Cloning and Characterization of a Coronatine-Regulated Tyrosine Aminotransferase from Arabidopsis[1]

Anna Lopukhina, Marcus Dettenberg, Elmar W. Weiler, and Heike Holländer-Czytko*

Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität, D–44780 Bochum, Germany

In plants, the phytotoxin coronatine, which is an analog of the octadecanoids 12-oxo-phytodienoic acid and/or jasmonic acid, gives rise to a number of physiological responses similar to those of octadecanoids. To further elucidate the physiological role of these compounds, the differential RNA display technique was used to isolate a number of novel octadecanoid-inducible genes expressed in coronatine-treated Arabidopsis. Among these, a cDNA clone was identified that was similar to known tyrosine aminotransferases (TATs). The function was verified with the expressed recombinant protein. In Arabidopsis, the protein is present as a multimer of 98 kD, with a monomer of an apparent molecular mass of 47 kD. *TAT* mRNA could be induced within 2 h by various octadecanoids and by wounding of the plants. Accumulation of the TAT protein and a 5- to 7-fold increase in its enzymatic activity was observed 7 to 9 h after application of octadecanoids, coronatine, or wounding. The potential role of TAT in the defense response to herbivores and pathogens is discussed.

Plants are exposed to various environmental influences during their life cycle, which may present different forms of stress, e.g. attacks by herbivores that lead to mechanical injuries but also mechanical stress by wind or touch. Invasion of pathogens has to be prevented and stress by high light intensities or UV light has to be met. Most plants react by activating the transcription of a set of appropriate genes whose gene products either have a defensive role per se or help to repair the effects resulting from stress. The particular reaction is dependent on the kind of stress and the developmental stage of the plant and can involve the strengthening of the cell wall (Hause et al., 1994; Kaiser et al., 1994) or the production of secondary plant products, such as phytoalexins or alkaloids (for overview, see Harborne, 1988).

Many of these induced plant responses are mediated by octadecanoids, of which jasmonic acid (JA) and its precursor 12-oxo-phytodienoic acid (OPDA) were identified as the major signaling compounds (Farmer and Ryan, 1990; Weiler et al., 1994; Parchmann et al., 1997). Upon wounding, the endogenous level of JA increases (Bowles, 1991; Berger et al., 1996; Laudert et al., 1996; Laudert and Weiler, 1998), a phenomenon that can be observed also in plant tissue cultures upon elicitation (Gundlach et al., 1992), whereas tendril coiling in *Bryonia dioica* is accompanied by an increase of OPDA (Blechert et al., 1999). Although the biosynthesis of jasmonates from linolenic acid is well understood (Vick and Zimmerman, 1984) and many of the enzymes of the pathway have been cloned and characterized (for compilation, see Weiler et al., 1999), the mechanisms of regulating octadecanoid production and the octadecanoid signaling pathway are less well understood.

The non-host-specific phytotoxin coronatine is produced by several cultivars of *Pseudomonas syringae* and induces leaf chlorosis, inhibits root growth, and induces the production of ethylene and of several proteins, e.g. proteinase inhibitors (Pautot et al., 1991; Bohlmann et al., 1998). Coronatine has been shown to be an essential factor in the early stages of infection of Arabidopsis and presumably acts by suppressing defense-related genes (Dong et al., 1991; Mittal and Davis, 1995). On the other hand, coronatine seems to mimic several effects of octadecanoids in plants and is thought to be an octadecanoid analog mediating and inducing defense reactions (Weiler et al., 1994). Mutants of Arabidopsis (*coi1)* that are insensitive to coronatine are also insensitive to methyl jasmonate (MeJA) and resistant to infection with *P. syringae.* The MeJA-insensitive mutants *jin1* and *jin4* show reduced responses to wounding (Feys et al., 1994; Benedetti et al., 1995; Benedetti et al., 1998). Thus, there seems to be a common site of action for coronatine and octadecanoids. An identification of genes induced or suppressed by coronatine therefore might yield new genes that are regulated by octadecanoids as well and further enhance our understanding of the physiological role of the octadecanoids in the plant.

In this paper, we report the isolation of a Tyr aminotransferase (TAT) from Arabidopsis obtained by differential RNA display from coronatine-treated plants, the first characterization of the heterologous overexpressed protein and the regulation of the enzyme's activity in the plant.

[1] This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, Germany (grant to E.W.W.).

* Corresponding author; e-mail Heike.Hollaender-Czytko@ruhr-uni-bochum.de; fax 49–234–3214187.

RESULTS

Identification of Coronatine-Regulated Genes

The differential RNA display originally identified 28 differential bands from coronatine-treated plants. Eight clones, designated *CORI2* to *CORI9* (CORI = coronatine induced), turned out to represent mRNAs, which show different levels of expression after treatment of plants, one of which, *CORI3*, is characterized in detail in this paper.

Isolation of a cDNA Clone Homologous to TAT

The original *CORI3* fragment had a size of 250 bp and thus was relatively short. Screening of the cDNA library from Arabidopsis yielded the clone pZL1*pCORI3* (1.1 kb) which lacked the 5′ end. The full-length clone pZL1*CORI3* was generated via PCR and had a size of 1,460 bp, encoding a putative protein of 422 amino acids (AtTAT1; Fig. 1; gene bank accession no. AF268090).

BLAST (Altschul et al., 1990) database searches (National Center for Biotechnology Information, Bethesda, MD) revealed that the amino acid sequence deduced from the nucleotide sequence of pZL1*CORI3* exhibited a 35% identity to TATs from humans and rats (Fig. 1; Rettenmeier et al., 1990).

In addition, the Arabidopsis genome sequencing project released a gene sequence (accession no. CAA 23026) of a TAT-like gene which encodes the *CORI3* sequence. The gene has seven exons and six introns and the nucleotide sequence of the gene and the cDNA is identical except for four nucleotides. These changes do not lead to different amino acids. The PSORT program (Nakai and Horton, 1999) predicted a putative transit peptide of the CORI3 protein (AtTAT1) with a cleavage site between amino acid 36 (C) and 37 (D; Fig. 1) and suggested a localization of the protein in the chloroplast.

DNA-Blot Analysis

DNA-blot analysis was conducted to examine the number of copies of the TAT gene in Arabidopsis. Total DNA was digested with restriction enzymes *Xho*I and *Bgl*I, which do not cut, with *Xba*I, which cuts once and with *Hin*dIII, which cuts twice within the cDNA of the *CORI3* cDNA. Hybridization of the blot with ^{32}P-labeled pZL1*CORI3* cDNA was done at different stringencies with the following results: As expected, restriction with *Xho*I and *Bgl*I resulted in only one band each and *Xba*I and *Hin*dIII gave two and three bands, respectively. However, as can be seen in Figure 2, there was one additional weak band after restriction with *Xho*I and two weak bands with *Xba*I, possibly from other *TAT*-like genes.

