Creation of a Metabolic Sink for Tryptophan Alters the Phenylpropanoid Pathway and the Susceptibility of Potato to *Phytophthora infestans*

Kening Yao,[a] Vincenzo De Luca,[b] and Normand Brisson,[a,1]

[a] Department of Biochemistry, Université de Montréal, Montréal, Québec, H3C 3J7 Canada
[b] Department of Biological Sciences, Université de Montréal, Montréal, Québec, H3C 3J7 Canada

The creation of artificial metabolic sinks in plants by genetic engineering of key branch points may have serious consequences for the metabolic pathways being modified. The introduction into potato of a gene encoding tryptophan decarboxylase (TDC) isolated from *Catharanthus roseus* drastically altered the balance of key substrate and product pools involved in the shikimate and phenylpropanoid pathways. Transgenic potato tubers expressing the *TDC* gene accumulated tryptamine, the immediate decarboxylation product of the TDC reaction. The redirection of tryptophan into tryptamine also resulted in a dramatic decrease in the levels of tryptophan, phenylalanine, and phenylalanine-derived phenolic compounds in transgenic tubers compared with nontransformed controls. In particular, wound-induced accumulation of chlorogenic acid, the major soluble phenolic ester in potato tubers, was found to be two- to threefold lower in transgenic tubers. Thus, the synthesis of polyphenolic compounds, such as lignin, was reduced due to the limited availability of phenolic monomers. Treatment of tuber discs with arachidonic acid, an elicitor of the defense response, led to a dramatic accumulation of soluble and cell wall–bound phenolics in tubers of untransformed potato plants but not in transgenic tubers. The transgenic tubers were also more susceptible to infection after inoculation with zoospores of *Phytophthora infestans*, which could be attributed to the modified cell wall of these plants. This study provides strong evidence that the synthesis and accumulation of phenolic compounds, including lignin, could be regulated by altering substrate availability through the introduction of a single gene outside the pathway involved in substrate supply. This study also indicates that phenolics, such as chlorogenic acid, play a critical role in defense responses of plants to fungal attack.

INTRODUCTION

Phenylpropanoid metabolism in plants leads to the formation of numerous phenolic compounds that are believed to have important functions in plant defense responses to wounding and pathogen infection (Hahlbrock and Scheel, 1989; Nicholson, 1992). The phenylpropanoid pathway is initiated by the enzymatic conversion of phenylalanine to cinnamic acid, which is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL; Koukol and Conn, 1961). The hydroxylation of cinnamic acid to *p*-coumaric acid and the formation of coenzyme A thioesters of hydroxycinnamoyl intermediates provide precursors for branch pathways leading to the production of a number of phenolics, including lignin, soluble esters, coumarins, and flavonoids (Mann, 1987; Hahlbrock and Scheel, 1989).

Most of the enzymes of the central phenylpropanoid pathway are now well characterized. It is believed that the level of PAL activity is the key factor in the regulation of this pathway (Elkind et al., 1990; van der Krol et al., 1990; Nicholson,

1992). For example, the transient increase in PAL activity observed in potato tubers when treated with an elicitor has been correlated with the increase in the level of phenolics (Maina et al., 1984). Similar increases in phenylpropanoid metabolism have been observed in cultured pine and soybean cells after treatment with a fungal elicitor and have also been correlated with increased PAL activity (Farmer, 1985; Campbell and Ellis, 1992). By contrast, inhibition of PAL activity by the mechanism of cosuppression (Elkind et al., 1990; Bate et al., 1994) reduces the accumulation of chlorogenic acid and rutin in transgenic tobacco plants. Moreover, studies with potato (Hahlbrock and Scheel, 1989), bean (Hahlbrock and Scheel, 1989), and tobacco (Maher et al., 1994) have demonstrated that inhibition of phenylpropanoid metabolism by inhibiting PAL activity abolishes the typical resistance response of host tissue to fungal infection.

The shikimate pathway may also play a crucial role in the regulation of phenylpropanoid metabolism by controlling the supply of phenylalanine (for review, see Bentley, 1990). The shikimate pathway is initiated by condensation of phosphoenol-

pyruvate and erythrose 4-phosphate (E-4-P), followed by several reactions to yield shikimate. The condensation of shikimate with another molecule of phosphoenolpyruvate results in the formation of chorismate, where the pathway bifurcates to produce phenylalanine and tyrosine from prephenate and tryptophan through anthranilate (Figure 1). Metabolite level control of substrate supply could occur by feedback regulation of chorismate mutase and anthranilate synthase. Tryptophan has repeatedly been shown to be a negative feedback regulator of its own synthesis and a positive feedback activator of phenylalanine and tyrosine biosynthesis, whereas phenylalanine is a negative feedback regulator of its own synthesis (Bentley, 1990).

Genetic engineering of plants has been used to alter the level of expression of certain genes involved in a pathway or to redirect branch point substrates. Recent reports on the sense suppression of PAL (Elkind et al., 1990; Bate et al., 1994) and antisense suppression of cinnamyl alcohol dehydrogenase (Halpin et al., 1994) have provided useful examples of alteration of gene expression that result in plants containing decreased levels of phenols and lignin, respectively. An approach involving the redirection of branch point substrates has been successfully used to control ethylene levels in tomato plants (Klee et al., 1991). Recent studies with a tryptophan decarboxylase (TDC) isolated from the medicinal plant *Catharanthus roseus* (De Luca et al., 1989) have demonstrated that transgenic canola plants expressing this heterologous gene redirect tryptophan into tryptamine, leading to a drastic reduction in the level of indoleglucosinolates (Chavadej et al., 1994). The lack of indoleglucosinolate accumulation was caused by a probable depletion of the available tryptophan pool through direct competition for that pool. The expression of TDC in transgenic canola appeared to redirect tryptophan into tryptamine rather than into tryptophan-derived indoleglucosinolates.

The effective use of the TDC gene to create an artificial metabolic sink led us to question whether the available tryptophan pool was actually decreased in transgenic plants expressing this gene. In this study, we report the effect of branch point substrate competition on aromatic amino acid and phenylpropanoid metabolism in transgenic potato plants expressing TDC. Redirection of tryptophan into tryptamine in tubers of transgenic plants resulted in a corresponding decrease in the levels of tryptophan and phenylalanine, which suggests that the metabolite level control of substrate supply actually occurs in vivo and that it responds to a gene that creates an artificial metabolic sink. The decreased level of phenylalanine in turn led to a decrease in the accumulation of soluble phenolic compounds as well as those associated with the cell wall. Tubers from these transgenic plants were also more susceptible to infection by *Phytophthora infestans*. The results illustrate the delicate modulation occurring between the shikimate pathway and phenylpropanoid metabolism and suggest that the phenylpropanoid pathway can be down-regulated by the control of substrate supply created by the introduction of a single gene outside the pathway.
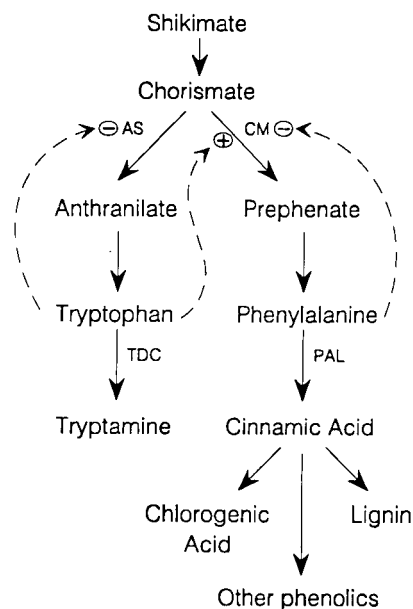

Figure 1. A Simplified Diagram Illustrating the Shikimate Pathway and the Phenylpropanoid Pathway.

