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Characterization and Expression of Caffeoyl-Coenzyme A 3-*O*-Methyltransferase Proposed for the Induced Resistance Response of *Vitis vinifera* L.

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Cell-suspension cultures of *Vitis vinifera* L. cv Pinot Noir accumulated resveratrol upon fungal elicitation, and the activity of *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT), yielding feruloyl-CoA, increased to a transient maximum at 12 to 15 h. CCoAOMT cDNA was cloned from the elicited cells and was shown to encode a polypeptide highly homologous to CCoAOMTs from cells of *Petroselinum* species or *Zinnia* species. The expression of the cDNA in *Escherichia coli* revealed that grapevine CCoAOMT methylates both caffeoyl- and 5-hydroxyferuloyl-coenzyme A and is probably involved in phenolic esterification and lignification. Commercial plant activators induce the disease-resistance response of test plants and are considered to mimic the action of salicylic acid. Among these chemicals, 2,6-dichloroisonicotinic acid and benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester provoke systemic acquired resistance (SAR) and were also shown to induce the expression of class III chitinase in grapevine. The SAR response is classified by an unchanged phenotype of tissues, but the mechanistic basis is unknown. Treatment of the cultured *V. vinifera* cells with either fungal elicitor or low concentrations of salicylic acid and 2,6-dichloroisonicotinic acid, respectively, raised the CCoAOMT or stilbene synthase transcript abundance, suggesting that grapevine is capable of the SAR response, whereas benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester was ineffective. The data imply for the first time (to our knowledge) that the expression of phenylpropanoid genes in grapevine is induced by SAR activators without phenotypic consequences and suggest a role for CCoAOMT and stilbene synthase in the disease-resistance response leading beyond the level of pathogenesis-related proteins as markers of the SAR.

Plants respond to local fungal infection by the activation of various defense measures in the challenged tissues, and the induction of phenylpropanoid pathways appears to play a crucial role in this response (Hahlbrock and Scheel, 1989). The activation causes the short-term accumulation of phenolic metabolites, which might possess potent antimycotic activity as such or may protect the cells indirectly after incorporation into the cell wall. The phenolic materials strengthening the cell wall under these conditions have been collectively addressed in the literature as "lignin-like" compounds, but there is increasing evidence that the reinforcement is based primarily on coumaric and ferulic esters of cell wall polysaccharides or callose (Matern and Kneusel, 1988; Matern, 1991; Iiyama et al., 1994). The reinforcement triggered locally by pathogen invasion (Niemann et al., 1991) or fungal elicitation (Graham and Graham, 1991) causes drastic changes in the rigidity and digestibility of the cell wall and presents a major obstacle to the activities of lytic fungal enzymes (Nicholson and Hammerschmidt, 1992; Matern et al., 1995).

Point inoculations of cucumber plants with *Colletotrichum* ssp. have revealed that fungal challenge, in addition to triggering the local resistance response, is capable of mobilizing the plants' resistance capacities in tissues in advance of the fungus (Kuc, 1982). This "induced immunity" of remote tissues was not associated with phenotypic changes but alerted and predisposed the tissues to a more intensive response upon subsequent infection. The state of enhanced resistance was termed systemic acquired resistance (SAR; Lawton et al., 1993). The communication of tissues in the SAR requires a systemic signaling mechanism, the nature of which has not been elucidated. SA was suggested as an endogenous signal substance in this process (Malamy et al., 1990; Metraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Raskin, 1992), and recent reports corroborated the local requirement of SA at the site of the challenge inoculation (Gaffney et al., 1993). However, grafting experiments with transgenic tobacco conceivably ruled out SA as the mobile signal for SAR transmission, and its exact role is still under controversial discussion (Vernooij et al., 1994; Shulaev et al., 1995).

The principal phenomenon of SAR has been confirmed for several plants such as tobacco and Arabidopsis (Ward

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Abbreviations: BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; CCoAOMT, *S*-adenosyl-L-Met:*trans*-caffeoyl-coenzyme A *O*-methyltransferase (EC 2.1.1.104); COMT, caffeate *O*-methyltransferase (EC 2.1.1.6); INA, 2,6-dichloroisonicotinic acid; OMT, *O*-methyltransferase; PAL, Phe ammonia-lyase (EC 4.3.1.5); SA, salicylic acid; SAR, systemic acquired resistance; STS, stilbene synthase (EC 2.3.1.95); VCH3, *Vitis* acidic class III chitinase (EC 3.2.1.14); WP, wettable powder.

et al., 1991; Uknes et al., 1992) and the transient expression of pathogenesis-related proteins, e.g. glucanase and chitinase, was correlated with the SAR in remote tissues (Binder et al., 1989). Although the experimental proof is still lacking for major crop plants, the application of the SAR concept for crop protection is appealing, and chemicals like INA or BTH, which mimic the action of SA and trigger the SAR response at micromolar concentrations in cucumber, tobacco, or Arabidopsis (Metraux et al., 1991; Kessmann et al., 1994; Friedrich et al., 1996; Lawton et al., 1996) were developed commercially. Plants sprayed with these chemicals revealed no signs of metabolic changes unless inoculated subsequently with a fungus. The incubation of cucumber hypocotyls with INA, for example, caused the enhanced incorporation of cell wall-associated phenolics only upon subsequent inoculation with *Colletotrichum lagenarium* (Siegrist et al., 1994). Similarly, incubation of parsley cell-suspension cultures with INA followed by fungal elicitor treatment initiated an enhanced rate of transcription of PAL and 4-coumarate:CoA ligase (EC 6.2.1.12) mRNAs (Kauss et al., 1992) and these changes were succeeded by phenolic reinforcement of the cell wall (Kauss et al., 1993). Most notably, however, INA alone failed to induce PAL activity (Kauss et al., 1992).

The accumulation of lignin-like materials occurred more rapidly and to a greater extent in leaf tissues in the state of SAR than in control leaves (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1985) and the ready incorporation of phenylpropanoid compounds into the polymers under these conditions was demonstrated by Dean and Kuc (1987) on feeding radiolabeled cinnamic acid to cucumber plants challenged with *C. lagenarium*. These results already point to a close link between SAR and the sensitization of phenylpropanoid metabolism, although the fact that the tissue phenotype in the SAR state was unchanged and required an additional fungal inoculation to release the enhanced lignification remains puzzling. The reinforcement with lignin-like materials requires the synthesis of feruloyl- and sinapoyl-CoAs (Walter, 1992; Boudet et al., 1995) and a novel route to these substrates was reported recently based on the sequential methoxylations of 4-coumarate:CoA ligase (Kneusel et al., 1989; Pakusch et al., 1989, 1991; Pakusch and Matern, 1991; Matern et al., 1995). The methyltransferase involved, CCoAOMT, was cloned, and the expression of the enzyme activity appeared to be controlled by multiple parameters (Schmitt et al., 1991; Ye et al., 1994; Grimmig and Matern, 1997). The complex pattern of regulation nourished the idea that CCoAOMT might be a target of modulation in the SAR predisposition of tissues for enhanced lignification, which is compatible also with the fact that the pathway beyond PAL must be particularly considered for SAR regulation (Kauss et al., 1992).