A comparison of *TAT*-like genes published from the Arabidopsis genomic sequencing project shows that another gene with 78% similarity of the amino acid sequence of AtTAT1 could be detected (accession no. CAA 23025, chromosome 4). Four other *TAT*-like genes (accession nos. CAA 16881, chromosome 4; AAD 23027, chromosome 2; AAD 21706, chromosome 2; and BAA 96891, chromosome 5) have been identified with 55% to 44% homologies to the AtTAT1 amino acid sequence. For the coding regions of CAA 23025 and AAD 23027, the PSORT program predicts putative transit peptides and plastidic localization.

Expression of the *CORI3* Gene by Treatment with Octadecanoids and Hormones

Accumulation of *CORI3*-specific mRNA was found not only for plants sprayed with coronatine but also with methyl-OPDA (OPDAMe) and MeJA and was dosage dependent. Whereas untreated control plants gave only a weak signal of a size of 1.7 kb, the treated plants were induced already at concentrations as low as 0.5 μM, with maximum induction at 5 μM for coronatine, 20 μM for OPDAMe, and 50 μM for MeJA (Fig. 3).

When other hormones were applied, only abscisic acid gave a slight induction, whereas gibberellic acid, cytokinin, indolylbutyric acid, and ethephon did not cause any reaction at the given concentrations (Fig. 4). When Arabidopsis leaves were wounded a strong accumulation of the *CORI3* mRNA was observed.

Heterologous Expression of TAT

CORI3 protein was expressed in *Escherichia coli* to conduct a functional characterization of the putative TAT. Cloning of the full-length *CORI3* cDNA from pZL1*CORI3* into the expression vector pET21a(+) with a C-terminal His-tag led to the construct pET21a(+)*CORI3*. Another plasmid [pET21a(+)*pCORI3*] was made, in which the potential transit peptide sequence in *CORI3* had been removed. Both constructs were transformed into BL21p-Lys *E. coli* cells. Coomassie staining of SDS-PAGE gels containing total protein extract from induced bacterial cells did not detect overproduction of the protein (Fig. 5A). However, immunoblot analysis using the αAtCORI3 antiserum clearly showed a band of the expected size of 47 kD for pET21a(+)*CORI3* and a band of approximately 43 kD for pET21a(+)*pCORI3* (Fig. 5B).

As the proteins possessed a His-tag, they were purified on nickel-nitriloacetic acid (Ni-NTA) agarose columns under native conditions. Figure 5, C and D, show that the 47-kD full-length CORI3 protein was 75% to 80% pure, whereas the 43-kD protein was enriched to a lesser extent after purification. Activity measurements for TAT of the purified proteins from both constructs showed that, when expression of the protein after induction with IPTG was done at 32°C for 3 to 4 h, activity was relatively low with 5 to 12 nanokatals (nkat) mg^{-1} protein (Table I). Longer incubations at this temperature caused a decrease of the