The dashed-line arrows indicate feedback activation (+) or inhibition (−). AS, anthranilate synthase; CM, chorismate mutase; PAL, phenylalanine ammonia-lyase; TDC, tryptophan decarboxylase.

RESULTS

Analysis of Amino Acid and Phenolics Pools in Untransformed and Transgenic Tubers

The expression of TDC was examined in untransformed control and transgenic tubers of line M-9-D-25 upon wounding and elicitor treatment. Untransformed control tubers showed no detectable TDC activity over a period of 72 hr after wounding (Figure 2A) or after treatment with the elicitor arachidonic acid (Figure 2B). In transgenic tubers, TDC activity increased transiently, with maximal induction occurring at 24 and 36 hr, respectively, after wounding (Figure 2A) or elicitation (Figure 2B). The expression of heterologous TDC in wounded (Figure 2C) or elicited (Figure 2D) tubers of transgenic potatoes resulted in the accumulation of tryptamine, the immediate product of the TDC reaction.

Amino acid levels were also analyzed in untransformed and transgenic tubers (line M-9-D-25). Although the concentrations of most amino acids did not vary between unwounded control and transgenic tubers (data not shown), the tryptophan (Figure 2E, time 0) and phenylalanine (Figure 2G, time 0) pools were consistently 40 and 50% lower, respectively, in transgenic tubers compared with untransformed controls. The differences between the tryptophan (Figure 2E, times 0 to 36 hr) and
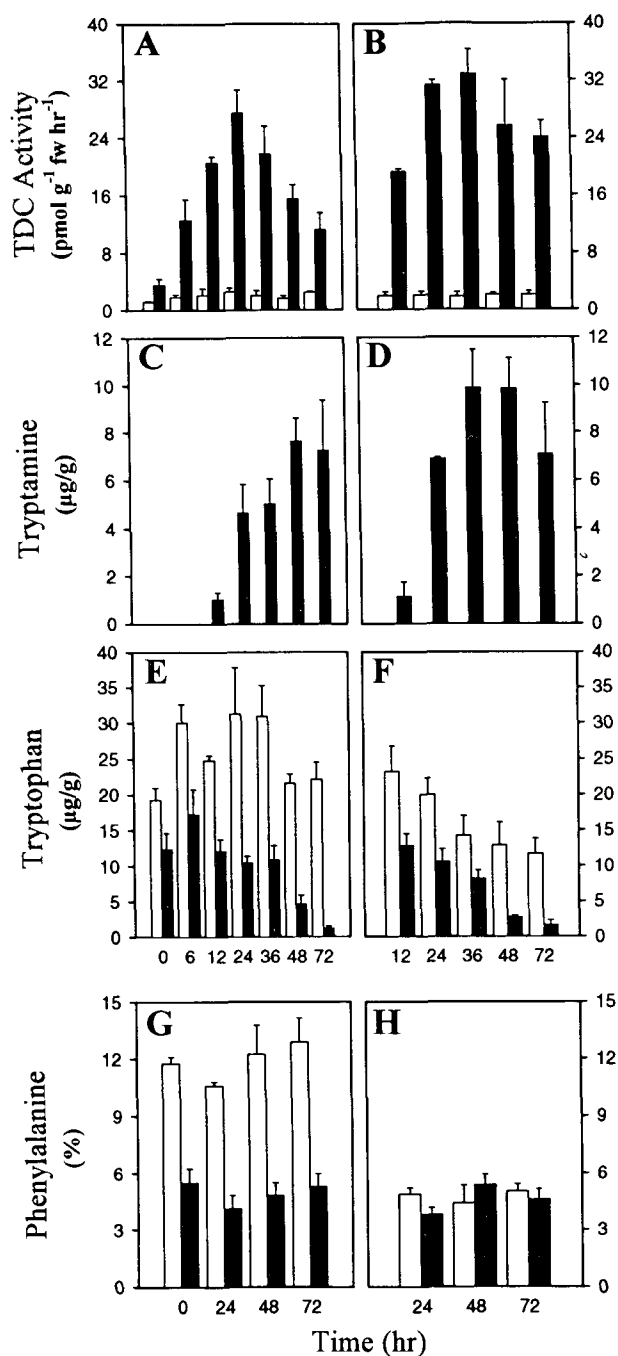
phenylalanine (Figure 2G, times 0 to 72 hr) pools were maintained after wounding of untransformed control and transgenic tubers, but the tryptophan pool in transgenic tubers decreased to 6% of that found in untransformed controls after 72 hr of wounding. Elicitor treatment caused a continuous decrease in the tryptophan (Figure 2F) pool in both transgenic and untransformed control tubers, whereas in untransformed control tubers the pool of phenylalanine declined by >50% within 24 hr to reach the levels found in transgenic tubers (Figures 2G and 2H). The important changes observed in the tryptophan and phenylalanine pools in tubers did not significantly affect protein synthesis after 72 hr of wounding or elicitor treatment, as shown by in vivo labeling studies (data not shown). Transgenic tubers from another line (M-9-D-11) were also analyzed and showed a reduction in the levels of TDC activity, tryptamine, and phenylalanine that was similar to that observed for line M-9-D-25 (data not shown). Furthermore, recent experiments with *TDC*-expressing transgenic tobacco lines (Songstad et al., 1990) have demonstrated that developing seedlings have greatly reduced tryptophan levels compared with those of untransformed controls (V. De Luca, unpublished results).

Chlorogenic acid, a major soluble phenolic ester that accumulates at wound sites in potato (Gans, 1978), is produced from caffeic and quinic acid precursors that are derived from the shikimate pathway intermediates phenylalanine and dehydroquinate, respectively. The decreased levels of phenylalanine and tryptophan occurring in transgenic tubers compared with untransformed controls indicated that lack of phenylalanine supply could affect the production of chlorogenic acid as well as other phenols in wounded or elicited transgenic tubers. Figure 3A shows that the levels of chlorogenic acid increased rapidly after wounding in both transgenic and untransformed tubers, but its concentration in transgenic tubers was <30% that in untransformed tubers 24 hr after wounding. Treatment of wounded tubers with the arachidonic acid elicitor promoted only a transient and slight increase in levels of chlorogenic acid (Figure 3B) rather than the larger accumulations (Figure 3A) occurring in wounded transformed and untransformed tubers.

Other soluble phenolics composed of free phenolic acids and phenolic acid esters were also detected by HPLC and quantified as equivalents of ferulic acid. The results showed that wounding of transgenic and untransformed tubers affected

Figure 2. Effects of the Expression of the *TDC* Gene on Accumulation of Tryptamine and Changes in the Level of Tryptophan and Phenylalanine.