In the course of studies of *Vitis vinifera* L., which is cultivated worldwide with an estimated acreage of 10 million ha (Food and Agriculture Organization of the United Nations, 1987) and devastated frequently by fungal diseases (Agrios, 1997), we became aware of the surprisingly little research dedicated to the resistance mechanisms other than phytoalexin production (Langcake and Pryce, 1976; Melchior and Kindl, 1990; Liswidowati et al., 1991) and the description of lignin-like cell wall polymers (Weber, 1992). Grapevine tissues are recalcitrant to biochemical analysis because of their stiff, leathery texture, as well as their high content of phenolic polymers and organic acids. These difficulties can be overcome, however, by developing appropriate cell cultures. We therefore established a number of cell cultures from hypocotyls of different cultivars, and the cv Pinot Noir turned out to be particularly suitable and was chosen for model studies of the regulation of the phenylpropanoid pathway. The induction of CCoAOMT by fungal elicitation or treatment with SA, INA, or BTH was compared in these cultures with the induction of STS mRNA, which encodes the key enzyme of grapevine phytoalexin biosynthesis (Melchior and Kindl, 1990), or to the expression of VCH3 (Busam et al., 1997), postulated previously as an SAR marker gene in tobacco and cucumber (Ward et al., 1991; Lawton et al., 1994). Based on these studies, we propose that grapevine is also capable of the SAR response and we present a plausible explanation for the mode of predisposition to enhanced phenylpropanoid synthesis and cell wall reinforcement.

MATERIALS AND METHODS

Chemicals, Enzymes, and Materials

Sources of restriction enzymes, vectors, *Escherichia coli* host strains, biochemicals, and chemicals are the same as used by Busam et al. (1997). Caffeoyl-CoA, 5-hydroxyferuloyl-CoA, and 5-hydroxyferulate were synthesized according to the method of Stöckigt and Zenk (1975) and Banerjee et al. (1962). *S*-Adenosyl-L-[*methyl*-^{14}C]Met (1.85–2.29 GBq mmol^{-1}) was purchased from Amersham.

Cell Cultures

Pigmented tissue cultures of *Vitis vinifera* cv Gamay Fréaux were obtained from F. Cormier (FRDC, Agriculture Canada, St. Hyacinthe, Quebec). Unpigmented callus cultures were initiated in dark/light intervals (12/12 h) from *V. vinifera* L. cv Pinot Noir stems plated on SH-agar (Schenk and Hildebrandt, 1972) supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} *p*-chlorophenoxyacetic acid, and 0.1 mg L^{-1} kinetin. The calli were transferred to B5 agar (Gamborg et al., 1968) containing 0.1 mg L^{-1} 1-NAA, 0.2 mg L^{-1} kinetin, and 250 mg L^{-1} casein hydrolysate, and suspension cultures were established in the modified B5 medium (40 mL in 200-mL Erlenmeyer flasks) and propagated at 110 rpm on a rotary shaker at 25°C in 12-h dark/light cycles. The rapidly growing cells were routinely subcultured every 12 d. Induction of the cell cultures with commercial yeast extract or plant activators (SA, INA, and BTH) and treatment with WP were described by Busam et al. (1997).

CCoAOMT cDNA Cloning and Sequencing

A *Vitis* sp. cDNA library of 2×10^5 recombinants (Busam et al., 1997) was screened by plaque hybridization at low

stringency using a ^{32}P-labeled CCoAOMT cDNA probe from parsley (Schmitt et al., 1991). Two clones were selected in three rounds of plaque purification, and plasmids (pBluescript SK$^+$) harboring inserts of approximately 1.0 kb were rescued from these clones by the in vivo excision protocol for λZAP (Stratagene). Both strands of double-stranded template DNA were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using modified T7 DNA polymerase (Sequenase, United States Biochemical) and the universal (M13) and reverse sequencing primers (RP) in addition to sequence-derived primers.

Southern-Blot Hybridization

Genomic DNA was isolated from the leaves of young *V. vinifera* plants (Steenkamp et al., 1994) and the DNA (10 μg) was digested with *Pst*I, *Eco*RI, *Hin*dIII, or *Bam*HI restriction enzymes. The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and blotted to nylon membranes by downward capillary transfer (Zhou et al., 1994). The blots were hybridized at 65°C overnight with a ^{32}P-labeled *V. vinifera* CCoAOMT cDNA (VCCoAOMT) probe, washed under stringent conditions, and subjected to autoradiography (Sambrook et al., 1989).

Northern-Blot Hybridization

Equivalent amounts of either total or poly(A$^+$) RNA, isolated from the cultured *V. vinifera* cells (Busam et al., 1997) and quantified spectrophotometrically (Gene-quant, Pharmacia), were used for hybridization. The hybridization of northern blots (Busam et al., 1997) was carried out overnight at 42°C with digoxigenin-labeled VCCoAOMT or STS (Melchior and Kindl, 1990) cDNA probes in the presence of 50% formamide.

Heterologous Enzyme Expression

The grapevine CCoAOMT cDNA was 5′ truncated by introduction of an *Nde*I restriction site at the translational start codon and PCR amplification with VCCoAOMT cDNA in pBluescript as template. The PCR product was digested with *Nde*I and *Xho*I and inserted into *pET-21b* (Novagen, Madison, WI) for the expression in *E. coli*. The plasmids containing the correct insert were propagated in *E. coli* BL21(DE3) in the presence of 100 μg mL^{-1} carbenicillin according to the work of Studier et al. (1990). Expression of the *V. vinifera* CCoAOMT was induced by the addition of 1 mM isopropyl-β-thiogalactopyranoside, and the cells were harvested by centrifugation (5000*g*, 10 min) after an additional 3 h of incubation. The bacterial pellet was resuspended in 50 mM sodium phosphate extraction buffer, pH 7.5, containing 1 mM $MgCl_2$, 2 mM DTT, and 10% (v/v) glycerol.

CCoAOMT and COMT Assays

CCoAOMT activity was assayed according to the method of Pakusch et al. (1989) using caffeoyl-CoA or 5-hydroxyferuloyl-CoA as a substrate, and COMT activity was measured under equivalent conditions with caffeate or 5-hydroxyferulate as substrate. Crude enzyme extracts were prepared by homogenization of deep-frozen *V. vinifera* cells in extraction buffer and clearing by centrifugation (10,000*g*, 10 min).

Purification of *V. vinifera* CCoAOMT Expressed in *E. coli*

Crude enzyme extracts from *E. coli*, prepared by sequential addition of lysozyme and DNase and cleared by centrifugation (15,000*g*, 15 min), were subjected to ammonium sulfate fractionation (30–80%). The pellet was resuspended in extraction buffer, cleared by centrifugation, the supernatant was applied to a Sephracryl S-200 HR column (100 × 5 cm), and CCoAOMT was eluted in extraction buffer. Fractions containing CCoAOMT were concentrated by ultrafiltration and applied to a Mono-Q HR 5–5 column (Pharmacia). The CCoAOMT was eluted with a linear NaCl gradient (0–500 mM). The relative molecular mass and apparent purity of the CCoAOMT was monitored by SDS-PAGE on 14% gels, and proteins in the gel were stained with Coomassie blue. The enzyme purified to homogeneity was used for the determination of substrate specificities.

RESULTS

Growth and Elicitation of *V. vinifera* Cell-Suspension Culture

Preliminary RNA and protein extractions of different grapevine tissues and pigmented cell-suspension culture (F. Cormier, Agriculture Canada, Quebec) resulted in poor yields, since high buffer concentrations (1–1.5 M) were required to neutralize the endogenous acid. Therefore, tissue and cell-suspension cultures were initiated from the leaves and hypocotyls of different grapevines such as *V. vinifera* L. cv Pinot Noir, V. *vinifera* cv Cabernet Franc, or *Vitis rupestris*. An unpigmented cv Pinot Noir culture was eventually chosen for further investigations because the cells showed a fine yellow-white appearance, grew rapidly in suspension, and did not accumulate unusually high amounts of phenols or acids. Accordingly, active enzymes and intact RNA were extracted in satisfactory yields from the cultures using 0.05 to 0.1 M buffers. To our knowledge, cell cultures of this cultivar and the induction characteristics have not been reported previously. The inducibility of the cell-suspension culture was therefore tested with various biotic elicitors, taking the accumulation of the stilbene-phytoalexin resveratrol as a marker of the positive response (Langcake and Pryce, 1976). The cell culture reacted rapidly to the addition of live *Pseudomonas syringae* pv *syringae* cells or commercial yeast extract. Resveratrol and related phenolics started to accumulate after about 6 to 9 h under these conditions, and the yellow-white cells and the culture broth turned brown (data not shown). In contrast to many other plant cells (Tietjen et al., 1983; Graham and Graham, 1991), however, the *Vitis* sp. cells apparently failed to respond to the addition of *Phytophthora megasperma* f. sp. *glycinea* cell wall elicitor. Nevertheless, the results documented that *V. vinifera* L. cv Pinot Noir cell

culture can be used for model studies of the molecular mechanism of the grapevine defense response.