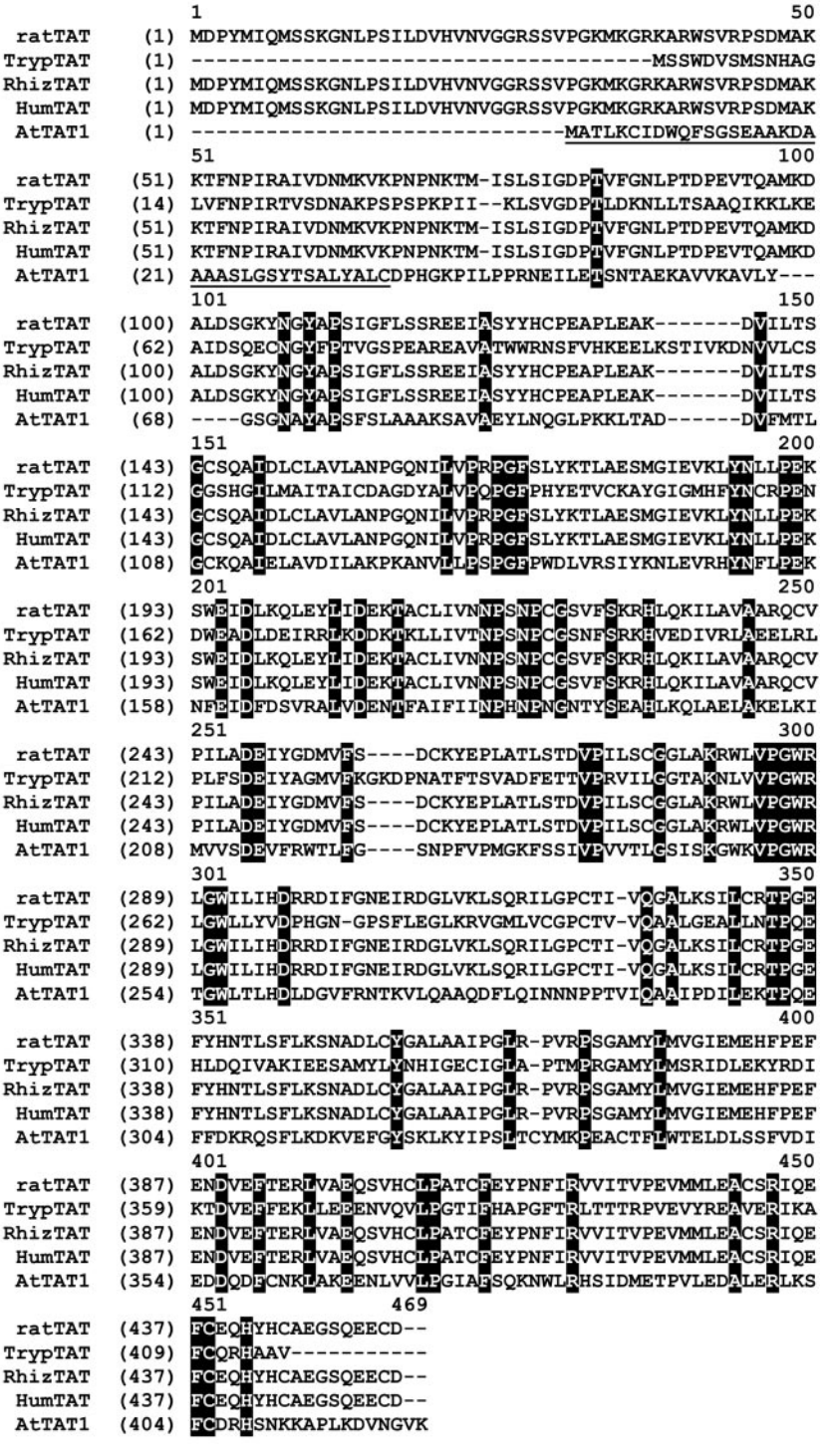

Figure 1. Alignment of the deduced amino acid sequence for the TAT from Arabidopsis with those from different species using CLUSTALW multiple sequence alignment (Higgins et al., 1996). Regions identical to all TATs are boxed in black. AtTAT1, TAT from Arabidopsis, this work; ratTAT, rat TAT (Hargrove et al., 1989); TrypTAT, TAT from *Trypanosoma cruzi* (Botempi et al., 1993); RhizTAT, TAT from *Rhizobium meliloti* (Rastogi and Watson, 1991); HumTAT, human TAT (Rettenmeier et al., 1990). The putative transit peptide of AtTAT1 is underlined.

activity, probably due to proteolysis of the protein and lysis of the cells. When temperatures during induction were lowered to 6°C and induction times prolonged, the amount of soluble, full-length protein increased considerably, with a specific activity up to 55 nkat mg^{-1} protein. The amount of protein without the putative transit peptide remained lower (Table I).

Native gel electrophoresis followed by immunodetection revealed that at 32°C only a small amount of the protein was present as a dimer. The majority was detected as higher M_r multimers or aggregates (data not shown).

Regulation of TAT in Arabidopsis Plants

Immunodetection of proteins after SDS-PAGE of plant extracts from Arabidopsis leaves with the αAt-

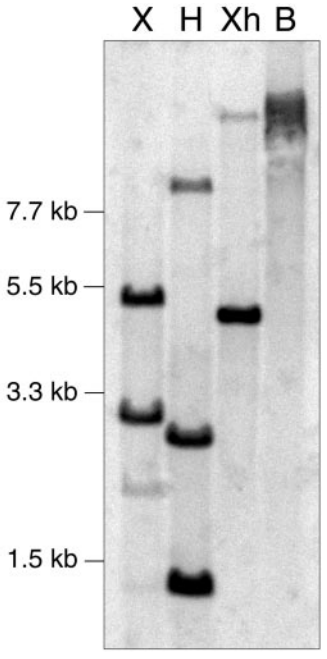

Figure 2. DNA blot of Arabidopsis genomic DNA. DNA (20 μg) was digested with *Xba*I (X), *Hin*dIII (H), *Xho*I (Xh), or *Bgl*I (B) and resolved on 0.6% (w/v) agarose gel. Hybridization was with the ^{32}P-labeled pZL1*CORI3* cDNA clone at 65°C. The positions of size markers (in kilobase pairs) are indicated at the left.

CORI3 antiserum revealed a positive signal of a protein with an apparent molecular mass of 47 kD (Fig. 6A), which would correspond to the size of the protein deduced from the full-length *CORI3* cDNA. The plant protein had the same size as the recombinant, full-length protein produced in *E. coli* (data not shown). This result suggests that in plants the putative transit peptide is not processed. Under native conditions a band of a molecular mass of approximately 98 kD was detected, suggesting that in plants TAT is active as a dimer (Fig. 6B). The monomer was not detectable (data not shown).

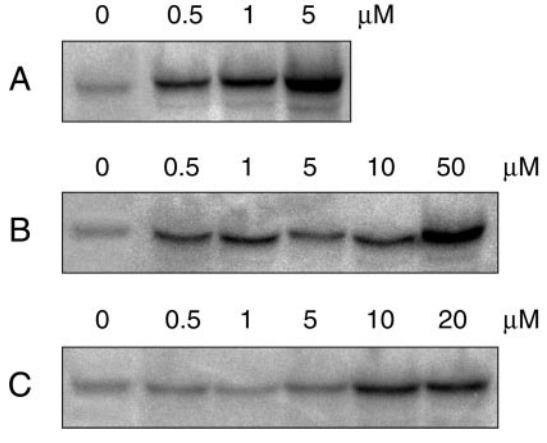

Figure 3. *CORI3* mRNA accumulation after treatment with octadecanoids. RNA-blot analysis of total RNA (20 μg per lane), isolated from Arabidopsis plants 2 h after treatment with different concentrations of coronatine (A), MeJA (B), and OPDAMe (C). Concentrations are given in micromoles on top of the blots. Hybridizations were carried out with the ^{32}P-labeled pZL1*CORI3* cDNA. Uniform loading was checked by comparing RNA intensities after ethidium bromide staining (not shown).

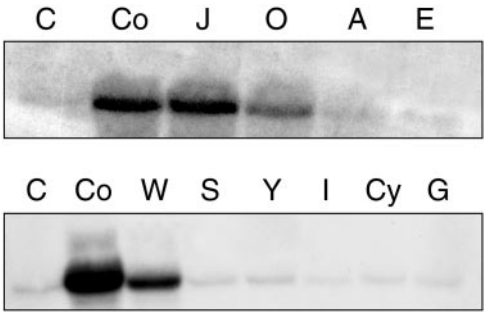

Figure 4. *CORI3* mRNA accumulation. Conditions are given in Figure 3. Results are from two different blots. C, Control plants; Co, 5 μM coronatine; J, 50 μM MeJA; O, 20 μM 12-OPDAMe; A, 100 μM ABA; E, 7 mM ethephon; W, wounded plants; S, 1 mM salicylic acid; Y, 25 μg mL^{-1} yeast elicitor; I, 50 μM IBA; Cy, 50 μM cytokinin; G, 50 μM gibberellic acid.

When expression of TAT was checked at the protein level, in non-treated leaves from Arabidopsis rosettes, a weak, basal level could be detected. Also, low basal levels of activity in the range of 0.2 to 0.3 nkat mg^{-1} protein could be measured (Fig. 7). As had been shown earlier (Fig. 3), levels of *TAT* mRNA accumulation increased after treatment of plants with coronatine. Activity levels for TAT were enhanced 3 h after application of coronatine and showed a maximum, with a 6- to 7-fold increase, at 9 h to decline slowly afterward (Fig. 7A). Immunodetection of the protein also showed an increase. In this case, the level of the protein was maximal at 12 h and remained constant for about 48 h (Fig. 7C). Activity kinetics and protein level measurements were quite similar when the octadecanoid MeJA was applied, whereas for OPDAMe maximal activity was observed around 7.5 h after application, followed by a dramatic decrease of activity (Fig. 7B). Also, protein accumulation was lower.