(A) and **(B)** TDC activity.
(C) and **(D)** Accumulation of tryptamine.
(E) and **(F)** Level of tryptophan.
(G) and **(H)** Level of phenylalanine.
Untransformed tubers are represented by open bars, and transgenic tubers (line M-9-D-25) are represented by closed bars. **(A)**, **(C)**, **(E)**,

and **(G)** show results from tubers after wounding. **(B)**, **(D)**, **(F)**, and **(H)** show results from tubers after elicitation. Arachidonic acid (elicitor) was applied to tuber discs 6 hr after wounding. Tryptamine and tryptophan were quantified by HPLC using authentic standards. The level of phenylalanine was determined as a percentage of total amino acids. SEs are indicated; $n = 3$. The background TDC activities detected in untransformed tubers are artifacts of the assay. Similar results were obtained when transgenic tubers of line M-9-D-11 were analyzed (data not shown). fw, fresh weight.
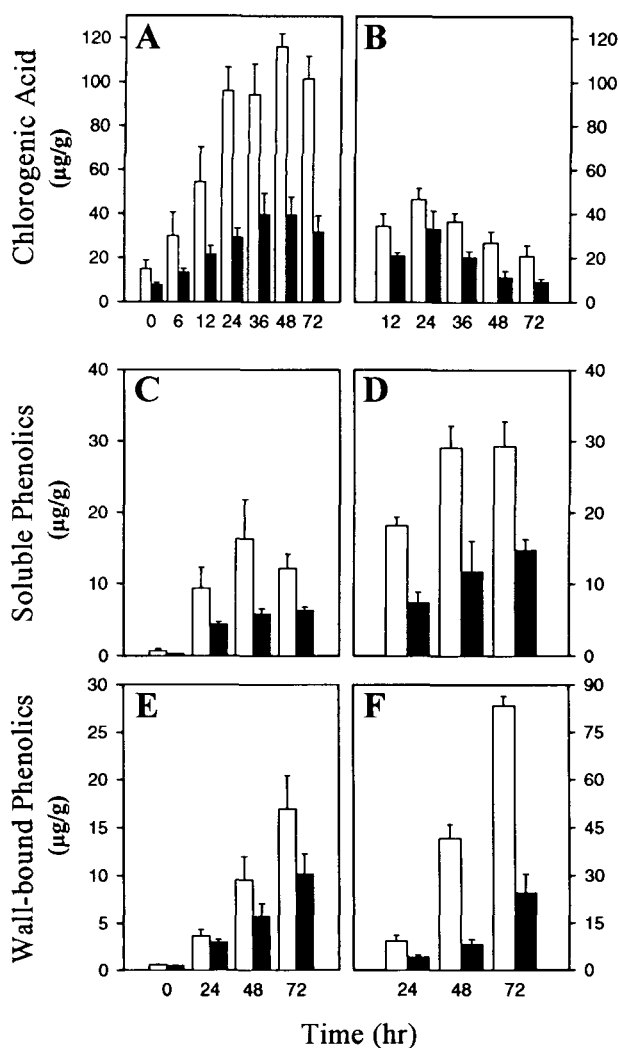
Figure 3. HPLC Analysis of the Accumulation of Phenolic Compounds in Tubers.

(A) and **(B)** Accumulation of chlorogenic acid.
(C) and **(D)** Accumulation of soluble phenolics.
(E) and **(F)** Accumulation of cell wall–bound phenolics.
Untransformed tubers are represented by open bars, and transgenic tubers (line M-9-D-25) are represented by closed bars. **(A)**, **(C)**, and **(E)** show results from tubers after wounding. **(B)**, **(D)**, and **(F)** show results from tubers after elicitation. Soluble and cell wall–bound phenolics include p-coumaric acid, ferulic acid, and other unidentified phenolics. The quantitation of these phenolics is expressed as equivalents of ferulic acid. SEs of the means are indicated; $n = 3$.

the accumulation of a number of other soluble phenolics whose concentrations increased to ∼15% (Figure 3C) of those of chlorogenic acid (Figure 3A). Elicitor treatment of wounded tubers caused a doubling of soluble phenol concentrations (Figure 3D) together with a decreased transient accumulation of chlorogenic acid (Figure 3B). When the levels of soluble phenols

accumulating in transgenic and in untransformed tubers were compared after wounding or elicitor treatment, the levels in transformed tubers were consistently <50% of those in untransformed controls. By comparing the HPLC traces, however, we found no qualitative differences in phenolics between untransformed and transgenic tubers. Although a number of phenols that accumulated remain to be identified, the accumulation of p-coumaric and ferulic acids was confirmed by using an HPLC column calibrated with authentic standards. Typically, the levels of p-coumaric acid and ferulic acid were 30 to 40% lower in transgenic tubers than in untransformed control tubers after wounding and elicitation.

Cell wall–bound phenols were also analyzed after their release by alkaline hydrolysis (Bolwell et al., 1985; Campbell and Ellis, 1992). The concentration of cell wall–bound phenols also increased in transgenic and untransformed control tubers after wounding (Figure 3E) or elicitor treatment (Figure 3F). Wounding caused a consistently smaller increase of cell wall–bound phenolics in transgenic tubers than in untransformed controls. These differences became much more dramatic when tuber discs were treated with the elicitor, because the levels of cell wall–bound phenols in transgenic tubers reached only 20 and 30% of those found in untransformed controls after 48 and 72 hr of elicitor treatment, respectively.

The effect of TDC on carbon flow through both the shikimate pathway and the phenylpropanoid pathway in transgenic tubers was also demonstrated by labeling the tuber discs with ^{14}C-shikimic acid. The incorporation of ^{14}C-shikimate into tryptophan, phenylalanine, and chlorogenic acid was measured in tissues fed for 24 and 48 hr. As shown in Table 1, the incorporation of labeled shikimate into these three metabolites decreased by >50% in transgenic tubers compared with untransformed controls. These results are consistent with the results of the HPLC analysis (Figures 2 and 3), which indicated that TDC caused a decrease in the accumulation of tryptophan, phenylalanine, and chlorogenic acid in transgenic tubers.

Examination of UV Autofluorescence and Lignin Content of Tubers

The differences in cell wall–bound phenolics that were observed after wounding and elicitor treatment prompted the microscopic examination of the UV autofluorescence of tuber sections. This method has been useful for the detection of cell wall–bound phenolic amides (Clarke, 1980), ferulic acid (Nicholson, 1992; Kato et al., 1994), and lignin and suberin (Monties, 1989; Schmutz et al., 1993). Tuber sections were either hand sectioned and viewed immediately under a fluorescence microscope without fixation and coverslip or fixed immediately after cutting into small blocks; 10-μm microtome sections were then produced for microscopic examination of the wound periderm. As shown in Figure 4, the wound-induced autofluorescence intensity of parenchyma cells from untransformed tubers (Figure 4A) was much stronger than that from

Table 1. Incorporation of ^{14}C-Shikimic Acid into Tryptophan, Phenylalanine, and Chlorogenic Acid

	Radioactivity (% Incorporation)[a]			
	Wild Type		Transgenic	
Metabolite	24 Hr	48 Hr	24 Hr	48 Hr
Tryptophan	3.4 ± 0.86	1.2 ± 0.25	1.3 ± 0.21	0.8 ± 0.14
Phenylalanine	1.5 ± 0.25	0.9 ± 0.27	0.8 ± 0.05	0.4 ± 0.06
Chlorogenic acid	2.8 ± 0.45	1.6 ± 0.50	0.5 ± 0.05	0.5 ± 0.08

[a] Tuber discs of transgenic line M-9-D-25 and untransformed controls were labeled with ^{14}C-shikimic acid (50,000 cpm per 0.95 g fresh weight) for 24 and 48 hr before analysis. The numbers represent the percentage of incorporation during the labeling period. Data are means of three independent experiments ± SE.

transgenic wounded tubers (Figure 4B). A more detailed microscopic analysis of 10-μm sections (Figures 4C and 4D) revealed that the major fluorescent compounds were localized in the cells of the wound periderm. Autofluorescence was much stronger in untransformed controls (Figure 4C) than in transgenic tubers (Figure 4D).