Induction of CCoAOMT and COMT Activities

COMT and, in particular, CCoAOMT activities have been commonly considered as indicators of phenylpropanoid synthesis and lignification (Ye et al., 1994; Boudet et al., 1995; Matern et al., 1995). Low constitutive activities of both CCoAOMT and COMT were determined for the cultured grapevine cells (Fig. 1). Following the addition of yeast extract, however, the CCoAOMT activity increased with a lag of about 6 h to reach approximately 3-fold levels within 24 h (Fig. 1A). COMT activity was not induced to a significant extent under these conditions but increased steadily over the time of the experiments by a factor of 1.5 to 2.0 (Fig. 1B). COMT and CCoAOMT from other plants, reportedly methylate caffeic and 5-hydroxyferulic substrates (Matern et al., 1995; Meng and Campbell, 1996), and the corresponding activities were therefore measured in the crude grapevine extracts. Caffeate and 5-hydroxyferulate were methylated by COMT(s) at a constant ratio of approximately 1:3 (Fig. 1B), whereas the CCoAOMT activities with caffeoyl-CoA and 5-hydroxyferuloyl-CoA were coincidentally induced, although the relative ratio varied over the time of the experiments from 0.7 to 0.5 (Fig. 1A). The differential ratio appears to be strongly influenced by the presence of unidentified factors in the crude extracts, since rebuffering of the enzyme by passage through a Sephadex G25 column inverted the ratio of specific activities while the basic time course of induction remained the same (data not shown).

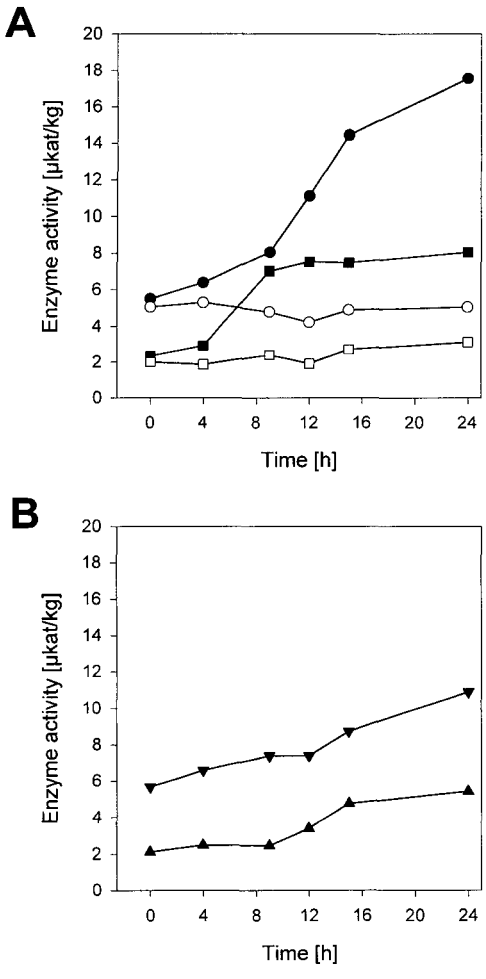

Figure 1. Induction of CCoAOMT (A) and COMT (B) activities in *V. vinifera* cell-suspension cultures. CCoAOMT assays were carried out with *S*-adenosyl-L-(*methyl*-^{14}C)-Met and caffeoyl-CoA (■, □) or 5-hydroxyferuloyl-CoA (●, ○), whereas caffeate (▲) and 5-hydroxyferulate (▼) were used as co-substrates in the COMT assays. The cell cultures were treated with yeast extract (1 mg mL^{-1} culture) (■, ●, ▲, ▼) and untreated cultures served as a control (□, ○).

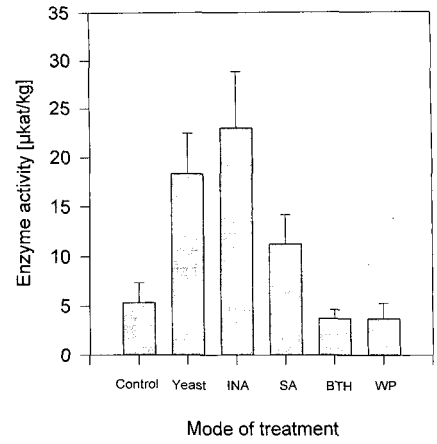

Figure 2. Specific CCoAOMT activities of noninduced (control) versus induced *V. vinifera* cells. The cell-suspension cultures were treated for 24 h with commercial yeast extract (Yeast, 1 mg mL^{-1} culture), 25 µM INA in WP, 20 µM SA, or 25 µM BTH in WP, and the enzyme activities were determined with caffeoyl-CoA. WP (0.15 mg/40 mL culture) lacking an active ingredient was used as a further control.

The induction of CCoAOMT activity in grapevine cells upon treatment with yeast extract served as a control for the induction of the cell cultures with chemicals (INA and BTH) that have been synthesized commercially as plant activators of the SAR response (compare Busam et al., 1997). The specific CCoAOMT activity was determined after 24 h of treatment or in nontreated cultures (Fig. 2), and treatment of the cells with low concentrations of SA served as a means of further control. In contrast to the elicitation with yeast extract, treatment with either INA or BTH did not cause the discoloration of the grapevine cultures. Formulated INA and BTH had to be used in the experiments and the formulation additive itself (WP) was used as an additional control (Fig. 2). Yeast extract or INA turned out to be the most potent elicitors of CCoAOMT activity, with average induction factors of 3 and 4, respectively, whereas SA caused a weaker elicitation. Treatment of the cell suspensions with BTH did not induce CCoAOMT activity. The data clearly indicated that grapevine cells can be induced, albeit in a differential mode, by typical inducers of the SAR response and suggested that the induction of phenylpropanoid pathways beyond the formation of cin-

namic acid (Kauss et al., 1992) might be involved in the response.

