min. Coincident with this increase in soluble hydroxycinnamates was a decrease in less soluble or conjugated sinapates, which were eluted after 20 min (Fig. 5). C4H plays a key role in the early phenylpropanoid pathway in response to stress, and biosynthesis of other simple phenylpropanoids is down-regulated from it (Dixon and Pavia, 1995; Leon et al., 2001). Our results also showed that wounding enhanced the accumulation of free hydroxycinnamates, possibly through the expression of *C4H*, while also stimulating the biosynthesis of other phenylpropanoids.

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