To investigate further the effects of altered metabolite pools caused by the expression of the *TDC* gene, intact transgenic and untransformed control tubers were also fixed, sectioned, and stained with aniline safranine O and astra blue (Gerlach, 1969). Microscope analysis of stained sections showed that the presence of lignin, which is associated particularly with vascular bundles, was greatly reduced in transgenic tubers (Figures 4E, untransformed, and 4F, transgenic). Similar results were obtained when sections were stained with toluidine blue, another lignin stain (Feder and O'Brien, 1968; data not shown). The decreased lignin content of transgenic tubers was also confirmed by HPLC quantitation. Two major degradation products of lignin monomers, syringaldehyde and vanillin, were identified after alkaline nitrobenzene oxidation of the cell wall residues. As shown in Table 2, the level of syringaldehyde from tubers of two transgenic lines was 40 to 50% lower than that from untransformed controls. Similarly, the level of vanillin from transgenic tubers was 20 to 30% lower than that from untransformed controls.

Susceptibility of *TDC*-Transformed Tubers to Infection

Oxidative cross-linking of cell wall proteins has been found to be induced shortly after elicitor treatment and has been proposed to increase the strength of the cell wall as a barrier to microbial ingress (Bradley et al., 1992). Phenolics also can provide covalent cross-links between polysaccharides and proteins in the cell wall, rendering the cell wall stronger and thus more resistant to hydrolytic enzymes produced by the invading pathogen (Iiyama et al., 1994). We therefore tested the cell wall digestibility of tuber discs by measuring the number of protoplasts released after incubation with the enzymes cellulase and pectinase (Brisson et al., 1994). Table 3 shows that

at the end of a 5-hr incubation period with these enzymes, the transgenic tubers had released twice as many protoplasts compared with the untransformed tubers. The increase in the yield of protoplasts from wounded transgenic tubers reflected the fact that their cell walls were more susceptible to the action of hydrolytic enzymes. Elicitor treatment of wounded tubers caused a dramatic decrease in the yield of protoplast from both transgenic and untransformed tubers.

To test whether the decreased accumulation of soluble and bound phenols in the wound periderm of transgenic tubers and the increased digestibility of their cell wall also correlated with an increased susceptibility to pathogen infection, transgenic and untransformed tubers were inoculated with zoospores of a virulent race of *P. infestans* and incubated at 18°C under darkness throughout the test period. Growth of fungal mycelium was detected by ELISA, which is designed for the quantitation of *Phytophthora* pathogens. As illustrated in Table 4, 6 days after inoculation there was a two- to fourfold increase in the ELISA value for tubers of both transgenic lines compared with untransformed controls, indicating that the transgenic tubers were indeed more susceptible to infection by *P. infestans.* However, treatment of tubers with the elicitor inhibited the growth of *P. infestans* on transgenic tubers as well as on untransformed controls (Table 4).

To demonstrate whether expression of *TDC* could also cause a reduced accumulation of phenolics in transgenic tubers in response to fungal infection, we analyzed the levels of tryptophan, chlorogenic acid, and total phenolics after inoculation with *P. infestans* of wounded tuber discs from two transgenic lines and the untransformed control. Results indicated that the pools of tryptophan (Figure 5A), chlorogenic acid (Figure 5B), soluble phenolics (Figure 5C), and cell wall–bound phenolics (Figure 5D) were smaller in inoculated tubers of the two transgenic lines when compared with those of inoculated untransformed controls. Moreover, inoculation caused a decrease in the tryptophan and chlorogenic acid pools in both transgenic and untransformed control tubers (Figures 5A and 5B). The decrease in the level of chlorogenic acid was paralleled by an increase in both soluble and cell wall–bound phenolics (Figures 5C and 5D).
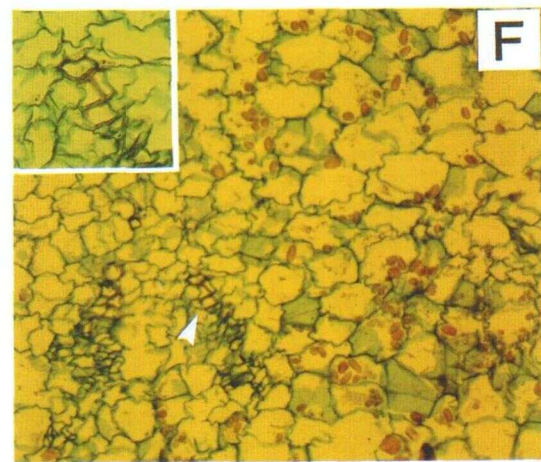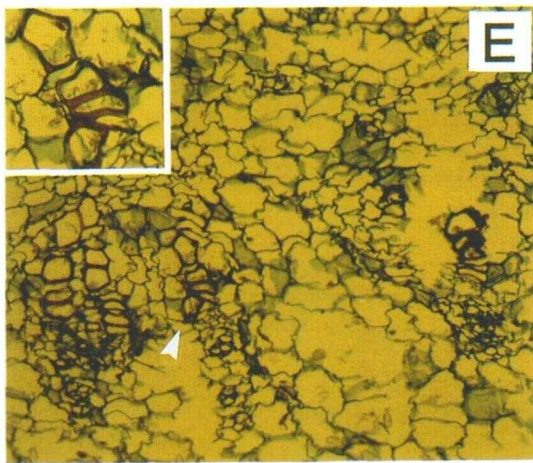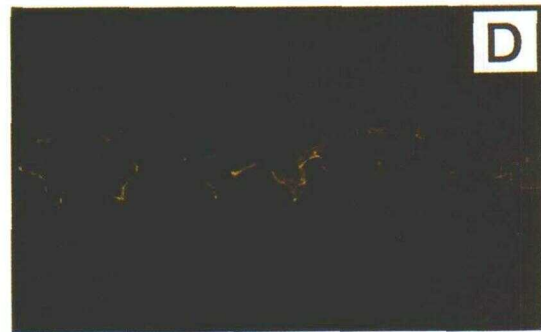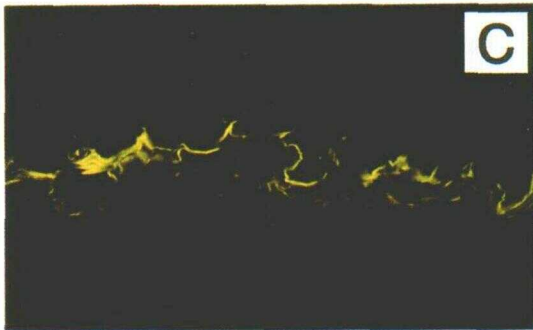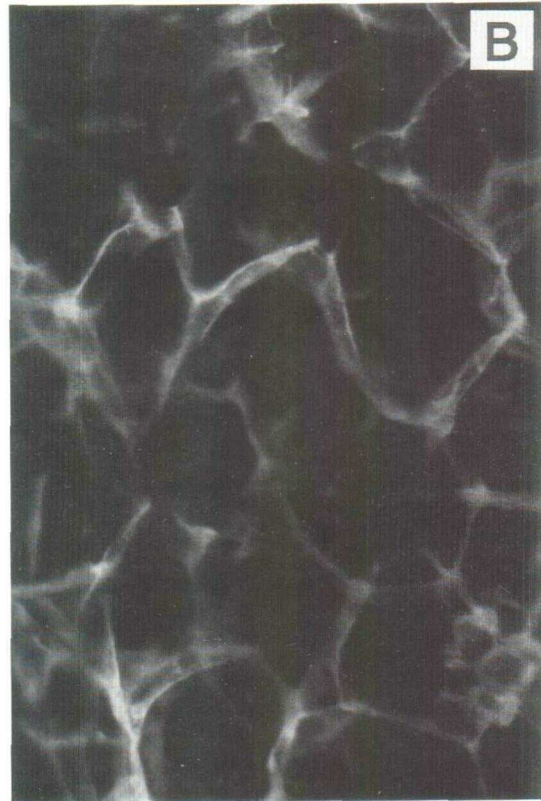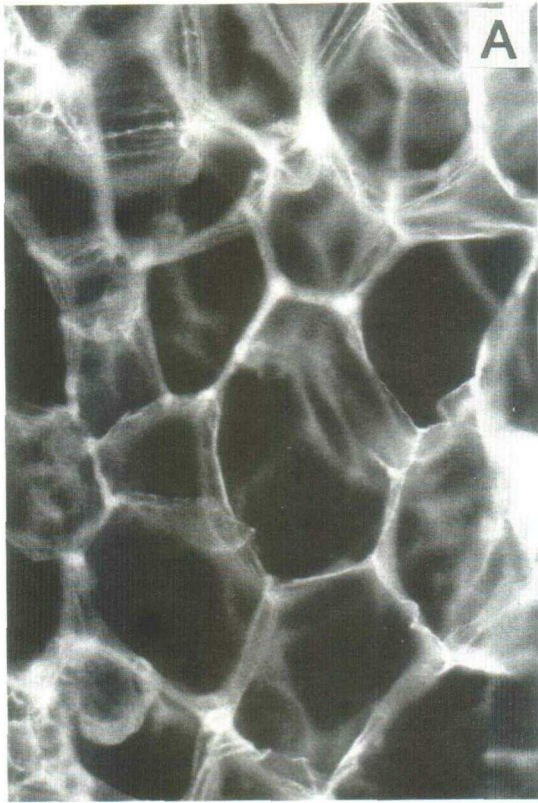
Figure 4. Histochemical Examination of Phenolic Compounds Accumulation in Tuber Sections.