CCoAOMT cDNA and Gene Copy Number

Based on the induction timing of CCoAOMT activity (Fig. 1), a cDNA library was generated from poly(A^+) RNA of grapevine cells that had been elicited for 4 h with yeast extract, and two clones were selected in three rounds of screening with a parsley CCoAOMT cDNA probe (Schmitt et al., 1991). These clones harbored an identical cDNA insert of 976 bp, designated VCCoAOMT. The insert appeared to represent the full-size cDNA encoding one polypeptide of 242 amino acids (Fig. 3) and containing 5′ and 3′ flanking regions of 59 and 191 bp, respectively, with several putative polyadenylation sites followed by a short stretch of poly(A) (Fig. 3). The size of the insert was compatible with the transcript length of *V. vinifera* mRNA of approximately 1.15 kb determined by northern hybridization, assuming an average poly(A) tailing. Furthermore, a mass of 27,233 D was calculated for the translated polypeptide, which compares favorably to CCoAOMTs from other plants, and database alignments with CCoAOMTs from

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1                                                          GGCA
5       CGAGGAGAAAGTTTCGGGCGAATACTAGAAGGAAAGGAACAGAAGATCTCTAGAA

60      ATG GCC ACG AAC CAA GAA GCT GGG AGG CAC CAG GAG GTT GGC
        M   A   T   N   Q   E   A   G   R   H   Q   E   V   G      14

102     CAC AAG AGC CTT TTG CAG AGT GAT GCT CTT TAT CAG TAT ATA
        H   K   S   L   L   Q   S   D   A   L   Y   Q   Y   I      28

144     CTT GAA ACC AGT GTG TAC CCA AGA GAG CCT GAA TCC ATG AAG
        L   E   T   S   V   Y   P   R   E   P   E   S   M   K      42

186     GAG CTC AGA GAG TTG ACT GCC CAG CAT CCA TGG AAC ATC ATG
        E   L   R   E   L   T   A   Q   H   P   W   N   I   M      56

228     ACT ACG TCT GCT GAT GAA GGG CAG TTC TTG AAC ATG CTT CTC
        T   T   S   A   D   E   G   Q   F   L   N   M   L   L      70

270     AAG CTC ATC AAT GCC AAG AAC ACC ATG GAG ATA GGC GTC TAC
        K   L   I   N   A   K   N   T   M   E   I   G   V   Y      84

312     ACT GGC TAC TCT CTT CTG GCC ACA GCC CTT GCT CTC CCC GAT
        T   G   Y   S   L   L   A   T   A   L   A   L   P   D      98

354     GAC GGA AAG ATC CTG GCT ATG GAC ATC AAC AAA GAA AAT TAC
        D   G   K   I   L   A   M   D   I   N   K   E   N   Y     112

396     GAG CTG GGT CTG CCA GTA ATT CAA AAG GCA GGG GTT GCC CAC
        E   L   G   L   P   V   I   Q   K   A   G   V   A   H     126

438     AAG ATT GAC TTC AAA GAA GGC CCT GCT TTG CCT GTT CTT GAT
        K   I   D   F   K   E   G   P   A   L   P   V   L   D     140

480     CAG ATG ATC GAA GAT GGC AAG TAT CAC GGG TCG TTC GAC TTC
        Q   M   I   E   D   G   K   Y   H   G   S   F   D   F     154

522     ATA TTC GTG GAC GCA GAC AAG GAC AAT TAT CTG AAC TAC CAC
        I   F   V   D   A   D   K   D   N   Y   L   N   Y   H     168

564     AAG AGA TTG ATC GAT TTG GTG AAG GTG GGG GGA ATC ATC GGC
        K   R   L   I   D   L   V   K   V   G   G   I   I   G     182

606     TAC GAC AAC ACC CTC TGG AAC GGG TCG GTG GTG GCT CCG CCC
        Y   D   N   T   L   W   N   G   S   V   V   A   P   P     196

648     GAT GCT CCG CTG CGG AAG TAC GTG AGG TAC TAC AGA GAC TTC
        D   A   P   L   R   K   Y   V   R   Y   Y   R   D   F     210

690     GTG TTG GAG CTG AAC AAG GCT CTT GCT GCT GAC CCA AGA ATC
        V   L   E   L   N   K   A   L   A   A   D   P   R   I     224

732     GAG ATC TGT ATG CTT CCG GTT GGT GAC GGG ATC ACC CTT TGC
        E   I   C   M   L   P   V   G   D   G   I   T   L   C     238

774     CGT CGG CTA AGC TGA ATCTCCTCGTCAACTCCACCGACGGTGGCTCTTTG
        R   R   L   S   *                                         242

824     ATCGGCGACTCAAAAACTCAATGGAAAATTGATACGACTATTTGCTTTCTTTAAT
879     TTTCCTCTTTTGCGACTTTAACTTTGGATTTGTATTTCAACGAAATGGATGATTT
934     TTTATAATAATAATAATAATAATTAAAAAAAAAAAAAAAAAAA
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Figure 3. cDNA and translated polypeptide sequences of *V. vinifera* CCoAOMT. The nucleotide and amino acid residues are numbered in the left and right margins, respectively. The first polyadenylation site is underlined, and the termination codon is marked by an asterisk.

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VvCCoAOMT   MATN-----Q EAGRHQEVGH KSLLQSDALY QYILETSVYP REPESMKELR     45
PcCCoAOMT   **S*GESKHS ------**** ********** ********** ****A*****     44
ZeCCoAOMT   ***PTGET-* P*-K****** ********** ********** ***QP*****     48
MsCCoAOMT   ****EDQKQT *S******** ********** ********F* **H*A*****     50
PtCCoAOMT   ****GEEQQS Q********* ********** ********** ****C*****     50

VvCCoAOMT   ELTAQHPWNI MTTSADEGQF LNMLLKLINA KNTMEIGVYT GYSLLATALA     95
PcCCoAOMT   *V**K****L ********** ********** ********** **********     94
ZeCCoAOMT   RI**K****L ********** **L******* ********** *****S****    100
MsCCoAOMT   *V**K***** ********** *S******** ********** **********    100
PtCCoAOMT   *V**K***** ********** *******V** ********** **********    100

VvCCoAOMT   LPDDGKILAM DINKENYELG LPVIQKAGVA HKIDFKEGPA LPVLDQMIED    145
PcCCoAOMT   ********** ***R****I* **I*E****G *****R**** *****H*L**    144
ZeCCoAOMT   **E******L ***R****I* **I******* *****R**** **L****LQ*    148
MsCCoAOMT   I*E******* ********** ****K****D *****R**** *****E**K*    150
PtCCoAOMT   I*E******* ***R****** ********** ********** **********    150

VvCCoAOMT   GKYHGSFDFI FVDADKDNYL NYHKRLIDLV KVGGIIGYDN TLWNGSVVAP    195
PcCCoAOMT   *****T***V *********I ********** *I**L***** *******AQ*    194
ZeCCoAOMT   E*C******* ********** ********** *F**V***** ******L***    198
MsCCoAOMT   E*N***Y*** ********** ********** ****V***** **********    200
PtCCoAOMT   ********** *********I *******E** ****L***** **********    200

VvCCoAOMT   PDAPLRKYVR YYRDFVLELN KALAADPRIE ICMLPVGDGI TLCRRLS.      242
PcCCoAOMT   A***M***** ******I*** ********** *********V *****I*.     241
ZeCCoAOMT   A********** ********** ****V***V* **Q******* *****I*.     245
MsCCoAOMT   ********** ********** ****V***** ********** *I***IK.     247
PtCCoAOMT   ****M***** ********** ********** ********** *****IQ.     247
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Figure 4. Alignment of CCoAOMT polypeptide sequences of *V. vinifera* L. (VvCCOAMT, GenBank accession no. Z54233), *P. crispum* (PcCCOAMT, GenBank accession no. M69184), *Z. elegans* (ZeCCOAMT, GenBank accession no. U13151), *M. sativa* (MsCCOAMT, GenBank accession no. U20736), and *P. tremuloides* (PtCCOAMT, GenBank accession no. U27116). Amino acid residues are numbered in the right margin. Asterisks indicate identical amino acid residues, and hyphens bridge the gaps introduced to maximize the alignment.

Petroselinum crispum (Schmitt et al., 1991), *Zinnia elegans* (Ye et al., 1994), *Medicago sativa* (Sewalt et al., 1995), or *Populus tremuloides* (Meng and Campbell, 1995; Fig. 4) revealed more than 70% identity at the nucleotide and 85 to 94% identity at the polypeptide sequence levels. Nonconservative replacements of amino acids were particularly distinct in the N-terminal decapeptide (Fig. 4). Much less identity, about 55% at the polypeptide level, was observed with CCoAOMTs from *Stellaria* spp. (Zhang et al., 1995) or Arabidopsis (Zou and Taylor, 1994) and no significant homology was observed to COMTs or any other plant OMT. The cDNA isolated from the elicited grapevine cells was thus likely to encode the CCoAOMT.