Wounding, which leads to an increase of endogenous OPDA and jasmonate levels, also led to induction of TAT activity with a maximum of a 5-fold increase and a time course similar to that shown after treatment with OPDAMe. Again, maximal activity was reached after 7.5 h and then dropped rapidly down to basal levels. TAT protein levels increased, although not as strong as after coronatine treatment, reaching maximal expression between 7 and 12 h and then remained either constant or in some experiments decreased slightly (data not shown).

DISCUSSION

Plants respond to mechanical injuries and pest attack by inducing genes activated by different signal transduction pathways, which can be jasmonate de-

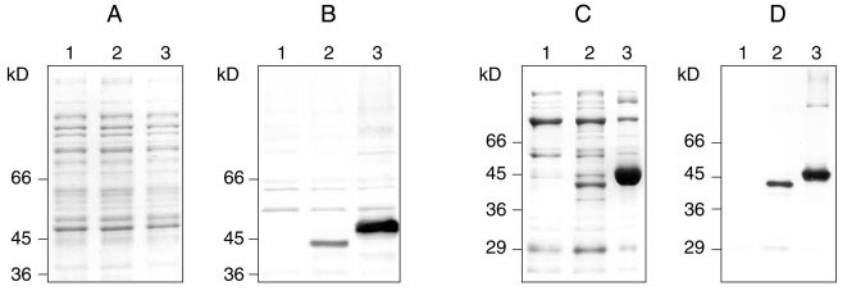

Figure 5. Expression of Arabidopsis TAT in *E. coli* cells. Soluble protein extracts were obtained from *E. coli* cells transformed with the plasmids pET21a(+) as control (lane 1), pET21a(+)*pCORI3* (without potential transit peptide; lane 2), and pET21a(+)*CORI3* (full-length TAT; lane 3). Crude protein extracts were from cells harvested 40 h after induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and grown at 6°C. They were subjected to SDS-PAGE (10% [w/v]; 10 μg protein/lane) and stained with Coomassie Blue (A) or analyzed by immunoblot with αAtCORI3 antiserum (B). Expressed, His-tagged proteins from these extracts were purified by Ni-NTA agarose chromatography, eluted with imidazole, separated by SDS-PAGE (10% [w/v]), and were either Coomassie stained (C, 5 μg of protein applied per lane) or analyzed by immunoblotting using αAtCORI3 antiserum (D, 5 μg of protein, lanes 1 and 2; 2 μg of protein, lane 3). Molecular mass markers are given in kilodaltons.

pendent or independent. Although the role of octadecanoids in herbivore attack seems indisputable, in plant-pathogen interactions their role is less well understood (McConn et al., 1997; Dong, 1998; Vijayan et al., 1998). With the aid of the non-host-specific phytotoxin coronatine, which mimics octadecanoids in several plant processes, it was possible to isolate and characterize several coronatine-inducible genes. The one reported in this study is *CORI3*, which encodes a TAT. This is the first full-length cDNA from plants for TAT reported. TAT activity of the clone could be verified when the cDNA was heterologously expressed and the protein purified over Ni-NTA agarose columns. The amount of soluble full-length protein increased considerably when induction with IPTG and growth prolonged for longer time at 6°C (Table I). In the plant, native TAT obviously forms a dimer of 98 kD (Fig. 6B) and folding of the protein and formation of the complex might be better at lower temperatures. Expression of proteins forming homo- or heterodimers often requires lower growth temperatures; e.g. geranyl diphosphate synthase, a heterodimer, is expressed in bacteria at 15°C (Burke et al., 1999).

CORI3 mRNA levels were increased by different octadecanoids of the jasmonate cascade (OPDAMe and MeJA) as well as by wounding (Figs. 3 and 4). TAT activity in Arabidopsis was also induced with a maximum at 7.5 or 9 h after treatment. TAT induction kinetics and the strength of induction are very similar for coronatine and MeJA with a maximum at 9 h and a relatively slow decrease. Protein levels of TAT increase as well, but then remain constant for at least 24 to 48 h (Fig. 7A). Wounding and treatment with OPDAMe result in very similar kinetics of activity with a maximum at 7.5 h and following rapid decrease to control levels. At the protein level, the accumulation is less strong when compared with coronatine and MeJA, with slight decreases in the amount over time (Fig. 7B). So in this case, coronatine seems to act more like MeJA, whereas wounding and OPDAMe display similar time courses. This is different from induction patterns of allene oxide synthase, an enzyme of the jasmonate biosynthesis (Laudert and Weiler, 1998). Also, allene oxide synthase mRNA accumulates in response to salicylate and ethephon, which is not the case for TAT (Fig. 4). Thus, although both enzymes are induced by treatment with octadecanoids and coronatine, there seem to be differences in induction patterns and separate defense responses because one enzyme belongs to the biosynthetic pathway of jasmonates and the other one is involved in a pathway leading from Tyr to potential defense compounds of the plant.

Table I. *Specific activities of recombinant Arabidopsis TAT*

Activities were measured in fractions of protein extracts from *E. coli* harboring the pET21a(+) plasmids and after induction with IPTG for different times and temperatures after Ni-NTA agarose chromatography and elution with imidazole.

Plasmids	Experiment I, Incubation Temperature 32°C, Incubation Time 3 h	Experiment II, Incubation Temperature 32°C, Incubation Time 3 h	Experiment III, Incubation Temperature 6°C, Incubation Time 18 h	Experiment IV, Incubation Temperature 6°C, Incubation Time 40 h
		nkat mg protein^{-1}		
pET21a(+), control	0.5	0.6	n.d.[a]	0.3
pET21a(+)*pCOR*/3	7.2	5.2	4.2	9.7
pET21a(+)*COR*/3	7.9	12.7	21.5	55.2

[a] n.d., Not detectable.

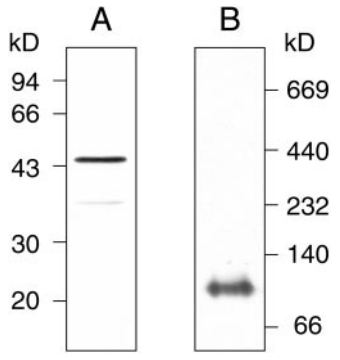

Figure 6. Molecular mass determination of TAT in Arabidopsis. On immunoblots of plant protein extracts (20 μg) after SDS-PAGE, the antiserum αAtCORI3 detected a single band of 47 kD (A). Blue native gel electrophoresis showed only one immunoreactive band of 98 kD (B). The enhanced chemiluminescence (ECL) kit from Amersham was used. Molecular mass markers are given in kilodaltons.