Table 2. Quantitation of Syringaldehyde and Vanillin Derived from Lignin of Untransformed Control and Transgenic Tubers

Plant	Syringaldehyde (μg/g)[a]	Vanillin (μg/g)[a]
Wild type	0.64 ± 0.04	0.32 ± 0.05
M-9-D-25	0.38 ± 0.03	0.20 ± 0.02
M-9-D-11	0.30 ± 0.05	0.25 ± 0.01

[a] Data are means of three independent experiments ± SE. μg/g, micrograms per gram fresh weight.

DISCUSSION

The Phenylpropanoid Pathway Is Affected by the Introduction of the *TDC* Gene in Transgenic Tubers

Transformation of potato with the *TDC* gene resulted in transgenic tubers that contained levels of tryptophan and phenylalanine that were 40 to 50% lower than those in untransformed control tubers. These results suggested that TDC activity has considerable effects on the pool sizes of these amino acids. Although unwounded transgenic tubers had little or no TDC activity and accumulated no tryptamine, leaves from 13 transgenic lines had TDC activity and accumulated varying levels of tryptamine (data not shown). The low level of TDC activity found in intact tubers might reflect the instability of this enzyme (Alvarez Fernandez and De Luca, 1994), preventing its accumulation in the absence of synthesis in dormant tubers. Therefore, the induction of TDC activity observed in wounded tubers could result from the wound-induced break of dormancy that induces polysome formation and protein synthesis (Kahl, 1974).

Tryptophan and phenylalanine are both derived from chorismate through a branch point controlled by the key enzymes anthranilate synthase and chorismate mutase (Figure 1). Chorismate is converted either to anthranilate and then to tryptophan or to prephenate and then to phenylalanine or tyrosine. The expression of *TDC* in transgenic potato appeared to decrease the concentration of tryptophan, as demonstrated in unwounded transformed tubers, wounded transformed tubers, or arachidonic acid–treated tubers (Figure 2). It is known that

tryptophan inhibits its own synthesis through feedback inhibition of anthranilate synthase and feedback activation of chorismate mutase (Bentley, 1990). An early study with cultured potato cells showed that tryptophan regulates anthranilate synthase by feedback inhibition (Carlson and Widholm, 1978). Our in vitro studies with crude desalted anthranilate synthase extracts from potato tubers also showed that this enzyme is feedback inhibited by tryptophan, and the concentration of tryptophan required for 50% inhibition was 75 μM. A decrease in the cellular concentration of tryptophan could therefore increase the flux of chorismate through the tryptophan branch of the pathway, concomitantly decreasing the production of phenylalanine and/or tyrosine. In fact, the concentration of phenylalanine was consistently lower by at least 50% in unwounded or wounded transgenic tubers than in their untransformed counterparts (Figure 2G).

These results are supported by the in vivo labeling experiment that demonstrated lower accumulation of tryptophan, phenylalanine, and chlorogenic acid in transgenic tubers after 24 and 48 hr labeling with ^{14}C-shikimic acid. Interestingly, treatment of wounded untransformed tubers with arachidonic acid resulted in a rapid decrease in the concentration of phenylalanine to the levels found in wounded and elicited transgenic tubers (Figure 2H). These results suggest that expression of *TDC* in transgenic tubers decreases the level of phenylalaline to the level that would normally be found if this amino acid was turning over rapidly, or that the remaining pool

Table 3. Release of Protoplasts from Untransformed and Transgenic Tubers

Plant	Treatment[a]	Protoplasts Released/g fw[b]
Wild type	−	1714 ± 324
M-9-D-25	−	3657 ± 587
M-9-D-11	−	4286 ± 700
Wild type	+	607 ± 193
M-9-D-25	+	678 ± 174
M-9-D-11	+	464 ± 135

[a] Numbers were obtained from three independent experiments ± SE. (+) and (−) represent elicitation and wounding, respectively.
[b] Protoplasts were counted after 5 hr of incubation with the cell wall–degrading enzyme solution.

Figure 4. (continued).

(A) and **(B)** UV autofluorescence microscopy of parenchyma cells from untransformed and transgenic tubers (line M-9-D-25), respectively, 12 hr after wounding. The tuber discs were not fixed before hand sectioning, and the sections were viewed immediately without coverslips. Magnification ×111.

(C) and **(D)** UV autofluorescence microscopy of wound periderms from untransformed and transgenic tubers, respectively, 72 hr after wounding. The tuber discs were fixed and were then cut into 10-μm sections. The sections were covered with coverslips before viewing. Magnification ×30.