The copy number of grapevine CCoAOMT genes was estimated by Southern hybridization of DNA isolated from leaves of young *V. vinifera* plants, and the patterns of *Pst*I, *Eco*RI, *Hin*dIII, or *Bam*HI restrictions were analyzed using VCCoAOMT cDNA as the hybridization probe (Fig. 5). VCCoAOMT cDNA lacks restriction sites for these endonucleases. The small number of restriction fragments, showing three bands in the case of *Hin*dIII and only two bands for *Pst*I, *Eco*RI, and *Bam*HI (Fig. 5), suggested that *V. vinifera* encodes only one or two CCoAOMT genes that may represent the alleles of the same gene. This assump-

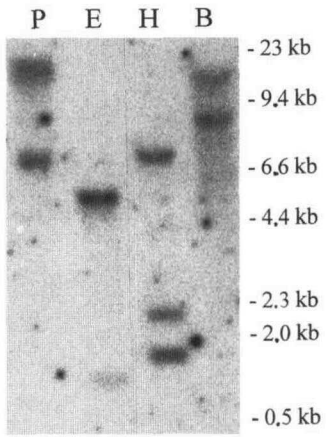

Figure 5. Southern hybridization of genomic DNA isolated from the leaves of young *V. vinifera* plants. The DNA (10 μg/lane) was restricted with *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), or *Bam*HI (B) prior to separation on a 0.7% agarose gel, and ^{32}P-labeled VCCoAOMT cDNA was used as a hybridization probe.

tion is relevant for the bifunctionality of the methyltransferase and the relative ratio of catalysis with the substrates caffeoyl- and 5-hydroxyferuloyl-CoA.

Heterologous Expression of *V. vinifera* CCoAOMT

The VCCoAOMT cDNA was 5′ truncated for the expression in *E. coli* as had been accomplished with enzymes from *Ruta* spp. and *Petroselinum* spp. (Junghanns et al., 1995; Matern et al., 1995; B. Grimmig, unpublished data) and an *Nde*I-restriction site was generated at the start of translation. The amplified cDNA construct was introduced into vector *pET-21b* and transformed into the host strain *E. coli* BL21 (DE3) for isopropyl-β-thiogalactopyranoside-induced expression of CCoAOMT. The heterologous expression yielded a highly active CCoAOMT that was purified on a preparative scale by conventional column chromatography. Subsequent examination by SDS-PAGE revealed one band of 27 ± 2 kD for the homogeneous enzyme (Fig. 6). The catalytic activity of the heterologously expressed grapevine CCoAOMT confirmed the identity of the VCCoAOMT cDNA clone and this pure enzyme was used for the re-evaluation of substrate specificity.

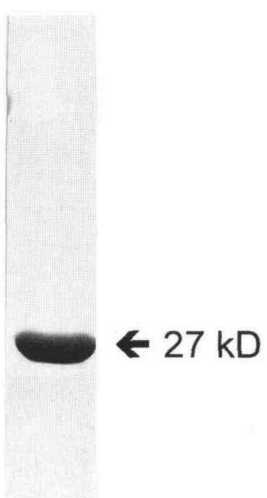

Figure 6. SDS-PAGE separation of purified *V. vinifera* CCoAOMT expressed in *E. coli* (approximately 4 μg). The gel (14%) was stained with Coomassie blue, and the arrow marks the mobility of a 27-kD reference protein.

Recent investigations in *Zinnia* spp. (Ye et al., 1994) revealed that CCoAOMT is also involved in the lignification of tissues during ontogenetic development. Woody plant species like *Zinnia* or *Vitis* produce syringyl/guaiacyl-type lignins, which require feruloyl-CoA as well as sinapoyl-CoA substrates for the synthesis of monolignols (Lewis and Yamamoto, 1990; Walter, 1992) and hence the methylation of caffeoyl- and 5-hydroxyferuloyl-CoA. The grapevine CCoAOMT expressed in *E. coli* efficiently methylated caffeoyl-CoA and 5-hydroxyferuloyl-CoA at a relative ratio of about 3:1 (36.5 versus 12.4 mkat/kg) (Table I), which is in contrast to the activities measured with crude *Vitis* sp. cell extracts. Most notably, neither caffeate nor 5-hydroxyferulate was accepted as a substrate, although the specific activity of the enzyme exceeded that of the enzyme isolated from grapevine cells by about 3 orders of magnitude (Table I). The bifunctionality toward caffeoyl- and 5-hydroxyferuloyl-CoA in vitro obviously also qualifies grapevine CCoAOMT for the lignification of tissues. Nevertheless, COMT may be additionally involved in vivo in the formation of syringyl units as has been shown in tobacco (Atanassova et al., 1995).

Expression of CCoAOMT and STS Genes in *V. vinifera* Cells Responding to SAR Activators

The cDNAs of CCoAOMT and STS (Melchior and Kindl, 1990) were used as specific probes to monitor changes in the amounts of transcripts. Treatment of the grapevine cell cultures with yeast elicitor increased the CCoAOMT and STS mRNA levels with equivalent kinetics to a transient maximum at 4 h (Fig. 7). The results are compatible with the time course of induction of the corresponding enzyme activities as well as with the accumulation of the stilbenphytoalexin resveratrol. The induction of grapevine cells with yeast extract again served as a control to compare the effect of plant activator chemicals on de novo transcription.

In parallel sets of experiments, the grapevine cell cultures were treated with low concentrations of INA or BTH, which reportedly induce the SAR response in plants (Metraux et al., 1991; Friedrich et al., 1996), as well as with SA, which is under examination as an endogenous signal sub-

Table I. *Specificity of V. vinifera CCoAOMT expressed in E. coli*[a]

Substrate	Enzyme Activity
	mkat kg^{-1}
Caffeoyl-CoA	36.5
5-Hydroxyferuloyl-CoA	12.4
Caffeate	0
5-Hydroxyferulate	0

[a] The assays were carried out with the enzyme purified to homogeneity and representing about 20% of the total *E. coli* protein.

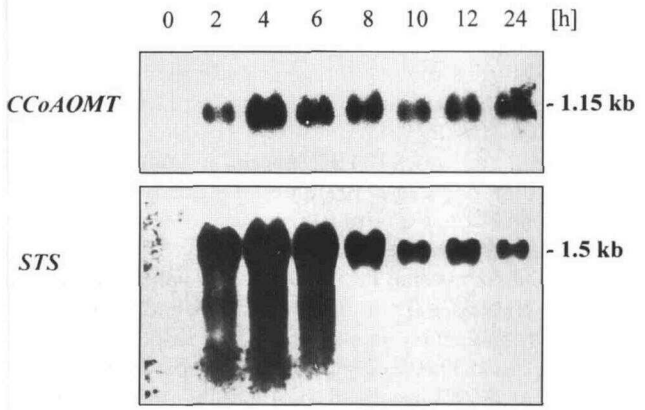

Figure 7. Relative abundance of CCoAOMT and STS transcripts in *V. vinifera* cell cultures induced with crude yeast extract (1 mg mL^{-1} culture). The cells were harvested at various times following the addition of yeast elicitor, and the total RNA was extracted and subjected to northern-blot analysis (7.5 μg/lane) using digoxigenin-labeled cDNAs of VCCoAOMT or STS as hybridization probes.