It is interesting that Titarenko et al. (1997), when using the differential display method to obtain jasmonate- and wound-inducible genes from Arabidopsis, were also able to isolate a cDNA fragment of 489 bp that showed homology to TAT from humans and rat. However, no sequence was published.

In plants, the product formed from Tyr by the reaction of TAT is 4-hydroxyphenyl-pyruvate (Fig. 8). Few reports on this plant enzyme have been published. De-Eknamkul and Ellis (1987) purified TAT from *Anchusa officinalis*. They could distinguish three different forms as homotetramers with native molecular weights of 180 to 220 kD and relatively high pH optima of 8.8 to 9.6. In *A. officinalis*, rosmarinic acid is formed via TAT, one of several phenolic compounds protecting the plant against peroxidative damage by scavenging free radicals (Liu et al., 1992). A similar function might well be mediated by TAT in Arabidopsis. It is well known that 4-hydroxyphenyl-pyruvate is converted to homogentisate by a dioxygenase in Arabidopsis and other plants (Löffelhardt and Kindl, 1979; Fiedler et al., 1982). The dioxygenase has been cloned from several plants (Garcia et al., 1997, 1999; Kleber-Janke and Krupinska, 1997; Norris et al., 1998). Homogentisate is the precursor of both tocopherols and plastoquinones in plants (Fig. 8). The synthesis of the prenylquinones has been shown to be localized at the inner envelope membrane of the chloroplast and both quinones can be found at the outer and inner membrane (Soll et al., 1985). α-Tocopherol, the main component of vitamin E and the reduced form of plastoquinone, are known as scavengers for reactive oxygen species (ROS; Hundal et al., 1995; Shintani and DellaPenna, 1998). Upon pathogen attack and stress, chlorophyll breakdown can occur and ROS can be formed that might have to be neutralized by prenylquinones. Higher demand for prenylquinones might lead to an increased activity of TAT, the first enzyme of the pathway.

In an alternate manner, a more direct role of TAT upon pathogen attack could be that phenolics formed from 4-hydroxyphenylpyruvate might serve as cross-linkers in the cell wall, thereby strenghtening this physical barrier (Hause et al., 1994; Schmidt et al., 1998). Future studies on localization and regulation of TAT under different stress conditions and the analysis of metabolites in transgenic plants exhibiting altered levels of TAT will help to elucidate the role of this enzyme in plant defense and its control by the octadecanoid pathway.

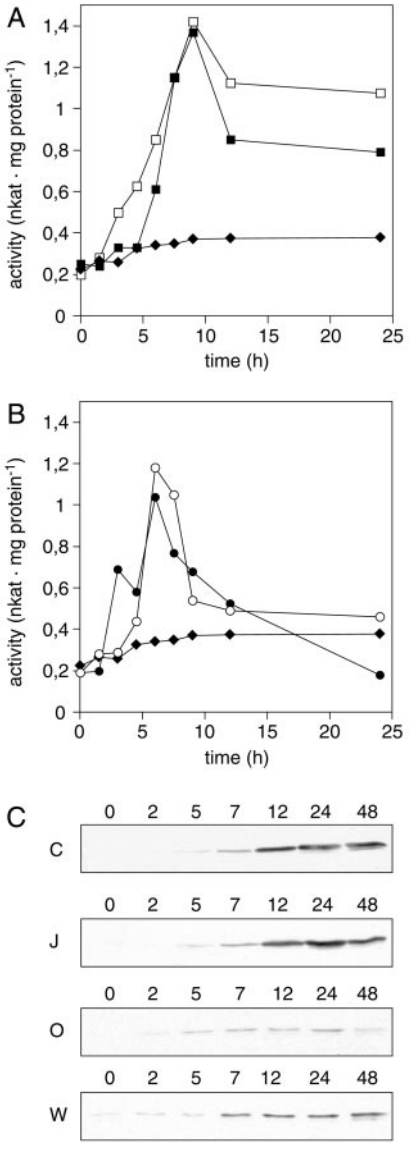

Figure 7. Regulation of TAT in Arabidopsis. TAT activity was measured at different time points after treatment of the plants with: A, 50 μM coronatine (white squares) or 100 μM MeJA (black squares); and B, 100 μM OPDAMe (white circles) or after wounding (black circles). Acitivity in untreated control plants is given for comparison in each graph (diamonds). C, Immunodetection of TAT in total protein extracts (20 μg/lane) of plants treated as given above after separation on SDS-PAGE. C, Coronatine; J, MeJA; O, OPDAMe; W, wounded. The time after treatment is given in hours. Detection was with the ECL system. Equal loading was checked by staining the blots with Ponceau (not shown).

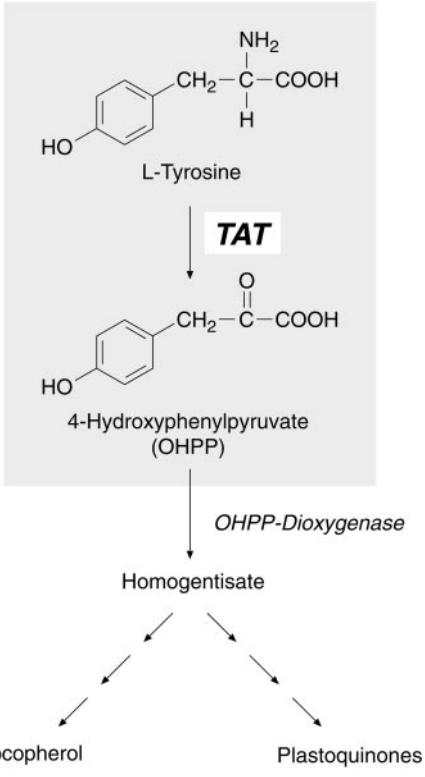

Figure 8. The biosynthetic pathway of tocopherols and plastoquinones.

MATERIALS AND METHODS

Plant Material and Growth Condition

Seeds of Arabidopsis, ecotype C24, were surface sterilized and grown on one-half-strength Murashige and Skoog medium with 0.8% (w/v) agar (Murashige and Skoog, 1962) in a growth chamber at 24°C and a 9-h-light/15-h-dark cycle with a light intensity of 80 μE m^{-2} s^{-1}. Nonsterile Arabidopsis plants were grown in the greenhouse in trays containing standard soil. Four- to 6-week-old plants were used in both cases for further experiments.