(E) and **(F)** Light microscopy of sections of intact tubers from untransformed and transgenic plants, respectively. Intact tubers were fixed immediately after cutting into small blocks. Ten-micron sections from fixed tissues were stained for lignin with aniline safranine O and astra blue. The stained sections were covered with coverslips before viewing; magnification ×15. The vascular bundle cells containing lignin are stained dark red. The inserts show the higher magnification of the vascular bundle cells marked by arrows; magnification ×30.

Table 4. ELISA Absorbance Values of Inoculated Tuber Discs[a]

| | Absorbance (410 nm) | | | |
| | Wounding | | Elicitor | |
Plant	I	II	I	II
Wild type	0.099	0.156	0.025	0.042
M-9-D-25	0.248	0.476	0.026	0.046
M-9-D-11	0.257	0.627	ND	ND

[a] The ELISA absorbance values were determined by using a *Phytophthora* detection kit. Tuber samples were taken 6 days after inoculation. I and II represent two independent experiments. ND, not determined.

of phenylalanine is inaccessible to modulation by the artificial metabolic sink. These results also provide important in vivo evidence that the cellular level of tryptophan may regulate flux through the shikimate pathway by the feedback mechanisms proposed from in vitro enzyme studies.

TDC-Induced Perturbation of Tryptophan and Phenylalanine Pools Alters the Ability To Accumulate the Antimicrobial Phenol Chlorogenic Acid in Transgenic Tubers

The creation of an artificial metabolic sink for tryptophan in transgenic tubers also severely affected their ability to produce wound-induced phenylpropanoid compounds that are derived from phenylalanine. The reduced availability of phenylalanine resulted in a large reduction in the accumulation of chlorogenic acid as well as several other minor soluble phenylpropanoids in wounded transgenic tubers. The reduced availability of soluble phenylpropanoids, in turn, caused a large reduction in the accumulation of cell wall–bound phenols that could be hydrolyzed by alkaline treatment in transgenic tubers. These results illustrate how the introduction of a single gene modifying the availability of tryptophan also alters the availability of phenylalanine and, thus, the ability of the plant to produce phenylalanine-derived phenylpropanoids. In addition, the results also illustrate that substrate control through changes in phenylalanine pool size could in fact regulate phenylpropanoid production in potato tubers.

Treatment of wounded tubers with the elicitor caused a doubling of soluble phenol concentrations and a large decrease in the transient accumulation of chlorogenic acid in both untransformed and transgenic tubers (Figure 3). This increased accumulation of phenolics after elicitor treatment indicates that phenylpropanoid metabolism is activated upon elicitation (Maina et al., 1984; Campbell and Ellis, 1992). These results support the proposal advanced by Friend (1981) that chlorogenic acid may act as a reservoir for the synthesis of other phenolics when phenylpropanoid metabolism is activated. Indeed, there are a number of reports indicating that chlorogenic acid could provide phenolic structures for the synthesis of
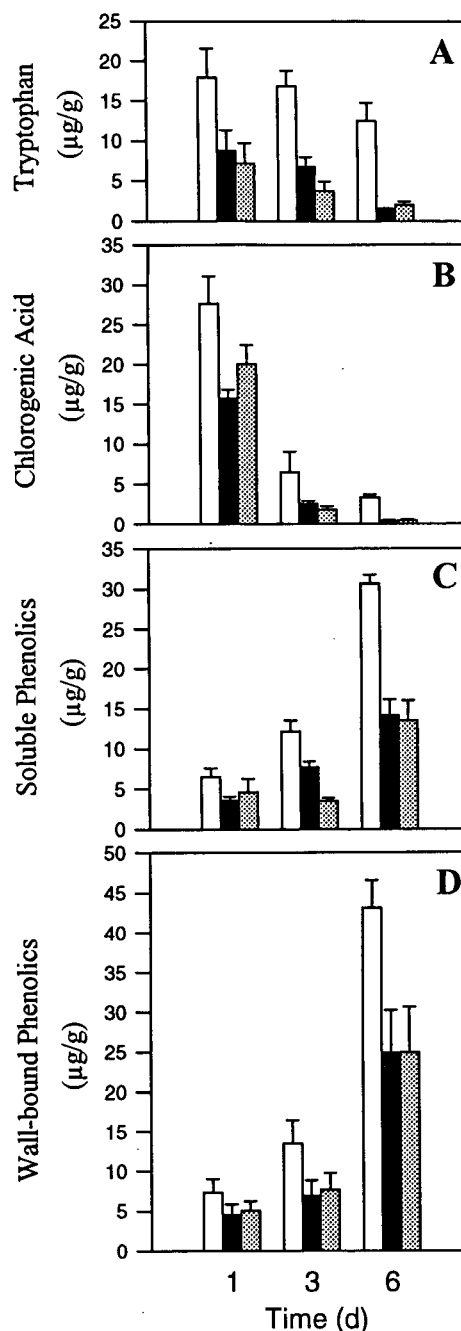

Figure 5. HPLC Analysis of Tryptophan and Phenolic Compounds in Tubers after Inoculation with *P. infestans*.

(A) Accumulation of tryptophan.
(B) Accumulation of chlorogenic acid.
(C) Accumulation of soluble phenolics.
(D) Accumulation of cell wall–bound phenolics.
Open bars represent untransformed tubers, closed bars represent tubers from transgenic line M-9-D-25, and stippled bars represent tubers from transgenic line M-9-D-11. The analysis was done after inoculation of tuber discs with zoospores of *P. infestans*. The quantitation of tryptophan, chlorogenic acid, and phenolics is as described in the legends to Figures 2 and 3, respectively. SEs are indicated; *n* = 3; d, days.

suberin (Zucker and Hankin, 1970; Kolattukudy, 1981). It is also possible that the decrease in the level of chlorogenic acid in elicited or inoculated tubers resulted directly from it becoming bound to the cell walls (Figures 2B and 5B; Friend, 1981).

Although numerous studies have suggested that PAL catalyzes a rate-limiting step in phenylpropanoid metabolism (Elkind et al., 1990; van der Krol et al., 1990; Nicholson, 1992), it has also been suggested that the availability of phenylalanine plays a critical role in the regulation of this pathway (Margna, 1976). Substrate level control of phenylpropanoid accumulation has been implied by the fact that many plants possess high PAL deaminating capacities, which markedly exceed the levels required for the phenylpropanoids produced, and by the frequent lack of correlation between the level of PAL in the cell and the accumulation of phenylpropanoids (Margna, 1976). More recent studies (Bate et al., 1994) have shown that PAL levels become rate limiting when PAL sense-suppressed transgenic tobacco plants express 20 to 25% of the activity normally found in untransformed controls. The data presented in this report provide in vivo evidence that an altered phenylalanine pool can seriously affect the production of phenylpropanoids in wounded and elicited potato tubers.