stance of the local resistance expression (Shulaev et al., 1995). Neither of these treatments caused visible signs of stress in the cultures, which is in contrast to the yeast or *Pseudomonas* sp. elicitor treatments. BTH failed to induce the de novo transcription of STS or CCoAOMT (Fig. 7), whereas treatment of the cell cultures with SA caused a transient, marked increase within 4 h in the amounts of STS and, to a lesser extent, CCoAOMT transcripts (Fig. 8). However, induction of the grapevine cells with INA resulted in the considerable, rapid increase of both CCoAOMT and STS transcript abundances, which reached maximal values after approximately 2 h. During the following 20 h, the CCoAOMT and STS mRNA levels decreased again at a slow rate (Fig. 8). Formulated WP of BTH and INA had to be used in these experiments and the control with formulation material lacking the active ingredient showed no inductive effect on CCoAOMT or STS transcription (data not shown). In tomato and cucumber, the systemically enhanced expression of pathogenesis-related proteins, in particular chitinase and glucanase, had been reported (Binder et al., 1989) and a class III chitinase was proposed as an SAR marker gene in tobacco and cucumber (Ward et al., 1991; Lawton et al., 1994). The plant activators INA and BTH also caused the induction of grapevine VCH3 transcript abundance (Busam et al., 1997). Overall, these results confirmed that grapevine cells respond to SA, INA, or BTH treatment like tobacco or cucumber and suggest that *V. vinifera* may be capable of expressing the SAR response.

DISCUSSION

Comparison of *V. vinifera* CCoAOMT to Heterologous CCoAOMTs

V. vinifera conceivably encodes only one CCoAOMT from one or two genes (Fig. 5) and the corresponding cDNA (VCCoAOMT) was cloned (Fig. 3) and identified by sequence alignments (Figs. 3 and 4) with CCoAOMTs from various other plants (Ye et al., 1994; Schmitt et al., 1991; Meng and Campbell, 1995; Sewalt et al., 1995) as well as by the catalytic activity of the heterologously expressed enzyme (Fig. 6; Table I). The sequence of *V. vinifera* CCoAOMT clearly differed from those of COMTs (Meng and Campbell, 1996) and other plant OMTs. The homologous sequence and the low gene copy number of *V. vinifera* CCoAOMT are reminiscent of the parsley CCoAOMT (Schmitt et al., 1991; Grimmig and Matern, 1997), but differ from *Zinnia* spp. (Ye et al., 1994), which encodes a family of genes, or *Stellaria* spp. and Arabidopsis (Zou and Taylor, 1994; Zhang et al., 1995), which share much lower homologies. The highly active VCCoAOMT, expressed in *E. coli* by a protocol that had been used for the preparative expression of CCoAOMTs from parsley or acridone synthase from the common rue (Junghanns et al., 1995; Matern et al., 1995), catalyzed the methylation of both 5-hydroxy-feruloyl- and caffeoyl-CoA but was inactive with caffeate or 5-hydroxy-ferulate, just like the parsley CCoAOMT (B. Grimmig, unpublished data). COMTs, for comparison, appear to be less specific and occasionally methylate also caffeoyl-CoA, as observed in poplar (Meng and Campbell, 1996) but not in alfalfa (Sewalt et al., 1995).

Crude extracts from grapevine cells apparently contained unidentified components that shift the relative ratio

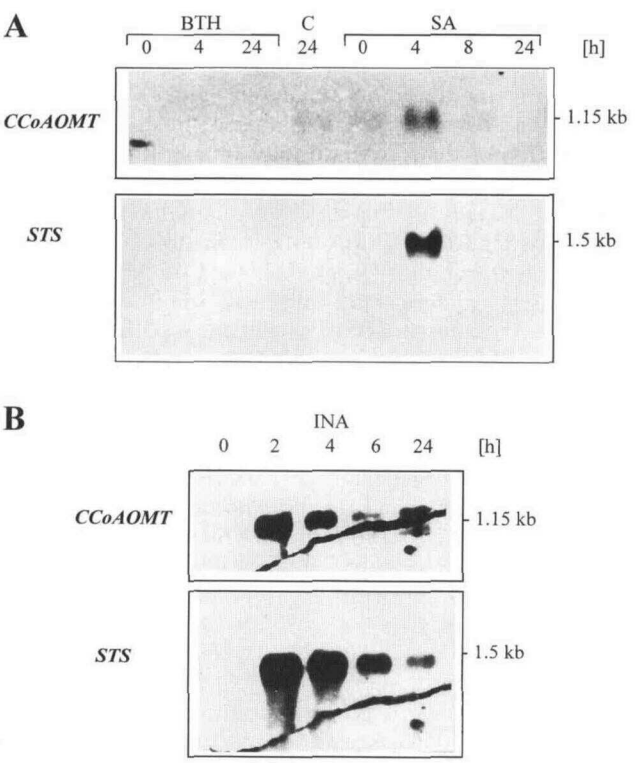

Figure 8. Relative amounts of CCoAOMT and STS transcripts in *V. vinifera* cell cultures treated with 25 μM BTH (A) or 20 μM SA and 25 μM INA (B). The cells were harvested at various times of treatment and the total RNA (A) or the poly(A^+) RNA (B) was extracted and subjected to northern-blot analysis (7.5 μg total or 0.7 μg poly$[A]^+$ RNA per lane) using digoxigenin-labeled cDNAs of VCCoAOMT or STS as hybridization probes. Control cultures were treated for 24 h with WP lacking BTH (lane C 24 h).

of substrate specificities in favor of 5-hydroxyferuloyl-CoA (Fig. 1), whereas the enzyme expressed in *E. coli* (Table I) or the crude enzyme after Sephadex gel filtration preferred caffeoyl-CoA over 5-hydroxyferuloyl-CoA by a factor of 3, and this effect deserves further attention. The gel-filtration experiments, in particular, ruled out that low-homology CCoAOMTs, which might have escaped the hybridization, were responsible for the observed shift in specificity, since low-molecular-weight factors rather than different enzyme entities determined the shift in specificity. The experiments indirectly also refuted the possibility of hydrolysis of the CoA-ester substrates during the assay prior to methylation of the acids by COMT activities (different inducibilities of COMT and CCoAOMT activities), although the definitive proof would require more sophisticated enzyme assays.

CCoAOMT Is Involved in the Induced Resistance Response of *V. vinifera*

Cell cultures of *V. vinifera* qualified for the model investigations by their selective inducibility with yeast extract and the convenient recovery of enzymes and RNA. Treatment of the cells with yeast elicitor coordinately induced the transcript abundance and activity of CCoAOMT (Figs. 1 and 7), and the mRNA induction pattern was very similar to that of the STS, which was monitored from the same filter blots. STS catalyzes the pivotal reaction in the synthesis of the *V. vinifera* phytoalexin resveratrol and has been extensively studied for its role in disease resistance and stress compensation (Hain et al., 1993). STS and CCoAOMT activities rely on the same substrates and the coordination of their induction documents the synergistic requirements for full expression of the disease resistance in grapevine. *V. vinifera* thus differs from alfalfa, in which de novo transcription of CCoAOMT seems to occur without consequent translation (Ni et al., 1996). The accumulation of lignin-like materials for cell wall reinforcement was correlated with resistance in several host-pathogen interactions (Hammerschmidt et al., 1985; Tiburzy and Reisener, 1990; Reimers and Leach, 1991), which appears to apply also to grapevine (Weber, 1992), and the physiological significance of CCoAOMT for cell wall reinforcement has been outlined in other plant systems (Schmitt et al., 1991; Matern et al., 1995). Thus, the induction of CCoAOMT is an integral part of the disease-resistance response of *V. vinifera* and follows the kinetics of other enzymes of the inducible phenylpropanoid pathway.