For induction experiments, plants from the greenhouse were sprayed with different hormones and octadecanoids at the given concentrations in 0.1% (w/v) Tween 20. Control plants were sprayed with 0.1% (w/v) Tween 20 only. Immediately after harvesting, the plant material was frozen in liquid nitrogen and stored at −75°C. For wounding experiments, 50% to 80% of the leaf area was crushed with a hemostat. Plant material was harvested, frozen in liquid nitrogen, and stored at −75°C. For enzymatic assays, fresh plant material was used. All experiments were done at least three times in independent experiments.

RNA Isolation and Differential RNA Display

For differential display, total RNA from control Arabidopsis plants and plants sprayed with 5 μM coronatine and incubated for 2 h was extracted as described by Barkan (1989). Poly(A^+) RNA was isolated using the Oligotex mRNA Kit (Qiagen, Hilden, Germany). Differential RNA display was performed as described by Liang and Pardee (1992). Purified poly(A^+) RNA (0.2 μg) was reverse transcribed in a 20-μL reaction containing 1 μM of the D collection of primers and 20 μM deoxyribonucleotide triphosphates (GeneExScreen Primer Kit, Biometra, Göttingen, Germany). The reaction was carried out as follows: The mixture was heated for 5 min at 65°C and then for 10 min at 37°C, after which 10 units of reverse transcriptase (Promega, Mannheim, Germany) were added. The reaction proceeded for another 60 min and the mixture was then heated at 95°C for 5 min and stored at −20°C until used for PCR.

Two-microliter aliquots were used as templates for PCR with the D primers in combination with an arbitrary primer U (GeneExScreen Primer Kit) in the presence of ^{32}S-labeled dATP (Amersham-Pharmacia, Freiburg, Germany). Reaction conditions for PCR were as follows: 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s for 40 cycles, followed by extension at 72°C for 5 min. Aliquots (4.5 μL) of the PCR reaction were run on a 6% (w/v) non-denaturing polyacrylamide gel to separate the amplified cDNAs. Duplicates of each reaction were subjected to electrophoresis and only bands that were amplified in a consistent manner were used for further analysis. Bands containing the differentially expressed cDNAs were excised from the gel, eluted in 100 μL of distilled, sterile water for 10 min, and then boiled for 15 min. The supernatant was ethanol precipitated. Each pellet was dissolved in 10 μL of distilled water and an aliquot (4 μL) was used for reamplification with the appropriate pair of primers. The primers that subsequently led to the isolation of the cDNA were D8 and U8 from the GeneExScreen Primer Kit. The PCR products were purified on 6% to 8% (w/v) non-denaturing polyacrylamide gels and subcloned into the pBluescript SK+ vector (Stratagene, Amsterdam). The partial cDNA fragment was further used for library screening and DNA- and RNA-blot analysis.

RNA-Blot Analysis

Total RNA (20 μg) was mixed with an equal volume of loading buffer {50% [v/v] formamide, 10% [v/v] formaldehyde, 0.1 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 8.0, and 50 μg mL^{-1} ethidium bromide}, separated by gel electrophoresis on a 1.3% (w/v) agarose-formaldehyde gel (10% [v/v]) and then transferred to Hybond-N membranes (Amersham-Pharmacia) using 20× SSPE (1× SSPE: 0.22 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4) (Sambrook et al., 1989). Hybridization was performed at 65°C with ^{32}P-random-labeled cDNA probes following standard procedures (Sambrook et al., 1989). For all blots, equal loading was confirmed by ethidium bromide staining of ribosomal RNAs (25S and 18S).

DNA-Blot Analysis

Genomic DNA was isolated from Arabidopsis leaves following the procedure of Pich and Schubert (1993). DNA (20–30 μg) was digested with the appropriate restriction enzymes, separated by gel electrophoresis on a 0.6% (w/v) agarose gel, and blotted onto Hybond-N membranes. Prehybridization and hybridization with ^{32}P-random-labeled

cDNA probes were performed at 65°C according to standard procedures (Sambrook et al., 1989).

Isolation and Cloning of the Full-Length cDNA Clone

The cDNA library (vector, Lambda ZIP LOX; BRL, Carlsbad, CA) from Arabidopsis, kindly provided by Dr. Stefan Trentmann (Ruhr-Universität Bochum, Germany) was screened by colony hybridization using the ^{32}P-labeled *CORI3* cDNA fragment from the differential RNA display as a probe. Approximately 150,000 plaques from the library were screened. Two positive clones were isolated and subcloned in vivo. The sequences of the two clones were determined and they turned out to be identical. The clone was named pZL1*pCORI3*.

To obtain the missing 5′ end of the clone, a Hybri-ZAP-cDNA library (Stratagene) was used and PCR was performed essentially as described in Chiang et al. (1995). The PCR was performed in a 50-μL reaction volume, containing 2.5 μL of the amplified cDNA Hybri-ZAP library with 50 pmol of each primer (primer A, 5′-end of the LambdaZAP-clone; 5′-AGGGATGTTTAATACCACTAC-3′, primer CORI3 R, specific for the partial *CORI3*cDNA; and 5′- TCAGAAACCACCACCATAATCTTGA-3′). The conditions for PCR were as follows: 94°C for 5 min, then 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min for 35 cycles, followed by incubation at 72°C for 10 min. The obtained PCR products were subcloned into the pBluescript vector and religated with pZL1*pCORI3*. The resulting full-length clone was named pZL1*CORI 3*.

Preparation of Polyclonal Antiserum

The immunization of a rabbit was done according to Weiler (1986). For overexpression in *Escherichia coli*, the cDNA from the partial clone pZL1*pCORI3* was subcloned into the pQE31 vector (Qiagen). Overexpression and purification under denaturing conditions using Ni-NTA agarose was done as described in the QIAexpressionist handbook (Qiagen). For pre-immunization, the rabbit was injected once per week subcutaneously with 150 μg of purified protein in two volumes of Freund's incomplete adjuvant for 4 weeks, followed by intramuscular injections of 150 μg of protein in Freund's incomplete adjuvant every 3 weeks. Serum was obtained 10 d after each boost and the antiserum αAtCORI3, which detected TAT in heterologous bacterial systems and in Arabidopsis, was stored at −20°C.