Strength of Cell Walls in Transgenic Tubers Is Modified Due to the Limited Availability of Phenolics

UV autofluorescence and histochemical analysis of tissue sections from the transgenic tubers confirmed the results of biochemical analysis and revealed that major modifications had taken place at the cell wall. First, wound-induced autofluorescence was markedly reduced in the cell wall of parenchyma cells in transgenic tubers. Second, autofluorescence of cell walls at the wound periderm of transgenic tubers was also dramatically reduced; and third, cells of vascular bundles from transgenic tubers stained only weakly for lignins as compared with similar cells from untransformed tubers. In fact, the levels of syringaldehyde and vanillin, the two major degradation products of lignin monomers formed during alkaline nitrobenzene oxidation, were 30 to 50% lower in transgenic tubers than in untransformed controls. Although it is very difficult to measure total lignin content by any method due to the resistance of the polymer to chemical degradation, the data obtained by alkaline nitrobenzene oxidation provide relative lignin contents of lignin monomers. These results suggest that the limited availability of phenolics in transgenic tubers yields cell walls with reduced strength, because phenolics are required for lignin and suberin biosynthesis (Kolattukudy, 1981; Cottle and Kolattukudy, 1982; Bolwell et al., 1985; Farmer, 1985; Schmutz et al., 1993), and they may also serve as cross-linkers between cell wall macromolecules (Iiyama et al., 1990, 1994; Lam et al., 1992). A direct link between the availability of phenolics and the strength of cell walls was provided from the results showing that more protoplasts were released from transgenic tubers than from untransformed control tubers after 5 hr of incubation with cell wall–degrading enzymes. It will be interesting in future studies to determine how this reduced

availability of phenolics affects the synthesis of lignin or suberin after inoculation with a pathogen.

The strong autofluorescence observed in the wound periderm of untransformed tubers in Figure 4C indicates that most of the phenolics are localized in this region after cutting the tuber. This is consistent with the finding that most of the wound-induced insoluble phenolics in potato tubers are localized in the suberin layer of wound periderm (Cottle and Kolattukudy, 1982). This mode of mobilization of phenolics in potato tubers upon wounding is thought to ensure the suberization of the periderm at the cut surface. It is well known that cells of wound surface become lignified and suberized. Therefore, because phenolics provide aromatic domains for suberization, the limited availability of phenolics in transgenic tubers would hinder this process in the wound periderm cells.

Transgenic Tubers Are More Susceptible to Infection by *P. infestans*

Accumulation of phenolics is generally accepted as an early or immediate response of plants to pathogen attack as well as to wounding or elicitor treatment (Friend, 1981; Hammerschmidt, 1984; Bolwell et al., 1985; Campbell and Ellis, 1992). In this study, we showed that potato tubers of both transgenic and untransformed controls accumulate phenolics after wounding, elicitor treatment, or inoculation with zoospores of *P. infestans*. The fact that *P. infestans* grew faster on wounded tuber discs from transgenic plants suggests that the reduced level of phenolics in these tubers may be responsible for the accelerated growth of the fungus. Indeed, an early report indicated that the amount of chlorogenic acid accumulating after cutting potato tuber tissue was directly related to field resistance of cultivars to *P. infestans* (Schöber, 1971). It has also been reported recently that suppression of PAL activity in transgenic tobacco plants resulted in a low accumulation of chlorogenic acid and, correspondingly, in more rapid and extensive lesion development than in wild-type plants after infection by the virulent fungal pathogen *Cercospora nicotianae* (Maher et al., 1994). Hohl and Suter (1976) have suggested that host cell wall dissolution is the earliest event in the formation of haustoria in the potato–*P. infestans* system. Indeed, we demonstrated that the cell wall of transgenic tubers is more prone to digestion by cell wall hydrolyzing enzymes. This is consistent with the hypothesis that the esterification of polysaccharides by phenolics would render them less susceptible to cell wall–degrading enzymes of pathogens (Ampomah and Friend, 1988). Finally, the fact that elicitor treatment inhibited the growth of the fungus on transgenic tubers while relatively low levels of phenolics accumulated in these tubers (Figure 2) suggests that other defense mechanisms are induced by treatment with the elicitor and are responsible for the fungal growth inhibition.

In this study, we showed that redirection of tryptophan in transgenic potato causes altered phenylpropanoid metabolism most likely by changing the flux of branch point substrates. This indicates that the synthesis and accumulation of phenolic

compounds, including lignin, can be regulated by altering substrate availability through the introduction of a single gene outside the pathway involved in substrate supply.

METHODS

Plant Material

The tryptophan decarboxylase (*TDC*) cDNA isolated from *Catharanthus roseus* was placed under the transcriptional control of the cauliflower mosaic virus 35S promotor (Chavadej et al., 1994). This gene construct was used to transform potato (*Solanum tuberosum* cv Désirée) by leaf disc transformation according to the protocol described by Matton et al. (1993). Both transgenic and untransformed plants were grown in a growth chamber under conditions of a 16-hr-light and 8-hr-dark cycle. The temperature was maintained at 21°C during the light period and 19°C during the dark period. Leaf samples from 13 independent lines of transgenic plants were all shown to accumulate tryptamine. Tubers were harvested and stored at 4°C until use. Tuber samples from two of those lines showing high TDC activity, M-9-D-25 and M-9-D-11, were used for this study.

Tuber Disc Treatments

Potato tubers were cut into discs (0.25 cm thick and 2 cm in diameter) and placed in Petri dishes on moistened sterile filter paper. Elicitor treatments were performed by spraying 40 μL of arachidonic acid (1 mg/mL) onto tuber disc surfaces after 6 hr of wounding. Control tuber discs were treated with sterile water and were kept at room temperature under darkness. Tuber disc samples were taken at specified time intervals for analysis.

Extraction of Soluble Phenolics

Tuber disc samples taken at specified time points were quickly ground in 100% methanol (1:1 [w/v]) at room temperature. The homogenate was incubated at 60°C for 30 min and then centrifuged for 15 min at 12,000g. The supernatant was collected, and the pellet was extracted once more with 50% methanol. The supernatant was combined and taken to dryness in a vacuum and redissolved in 200 μL of 50% methanol. This preparation was used for quantitation of tryptophan, tryptamine, chlorogenic acid, and total soluble phenols by HPLC. The total soluble phenolics include free phenolic acids and phenolic esters.

Alkaline Hydrolysis of Cell Walls

After extraction of soluble phenols, the residual pellet was washed two more times with 50% methanol, ensuring that no more soluble phenolics were present. The resulting pellet was resuspended in 0.5 mL of 1 N NaOH and incubated for 2 hr at room temperature, followed by centrifugation at 12,000g for 15 min. The supernatant was acidified with concentrated HCl and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness and redissolved in 200 μL of 50% methanol. This preparation was used for determination of cell wall–bound phenolics.

HPLC Analysis of Phenolics

Twenty microliters of the phenolic samples was analyzed by HPLC using a 3.9 × 300 mm C-18 reverse phase column (Waters, Milford, MA). Solvent A contained methanol/acetic acid/H_2O (15:0.5:84.5); solvent B contained methanol/acetic acid/H_2O (84.7:0.3:15). Gradient conditions are as follows: 0 to 3 min in 0% solvent B; 3 to 18 min in 0 to 40% solvent B; 18 to 25 min in 40 to 100% solvent B; 25 to 40 min in 100% solvent B; 40 to 50 min in 100 to 0% solvent B; 50 to 60 min in 0% solvent B. The solvent flow rate was constantly controlled at 0.5 mL min^{-1}. The eluant was monitored at 280 nm, and peak areas were determined by integration. The amount of tryptophan, tryptamine, and chlorogenic acid was determined using authentic standards (Sigma). Total phenolics (excluding chlorogenic acid) were determined as equivalents of ferulic acid.