Chemical Induction of the SAR

The development of synthetic plant activators that induce the SAR response has added a new perspective to the protection of commercial crops. The effects of INA or SA as chemical inducers have been studied experimentally (Metraux et al., 1990, 1991), whereas the more selective BTH was released only recently (Friedrich et al., 1996; Lawton et al., 1996) for the application in wheat, tobacco, banana, and tomato. The efficiency and mode of action of these chemicals in special crops such as grape, however, has not yet been investigated. In a preliminary study, grape plantlets were sprayed with INA 10 and 4 d prior to the inoculation with *P. viticola*, the causal agent of downy mildew disease, and these studies revealed a significantly enhanced resistance of the INA-pretreated plants as compared with water controls (G. Busam, unpublished). The slow growth of grapevine plants and the harsh conditions required for tissue extraction, however, are hurdles for biochemical investigations, and the experiments were therefore continued with the newly initiated *V. vinifera* L. cv Pinot Noir cell cultures. The expression of pathogenesis-related proteins, in particular chitinases (Legrand et al., 1987), had been recommended as a molecular marker of the SAR response in plants (Binder et al., 1989; Ward et al., 1991). Accordingly, VCH3 (Busam et al., 1997) was used to probe the induction. Treatment of the *Vitis* sp. cell cultures with low concentrations of INA, BTH, or SA induced rapid and long-lasting increases in the VCH3 transcript level (Figs. 8 and 9), whereas the general appearance of the cell cultures remained unchanged. This might be considered a first, preliminary indication that grapevine is capable of the SAR response.

Treatment of the cultures with INA but not with BTH triggered concomitantly the de novo expression of CCoAOMT and STS mRNAs (Figs. 8 and 9), whereas SA caused only a low-intensity, transient signal at 4 h, which may be due in part to rapid turnover, i.e. by glucosidation, as known from tobacco (Lee et al., 1995). The notion of CCoAOMT and STS induction by INA (Fig. 9) is particularly remarkable with respect to the unchanged phenotype of the cells. Furthermore, INA and BTH display differential modes of action, which overlap in the induction of VCH3 (Figs. 8 and 9) but differ greatly in the induction of phenylpropanoid enzymes such as CCoAOMT or STS (Figs. 8 and 9), which are indispensable for the expression of resistance (Dean and Kuc, 1987; Hammerschmidt and Kuc, 1982; Nicholson and Hammerschmidt, 1992; Hain et al., 1993). This questions the reliability of the induction of pathogenesis-related proteins as an indicator of the SAR of plants.

Putative Role of CCoAOMT in the SAR Response of *V. vinifera*

Previous studies by Kauss et al. (1992) had revealed that PAL was not induced by INA treatment of parsley cells. The combination of INA preincubation of cells followed by the treatment with fungal elicitor, however, caused the enhancement of PAL activity and mRNA amounts and increased considerably the accumulation of coumarin phytoalexins. Furthermore, the reinforcement of the plant cell wall with ferulic esters was greatly stimulated under these conditions as compared with controls that had not received the INA preincubation (Kauss et al., 1993). PAL can be regarded as an "early bottleneck" enzyme of the stress-inducible phenylpropanoid pathway and it is proposed that INA predisposition of tissues involves the induction of late rather than of early phenylpropanoid enzymes. This kind of induction may provide the key for a much stronger biosynthetic capacity when the early enzymes become induced by subsequent fungal elicitation. This report demonstrated clearly that CCoAOMT and STS are induced in grapevine cells without any visible consequence on incu-

bation with INA. The ferulic cell wall reinforcement is commonly associated with browning of the cells and depends on the hydroxylation of 4-coumaroyl- to caffeoyl-CoA as shown originally in parsley (Kneusel et al., 1989; Pakusch and Matern, 1991; Matern et al., 1995); this pathway has meanwhile been confirmed for many other plants. The hydroxylation is controlled by the cytoplasmic pH, which decreases upon fungal infection or elicitation (Kneusel et al., 1989). Under the premises that rate-limiting early enzymes for phytoalexin synthesis or cell wall reinforcement, e.g. PAL or 4-coumaroyl-CoA hydroxylase, are not induced by INA treatment, the induction of STS or CCoAOMT will not cause immediate effects without subsequent fungal challenge. Such a mechanism is reminiscent of a stretched resilient hunting bow and might explain in part the puzzling phenomenology of the SAR response of grapevine and other plants.

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The GenBank/EMBL accession number of the cDNA sequence of *V. vinifera* CCoAOMT (*V*CCoAOMT) reported in this article is Z54233.

LITERATURE CITED

Agrios GN (1997) Plant Pathology, Ed. 4. Academic Press, San Diego, CA

Atanassova R, Favet N, Martz F, Chabbert B, Tollier MT, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. Plant J **8:** 465–477

Banerjee SK, Manolopoulo M, Pepper JM (1962) The synthesis of lignin model substances: 5-hydroxyvanillin and 5-hydroxyacetoguaiacone. Can J Chem **40:** 2175–2177

Binder A, Baer G, Hofmann C, Kováts K (1989) Mechanisms in systemic induced disease resistance. NATO ASI Adv Sci Inst Ser Ser H Cell **36:** 267–272

Boudet AM, Lapiere C, Grima-Pettenati J (1995) Biochemistry and molecular biology of lignification. New Phytol **129:** 203–236

Busam G, Kassemeyer HH, and Matern U (1997) Differential expression of chitinases in *Vitis vinifera* L. responding to systemic acquired resistance activators or fungal challenge. Plant Physiol **115:** 1029–1038

Dean RA, Kuc J (1987) Rapid lignification in response to wounding and infection as a mechanism for induced systemic protection in cucumber. Physiol Mol Plant Pathol **31:** 69–81

Food and Agriculture Organization of the United Nations (1987) FAO Production Yearbook. Food and Agriculture Organization of the United Nations, Rome, Italy

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Gut Rella M, Meier B, Dincher S, Staub T, Uknes S, and others (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J **10:** 61–70

Gaffney T, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158

Graham MY, Graham TL (1991) Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissues following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. Plant Physiol **97:** 1445–1455

Grimmig B, Matern U (1997) Structure of the parsley caffeoyl-CoA O-methyltransferase gene, harbouring a novel elicitor-responsive *cis*-regulatory element. Plant Mol Biol **33:** 323–341

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Phytopathol **40:** 347–369

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier P, Stöcker R, and others (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361:** 153–156

Hammerschmidt R, Bonnen AM, Bergstrom GC, Baker KK (1985) Association of epidermal lignification with nonhost resistance of curcubits to fungi. Can J Bot **63:** 2393–2398

Hammerschmidt R, Kuc J (1982) Lignification as a mechanism for induced systemic resistance in cucumber. Physiol Plant Pathol **20:** 61–71

Iiyama K, Lam T, Stone BA (1994) Covalent cross-links in the cell wall. Plant Physiol **104:** 315–320

Junghanns KT, Kneusel RE, Baumert A, Maier W, Gröger D, Matern U (1995) Molecular cloning and heterologous expression of acridone synthase from elicited *Ruta graveolens* L. cell suspension cultures. Plant Mol Biol **27:** 681–692

Kauss H, Franke R, Krause K, Conrath U, Jeblick W, Grimmig B, Matern U (1993) Conditioning of parsley (*Petroselinum crispum* L.) suspension cells increases elicitor-induced incorporation of cell wall phenolics. Plant Physiol **102:** 459–466

Kauss H, Theisinger-Hinkel E, Mindermann R, Conrath U (1992) Dichloroisonicotinic and salicylic acid, inducers of systemic acquired resistance, enhance fungal elicitor responses in parsley cells. Plant J **2:** 655–660

Kessmann H, Staub T, Hofmann C, Maetzke T, Herzog J, Ward E, Uknes S, Ryals J (1994) Induction of systemic acquired disease resistance in plants by chemicals. Annu Rev Phytopathol **32:** 439–459