Cloning of the *CORI3* cDNA into the pET 21 Vector

For overexpression of the CORI3 protein in the pET 21a(+) vector (Novagen, Madison, WI), two constructs with and without a potential transit peptide (see "Results") were made. To generate the *Nde*I and *Xho*I restriction sites necessary for subcloning into the vector, the full-length pZl1*CORI3* clone was amplified using the primers pET21A and pET21R for the full-length construct (pET21A, 5′-GGGATTTCCATATGGCAACCCTTAAGTGCATTGAT-3′; and pET21R, 5′-CCGCTCGAGCTTAACACCATTGACGTCTTT-3′) and primers pET21B and pET21R for the construct without the potential transit peptide (pET21B, 5′-GGGATTTCCATATGGATCCTCATGGCAAACCCATT-3′). Both PCR products were restricted with *Nde*I and *Xho*I, and cloned using corresponding restriction sites in the multicloning site of the vector pET21a (+). The resulting clones were named pET21a(+)*CORI3* (primers pET21A and pET21R, full length), and pET21a(+)*pCORI3* (primers pET21B and pET21R, lacking the potential transit peptide). Both constructs contained a His_6-tag at the C terminus.

TAT Activity

For expression of the CORI3 protein (AtTAT1), clones pET21a(+)*CORI3* and pET21a(+)*pCORI3* were transformed into BL21(DE3) pLysS bacteria (Promega). Induction was with 1 mM IPTG. Cells then were grown for different times and different temperatures until harvest. Harvest and lysis followed the instructions of the manufacturer (pET manual, Novagen) and purification of the protein was done over a Ni-NTA-agarose column (Qiagen) according to the manufacturer's instructions under nondenaturing conditions. Activity of TAT was measured after Granner et al. (1970) at 35°C immediately after purification and protein determination as the protein proved to be unstable. Protein determination was done according to Bradford (1976), using bovine serum albumin as a standard.

For measurements of TAT activity, plants (5 g fresh weight) were homogenized in 2 volumes of ice-cold buffer, containing 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 8.0 mM α-ketoglutarate, 0.2 mM pyridoxal-5-phosphate, and 1 mM dithiothreitol. After filtration through cheesecloth and centrifugation at 6,000*g*, the supernatant was made 0.1% (v/v) with tergitol and a 15-min incubation period on ice followed. The supernatant of the following 30,000*g* centrifugation was used as crude enzyme extract. Immediately after protein determination, measurement of enzyme activity followed at 35°C, using 50 μg of total protein in an assay mixture containing 0.125 M potassium phosphate buffer (pH 7.5), 0.75 mM EDTA, 5.5 mM Tyr, 10 mM α-ketoglutaric acid, and 0.1 mM pyridoxal 5-phosphate. Addition of KOH converted 4-hydroxyphenylpyruvate, the product of TAT activity, to 4-hydroxybenzaldehyde, which was determined spectrophotometrically at 331 nm.

PAGE and Protein Analysis

Determination of protein concentrations was done according to Bradford (1976) with bovine serum albumin as a protein standard. For SDS-PAGE, proteins were separated on a 10% (w/v) separating gel (Laemmli, 1970).

Blue native gel electrophoresis was done according to Schägger and von Jagow (1991).

Transfer of proteins separated by SDS-PAGE onto nitrocellulose was done according to Towbin et al. (1979) and transfer of proteins after blue native PAGE according to Schägger and von Jagow (1991).

Immunodetection was performed after blotting using either the ECL western blotting analysis system (Amersham-

Pharmacia) or the alkaline phosphatase detection system (Promega). For ECL, the αAtCORI3 antiserum was used at a 1:15,000 dilution and the anti-rabbit secondary antibody was used at a dilution of 1: 5,000 (v/v). With the alkaline phosphatase system, the αAtCORI3 antiserum was used at a dilution of 1:20,000 (v/v) for proteins expressed in bacteria and 1:7,500 (v/v) for protein extract from plants. The second antibody, goat-anti-rabbit IgG (Promega) was used at a dilution of 1:7,500 (v/v). Detection was with the substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate.

ACKNOWLEDGMENTS

We thank Dr. Stefan Trentmann and Kathrin Hackstedt for help with initial differential RNA display experiments, Günter Nebe for help with computer alignments, and Klaus Hagemann for assistance and advice in preparing the figures.

Received February 20, 2001; returned for revision March 28, 2001; accepted May 7, 2001.

LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers E, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

Barkan A (1989) Tissue dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. Plant Cell **1:** 437–445

Benedetti CE, Costa CL, Turcinelli SR, Arruda P (1998) Differential expression of a novel gene in response to coronatine, methyl jasmonate, and wounding in the *coi1* mutant of *Arabidopsis*. Plant Physiol **116:** 1037–1042

Benedetti CE, Xie D, Turner JG (1995) *COI1*-dependent expression of an *Arabidopsis* vegetative storage protein in flowers and siliques and in response to coronatine and methyl jasmonate. Plant Physiol **109:** 567–572

Berger S, Bell E, Mullet JE (1996) Two methyl jasmonate-insensitive mutants show altered expression of *AtVsp* in response to methyl jasmonate and wounding. Plant Physiol **111:** 525–531

Blechert S, Bockelmann C, Füsslein M, von Schrader T, Stelmach B, Niesel U, Weiler EW (1999) Structure-activity analyses reveal the existence of two separate groups of octadecanoids in elicitation of the tendril-coiling response of *Bryonia dioica* Jacq. Planta **207:** 470–479

Bohlmann H, Vignutelli A, Hilpert B, Miersch O, Wasternack C, Apel K (1998) Wounding and chemicals induce the expression of the *Arabidopsis thaliana* gene *Thi2.1*, encoding a fungal defense thionin, via the octadecanoid pathway. FEBS Lett **437:** 281–286

Botempi EJ, Bua J, Aslund L, Porcel B, Segura EL, Henriksson J, Orn A, Pettersson U, Ruiz AM (1993) Isolation and characterization of a gene from *Trypanosoma cruzi* encoding a 46-kilodalton protein with homology to human and rat tyrosine aminotransferase. Mol Biochem Parasitol **59:** 253–262

Bowles D (1991) The wound response of plants. Curr Biol **1:** 165–167

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Burke CC, Wildung MR, Croteau R (1999) Geranyl diphosphate synthase: cloning, expression and characterization of this prenyltransferase as a heterodimer. Proc Natl Acad Sci USA **96:** 13062–13067

Chiang PW, Trbusek M, Osemlak-Hanzlik M, Kurnit DM (1995) Rapid PCR-based method to directionally pull out longer cDNA fragments from cDNA libraries. Biotechniques **18:** 36–40

De-Eknamkul W, Ellis BE (1987) Purification and characterization of tyrosine aminotransferase activities from *Anchusa officinalis* cell cultures. Arch Biochem Biophys **257:** 430–438

Dong X (1998) SA, JA, ethylene and disease resistance in plants. Curr Opin Plant Biol **1:** 316–323

Dong X, Mindrios M, Davis KR, Ausubel FM (1991) Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell **3:** 61–72