Amino Acid Analysis

One hundred microliters of the methanol-soluble preparation was taken to dryness and redissolved in 75 μL of dilution buffer containing 100 mM $NaHCO_3$ and 100 mM H_3BO_3, pH 8.5. Twenty microliters of the resuspended solution was mixed with 20 μL of 9-fluorenylmethyl chloroformate (20.7 mg/10 mL) (Varian, Mississauga, Ontario) and incubated at room temperature for 10 min to generate the fluorescent derivatives of amino acids. After extraction of the free fluorescent dye from the amino acid derivatives by the addition of 70 μL of pentane ethyl acetate (80:20), 20 μL of the amino acid derivatives was injected into an HPLC column equipped with an Amino Tag column (Varian) and a Fluorichrom detector (Varian). Each amino acid was quantified as a percentage of total amino acids.

Lignin Analysis

Lignin content of fresh tubers was analyzed by alkaline nitrobenzene oxidation according to Galletti et al. (1989). Two grams of freshly cut tuber discs was homogenized in 2 mL of methanol. After extraction of soluble and cell wall–bound phenols as described above, the final pellet was washed twice with H_2O, resuspended in 2 mL of 2 M NaOH, and then transferred to a stainless steel tube with a screw cap to which 80 μL of nitrobenzene was added. The tube was sealed and incubated at 160°C for 2 hr. After cooling to room temperature, the reaction mixture was diluted to 10 mL with H_2O and filtered. The filtrate was extracted three times with 10 mL CH_2Cl_2, and the organic phase was discarded. The aqueous phase was acidified with 6 N HCl and reextracted three times with 10 mL CH_2Cl_2. The combined organic phase was dried under vacuum and redissolved in 400 μL of 50% methanol. Fifty microliters was injected to the HPLC using a 4 × 250 mm LiChrosorb RP-8 column (Merck) and eluted in the solvent containing methanol/0.1% perchloric acid in water (15:85 [v/v]). The eluant was monitored at 280 nm, and peak areas were determined by integration. The amounts of vanillin, syringaldehyde, and 5-hydroxybenzaldehyde were determined using authentic standards (Sigma).

^{14}C-Shikimic Acid Labeling Experiments

^{14}C-shikimic acid was synthesized by culturing the *Escherichia coli* mutant JP 1636 (Ely and Pittard, 1979) with uniformly labeled ^{14}C-glucose and purified by column chromatography according to Srinivasan

et al. (1956). The purified shikimic acid was dissolved in H_2O, and 30 µL (50,000 cpm) was used to label tuber discs. At the end of labeling, tuber discs were ground in 100% methanol, and the homogenate was centrifuged at 12,000g for 10 min. The supernatant was dried and redissolved in 100 µL of H_2O. After acidification, chlorogenic acid was extracted twice with ethyl acetate and the combined organic phases were dried. The H_2O phase, which contained tryptophan and phenylalanine, was also dried after neutralization. Both preparations were redissolved in 50 µL of 50% methanol and separated by thin-layer chromatography on a silica GF plate (Merck) using ethyl acetate/formic acid/acetic acid/water (100:11:11:27) as solvent system. After development, tryptophan and chlorogenic acid were identified under UV light, and phenylalanine was identified by spraying the plate with 0.4% ninhydrin. The bands comigrating with standard tryptophan, chlorogenic acid, and phenylalanine were scraped off the plate, and the radioactivity was counted.

Cell Wall Digestibility Measurement

Cell wall digestibility was determined by measuring the release of protoplasts after enzymatic digestion. Tuber discs were treated with water or elicitor for 24 hr before digestion. Ten tuber discs were rinsed twice with H_2O and then placed in a Petri dish containing 10 mL of plasmolysis solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, 500 mM mannitol). After 2 hr of incubation, the plasmolysis solution was replaced by the enzymatic solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, 500 mM mannitol, 25 mg/mL cellulase, 3 mg/mL pectinase) and incubated for another 5 hr. At the end of incubation, the enzymatic solution was removed, and 10 mL of dense solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, 500 mM sucrose) was added to float protoplasts. After slightly agitating the Petri dish, the dense solution was centrifuged at 3000g for 5 min followed by careful removal of 9 mL from the top. The number of protoplasts in the 1-mL bottom solution was counted in a hemocytometer.

Enzyme Assay and Protein Labeling Assay

The TDC assay was as described previously (De Luca et al., 1988). Briefly, activity was determined by monitoring the tryptamine production from L-methylene-^{14}C-tryptophan. Anthranilate synthase was determined by measuring the formation of anthranilate from chorismate and glutamine according to Poulsen et al. (1991). The enzyme extract was desalted on a PD-10 column (Pharmacia, Uppsala, Sweden) to eliminate the endogenous tryptophan. Exogenous tryptophan was added to the incubation mixtures at various concentrations to determine the inhibition of enzyme activity. Protein synthesis after wounding was determined by labeling the tuber discs with 20 µCi of ^{35}S-methionine (1108 Ci/mmol; ICN, Montreal, Canada) as described by Ferguson et al. (1994). Protein was quantified according to Bradford (1976).

Histochemical Analysis of Cell Walls

After wounding, tuber discs were hand sectioned, and wound-induced phenolics were monitored without coverslips by UV autofluorescence (Zeiss Filter, Set 09; Carl Zeiss Canada, Don Mills, Ontario) using a Zeiss Axiophot microscope (Constabel et al., 1993). In other experiments, tuber discs were fixed in 0.5% chromic acid/3.5% acetic

acid/5.5% formalin, dehydrated in tert-butylalcohol, and then sectioned at 10 µm. After mounting the sections on slides with a coverslip, the accumulation of phenolics was viewed by UV autofluorescence as described above. For examination of the accumulation of lignin, intact tubers were cut into small blocks, fixed, dehydrated as described above, and then sectioned into 10 µm. The sections were then stained with aniline safranine O and astra blue (Gerlach, 1969) or toluidine blue O (Feder and O'Brien, 1968) for lignin examination.

Analysis of Inoculated Tuber Discs

Tuber discs from both transgenic and untransformed plants were inoculated with Phytophthora infestans to compare their susceptibility to infection by this pathogen. For this purpose, cultures of P. infestans (race 1, 2, 3, 4; American Type Culture Collection, Rockville, MD) were maintained on rye agar (Stolle and Schöber, 1982) and zoospores were induced as described by Rohwer et al. (1987). For inoculation, 40 µL of zoospores (20 to 30 zoospores per µL) was placed on the surface of the disc 8 hr after tubers were cut. Elicitor was applied 2 hr before inoculation as described above. Inoculated discs were incubated in Petri dishes at 18°C, and samples were taken for tryptophan and phenolics analysis by HPLC at the specified time. The growth of fungal mycelium on inoculated discs was evaluated by ELISA using a Phytophthora detection kit (Sigma) according to the manufacturer's instructions. Positive reactions were recognized by the obvious yellow coloration in the wells. The ELISA values, which are proportional to the amount of pathogen in tuber extracts, were determined using a microplate reader (model MR 5000; Dynatech Laboratories Inc., Chantilly, VA) and expressed as the absorbance at 410 nm.

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