Kneusel RE, Matern U, Nicolay K (1989) Formation of *trans*-caffeoyl-CoA from *trans*-4-coumaroyl-CoA by Zn-dependent enzymes in cultured plant cells and its activation by an elicitor-induced pH shift. Arch Biochem Biophys **269:** 455–462

Kuc J (1982) Induced immunity to plant diseases. Bioscience **32:** 854–860

Langcake P, Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the *Vitaceae* as a response to infection or injury. Physiol Plant Pathol **9:** 77–85

Lawton K, Uknes S, Friedrich L, Gaffney T, Alexander D, Goodman R, Metraux JP, Kessmann H, Ahlgoy P, Gutrella M, and others (1993) The molecular biology of systemic acquired resistance. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic, Dordrecht, The Netherlands, pp 422–432

Lawton KA, Beck J, Potter S, Ward E, Ryals J (1994) Regulation of cucumber class III chitinase gene expression. Mol Plant-Microbe Interact **7:** 48–57

Lawton KA, Friedrich L, Hunt M, Weymann,K, Kessmann H, Staub T, Ryals J (1996) Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J **10:** 71–82

Lee HI, Leon J, Raskin I (1995) Biosynthesis and metabolism of salicylic acid. Proc Natl Acad Sci USA **92:** 4076–4079

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol **41:** 455–496

Liswidowati MF, Hohmann F, Schwer B, Kindl H (1991) Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. Planta **183:** 307–314

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Matern U (1991) Coumarins and other phenylpropanoid compounds in the defense response of plant cells (supplement). Plant Med **57:** 515–520

Matern U, Grimmig B, Kneusel RE (1995) Plant cell wall reinforcement in the disease resistance response: molecular composition and regulation. Can J Bot **73:** 511–517

Matern U, Kneusel RE (1988) Phenolic compounds in plant disease resistance. Phytoparasitica **16:** 153–170

Melchior F, Kindl H (1990) Grapevine stilbene synthase cDNA only slightly differing from chalcone synthase cDNA is expressed in *E coli* into a catalytically active enzyme. FEBS Lett **268:** 17–20

Meng H, Campbell WH (1995) Cloning of aspen (*Populus tremuloides*) xylem caffeoyl-CoA 3-*O*-methyltransferase. Plant Physiol **108:** 1749

Meng H, Campbell WH (1996) Characterization and site-directed mutagenesis of aspen lignin-specific O-methyltransferase expressed in *Escherichia coli*. Arch Biochem Biophys **330:** 329–341

Metraux JP, Ahl-Goy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced systemic resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions. Kluwer Academic Press, Dordrecht, The Netherlands, pp 432–439

Metraux JP, Signer H, Ryals J, Ward E, Benz-Wyss M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardt B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250:** 1004–1006

Ni W, Sewalt VJH, Korth KL, Blount JW, Ballance GM, Dixon RA (1996) Stress responses in alfalfa. XXI. Activation of caffeic acid 3-*O*-methyltransferase and caffeoyl coenzyme A 3-*O*-methyltransferase genes does not contribute to changes in metabolite accumulation in elicitor-treated cell-suspension cultures. Plant Physiol **112:** 717–726

Nicholson RL, Hammerschmidt R (1992) Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol **30:** 369–389

Niemann GJ, van der Kerk A, Niessen WMA, Verhuis K (1991) Free and cell wall-bound phenolics and other constituents from healthy and fungus-infected carnation (*Dianthus caryophyllus* L.) stems. Physiol Mol Plant Pathol **38:** 417–432

Pakusch A-E, Kneusel RE, Matern U (1989) *S*-Adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-O-methyltransferase from elicitor-treated parsley cell suspension cultures. Arch Biochem Biophys **271:** 488–494

Pakusch A-E, Matern U (1991) Kinetic characterization of caffeoyl-coenzyme A-specific 3-*O*-methyltransferase from elicited parsley cell suspensions. Plant Physiol **96:** 327–330

Pakusch A-E, Matern U, Schiltz E (1991) Elicitor-inducible caffeoyl-coenzyme A 3-*O*-methyltransferase from *Petroselinum crispum* cell suspensions. Plant Physiol **95:** 137–143

Raskin I (1992) Salicylate, a new plant hormone. Plant Physiol **99:** 799–803

Rasmussen JB, Hammerschmidt R, Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol **97:** 1342–1347

Reimers PJ, Leach JE (1991) Race-specific resistance to *Xanthomonas oryzae pv oryzae* conferred by bacterial blight resistance gene Xa-10 in rice (*Oryza sativa*) involves accumulation of a lignin-like substance in host tissues. Physiol Mol Plant Pathol **38:** 39–55

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot **50:** 199–204

Schmitt D, Pakusch A-E, Matern U (1991) Molecular cloning, induction and taxonomic distribution of caffeoyl-CoA 3-*O*-methyltransferase, an enzyme involved in disease resistance. J Biol Chem **266:** 17416–17423

Sewalt VJH, Ballance GM, Ni W, Dixon RA (1995) Developmental and elicitor-induced expression of alfalfa COMT and CCOMT, methylating enzymes in parallel pathways for monolignol synthesis (abstract No. 334). Plant Physiol **108:** S-74

Shulaev V, Leon J, Raskin I (1995) Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7:** 1691–1701

Siegrist J, Jeblick W, Kauss H (1994) Defense responses in infected and elicited cucumber (*Cucumis sativus* L.) hypocotyl segments exhibiting acquired resistance. Plant Physiol **105:** 1365–1374

Steenkamp J, Wiid I, Lourens A, van Helden P (1994) Improved method for DNA extraction from *Vitis vinifera* L. Am J Enol Vitic **45:** 102–106

Stöckigt J, Zenk MH (1975) Chemical synthesis and properties of hydroxycinnamoyl-coenzyme A derivatives. Z Naturforsch **30C:** 352–358

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol **185:** 60–89

Tiburzy R, Reisener HJ (1990) Resistance of wheat to *Puccinia graminis f. sp. tritici*: association of the hypersensitive reaction with the cellular accumulation of lignin-like material and callose. Physiol Mol Plant Pathol **36:** 109–120

Tietjen KG, Hunkler D, Matern U (1983) Differential response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi. 1. Identification of induced products as coumarin derivatives. Eur J Biochem **131:** 401–407

Uknes S, Mauch-Mani B, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4:** 645–656

Vernooij B, Friedrich L, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959–965

Walter MH (1992) Regulation of lignification in defense *In* T Boller, F Meins, eds, Genes Involved in Plant Defense, Vol 8. Springer Verlag, New York, pp 327–352

Ward ER, Uknes S, Williams SC, Dincher SS, Metraux JP, Ryals J (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Weber B (1992) Phenolische Komponenten des Weinrebenblattes: Identität und phythopathologische Bedeutung. PhD thesis. University of Zürich, Switzerland

Yalpani N, Silverman P, Wilson TMA, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3:** 809–818

Ye ZH, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell **6:** 1427–1439

Zhang X-H, Dickson EE, Chinnappa CC (1995) Nucleotide sequence of a cDNA clone encoding caffeoyl-coenzyme A 3-*O*-methyltransferase of *Stellaria longipes* (*Caryophyllaceae*). Plant Physiol **108:** 429–430

Zhou MY, Yue D, Gomez-Sanchez P, Gomez-Sanchez CE (1994) Improved downward capillary transfer for blotting of DNA and RNA. Biotechniques **16:** 58–59

Zou J, Taylor DC (1994) Isolation of an *Arabidopsis thaliana* cDNA homologous to parsley (*Petroselinum crispum*) *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-O-methyltransferase, an enzyme involved in disease resistance. Plant Physiol Biochem **32:** 423–427