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759

Fiedler E, Soll J, Schultz G (1982) The formation of homogentisate in the biosynthesis of tocopherol and plastoquinone in spinach chloroplasts. Planta **155:** 511–515

Garcia I, Rodgers M, Lenne C, Roland A, Saillant A, Matringe M (1997) Subcellular localization and purification of a p-hydroxyphenylpyruvate dioxygenase from cultured carrot cells and characterization of the corresponding cDNA. Biochem J **325:** 761–769

Garcia I, Rodgers M, Pepin R, Hsieh T-F, Matringe M (1999) Characterization and subcellular compartmentation of recombinant 4-hydroxyphenylpyruvate dioxygenase from *Arabidopsis* in transgenic tobacco. Plant Physiol **119:** 1507–1516

Granner DK, Thompson EB, Tomkins GM (1970) Dexamethasone phosphate-induced synthesis of tyrosine aminotransferase in hepatoma tissue cell culture cells: studies of the early phases of induction and of the steroid requirement for maintenance of the induced rate of synthesis. J Biol Chem **245:** 1472–1478

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA **89:** 2389–2392

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA **87:** 7713–7716

Harborne J (1988) Introduction to Ecological Biochemistry, Ed 3. Academic Press, London

Hargrove JL, Scoble HA, Matthews WR, Baumstark BR, Biemann K (1989) The structure of tyrosine aminotransferase. J Biol Chem **264:** 45–53

Hause B, zur Nieden U, Lehmann J, Wasternack C (1994) Intracellular localization of jasmonate-induced proteins in barley leaves (*Hordeum vulgare* cv. Salome). Bot Acta **107:** 333–341

Higgins DG, Thompson JD, Gibson TJ (1996) Using CLUSTAL for multiple sequence alignments. Methods Enzymol **266:** 383–402

Hundal T, Forsmark-Andreé P, Ernster L, Andersson B (1995) Antioxidant activity of reduced plastoquinone in chloroplast thylakoid membranes. Arch Biochem Biophys **324:** 117–122

Kaiser IK, Engelberth J, Groth B, Weiler EW (1994) Touch- and methly-jasmonate-induced lignification in tendrils of *Bryonia dioica* Jacq. Bot Acta **107:** 24–29

Kleber-Janke T, Krupinska K (1997) Isolation of cDNA clones for genes showing enhanced expression in barley leaves during dark-induced senescence as well as during senescence under field conditions. Planta **203:** 332–340

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Laudert D, Pfannschmidt U, Lottspeich F, Holländer-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol **31:** 323–335

Laudert D, Weiler EW (1998) Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signaling. Plant J **15:** 675–684

Liang P, Pardee A (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967–971

Liu GT, Zhang TM, Wang BE, Wang YW (1992) Protective action of seven natural phenolic compounds against peroxidative damage to biomembranes. Biochem Pharmacol **43:** 147–152

Löffelhardt W, Kindl H (1979) Conversion of 4-hydroxyphenylpyruvic acid into homogentisinic acid at the thylakoid membrane of *Lemna gibba*. FEBS Lett **104:** 332–334

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA **94:** 5473–5477

Mittal S, Davis KR (1995) Role of the phytotoxin coronatine in the infection of *Arabidopsis thaliana* by *Pseudomonas syringae* pv. tomato. Mol Plant-Microbe Interact **8:** 165–171

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci **24:** 34–36

Norris SR, Shen X, DellaPenna D (1998) Complementation of the *Arabidopsis pds1* mutation with the gene encoding p-hydroxyphenylpyruvate dioxygenase. Plant Physiol **117:** 1317–1323

Parchmann S, Gundlach H, Mueller MJ (1997) Induction of 12-oxo-phytodienoic acid in wounded plants and elicited cell cultures. Plant Physiol **115:** 1057–1064

Pautot V, Holzer FM, Walling LL (1991) Differential expression of tomato proteinase inhibitor I and II genes during bacterial pathogen invasion and wounding. Mol Plant-Microbe Interact **4:** 284–292

Pich U, Schubert I (1993) Midiprep method for isolation of DNA from plants with a high content of polyphenolics. Nucleic Acids Res **21:** 3328

Rastogi VK, Watson RJ (1991) Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. J Bacteriol **173:** 2879–2887

Rettenmeier R, Natt E, Zentgraf H, Scherer G (1990) Isolation and characterization of the human tyrosine aminotransferase gene. Nucleic Acids Res **8:** 3853–3861

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

Schägger H, von Jagow G (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal Biochem **199:** 223–231

Schmidt A, Scheel D, Strack D (1998) Elicitor-stimulated biosynthesis of hydroxycinnamoyltyramines in cell suspension cultures of *Solanum tuberosum*. Planta **205:** 51–55

Shintani D, DellaPenna D (1998) Elevating the vitamin E content of plants through metabolic engineering. Science **282:** 2098–2100

Soll J, Schultz G, Joyard J, Douce R, Block M (1985) Localization and synthesis of prenylquinones in isolated outer and inner envelope membranes from spinach chloroplasts. Arch Biochem Biophys **238:** 290–299

Titarenko E, Rojo E, Leon J, Sanchez-Serrano J (1997) Jasmonic acid-dependent and -independent signaling pathways control wound-induced gene activation in *Arabidopsis thaliana*. Plant Physiol **115:** 817–826

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76:** 4350–4354

Vick BA, Zimmerman DC (1984) Biosynthesis of jasmonic acid by several plant species. Plant Physiol **75:** 92–97

Vijayan P, Shockey J, Levesque CA, Cook RJ, Browse J (1998) A role for jasmonate in pathogen defense of *Arabidopsis*. Proc Natl Acad Sci USA **95:** 7209–7214

Weiler EW (1986) Plant hormone immunoassays based on monoclonal and polyclonal antibodies. *In* HF Linskens, EF Jackson, eds, Immunology in Plant Sciences. Springer Verlag, Berlin, pp 1–17

Weiler EW, Kutchan TM, Gorba T, Brodschelm W, Niesel U, Bublitz F (1994) The *Pseudomanas* phytotoxin coronatine mimics octadecanoid signaling molecules of higher plants. FEBS Lett **345:** 9–13

Weiler EW, Laudert D, Stelmach B, Hennig H, Biesgen C, Kubigsteltig I (1999) Octadecanoid and hexadecanoid signalling in plant defense. *In* DJ Chadwick, JA Goode, eds, Insect-Plant Interactions and Induced Plant Defense. Wiley, Chichester (Novartis Foundation Symposium 223), West Sussex, UK, pp 191–204