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Decreased Cell Wall Digestibility in Canola Transformed with Chimeric Tyrosine Decarboxylase Genes from Opium Poppy[1]

Peter J. Facchini*, Min Yu, and Catherine Penzes-Yost

Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

Tyrosine decarboxylase (TYDC) is a common plant enzyme involved in the biosynthesis of numerous secondary metabolites, including hydroxycinnamic acid amides. Although a definite function has not yet been determined, amides have been proposed to form a physical barrier against pathogens because they are usually found as integral cell wall components. Canola (*Brassica napus*) was independently transformed with chimeric genes (*35S::TYDC1* and *35S::TYDC2*) under the transcriptional control of the cauliflower mosaic virus 35*S* promoter, and encoding two TYDC isoforms from opium poppy (*Papaver somniferum*). All T_0 plants displayed a suppressed level of wild-type TYDC activity, and transgene mRNAs were not detected. Silencing of *35S::TYDC1* was overcome in the T_1 progeny of self-pollinated T_0 plants, since high levels of TYDC1 mRNAs were detected, and TYDC activity increased up to 4-fold compared with wild-type levels. However, TYDC1 mRNA levels decreased in T_2 plants and were not detected in the T_3 progeny. TYDC activity also gradually declined in T_2 and T_3 plants to nearly wild-type levels. In contrast, silencing of *35S::TYDC2* was maintained through four consecutive generations. T_1 plants with a 3- to 4-fold increase in wild-type TYDC activity showed a 30% decrease in cellular tyrosine pools and a 2-fold increase in cell wall-bound tyramine compared with wild-type plants. An increase in cell wall-bound aromatic compounds was also detected in these T_1 plants by ultraviolet autofluorescence microscopy. The relative digestibility of cell walls measured by protoplast release efficiency was inversely related to the level of TYDC activity.

Plant responses to pathogens include the induction of numerous metabolic pathways that comprise an arsenal of biochemical and physical defenses. Induction of hydrolytic enzymes such as chitinases and glucanases and the production of low-M_r antimicrobial compounds known as phytoalexins are common biochemical defense responses that increase disease resistance in plants by directly inhibiting the growth of pathogens (Hahlbrock and Scheel, 1989). The deposition of lignin and the cross-linking of Hyp-rich glycoproteins within the polysaccharide matrix of the cell wall are examples of physical defense mechanisms that reduce plant cell susceptibility to penetration by invading pathogens (Showalter et al., 1985; Matern and Kneusel, 1988).

TYDC (EC 4.1.1.28) catalyzes the decarboxylation of Tyr to tyramine (Fig. 1) and is widespread in higher plants (Hosoi et al., 1970; Tocher and Tocher, 1972; Marques and Brodelius, 1988; Kawalleck et al., 1993; Trezzini et al., 1993; Facchini and De Luca, 1994). The rapid and transient induction of TYDC mRNAs in response to elicitors and/or pathogens in parsley (Schmelzer et al., 1989; Kawalleck et al., 1993), Arabidopsis (Trezzini et al., 1993), and opium poppy (*Papaver somniferum*; Facchini et al., 1996) suggests that tyramine serves as a precursor to an important class of plant defense-response metabolites. In opium poppy, TYDC is encoded by a family of 10 to 15 genes that can be categorized into two subgroups based on sequence identity (Facchini and De Luca, 1994; Facchini et al., 1998). Each subgroup consists of approximately six members that share approximately 90% identity at the nucleotide and amino acid levels. In contrast, comparison of subgroup members (represented by *TYDC1* and *TYDC2*) reveals sequence identities of <75%. Although the catalytic properties of the different TYDC isoforms are similar (Facchini and De Luca, 1994), the *TYDC* gene family exhibits differential and organ- and temporal-specific expression (Facchini et al., 1998).

Recent studies have shown that the biosynthesis of hydroxycinnamic acid amides of tyramine and their subsequent polymerization in the cell wall by oxidative enzymes are integral and ubiquitous components of the plant defense response to pathogen challenge (Clarke, 1982; Negrel and Martin, 1984; Negrel and Jeandet, 1987; Negrel and Lherminier, 1987; Negrel et al., 1993a; Schmidt et al., 1998). These amides, together with other cell wall-bound phenolics, are believed to create a barrier against pathogens by reducing the digestibility of the cell wall and/or by directly inhibiting the growth of fungal hyphae. Hydroxycinnamic acid amides, which have been found in a variety of plants (Martin-Tanguy et al., 1978), are formed by the condensation of hydroxycinnamoyl-CoA esters with various amines such as polyamines (e.g. putrescine and spermidine) or tyramine. THT (EC 2.3.1.110) catalyzes the condensation of tyramine and select derivatives of hydroxycinnamoyl-CoA (Fig. 1) and is induced in response to pathogens (Fleurence and Negrel, 1987), elicitor treatment (Villegas and Brodelius, 1990; Schmidt et al., 1998; Yu and Facchini, 1999), and wounding (Negrel et al., 1993a). The enzyme was first

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* Corresponding author; e-mail pfacchin@ucalgary.ca; fax 1–403–289–9311.

Abbreviations: CaMV, cauliflower mosaic virus; NPT, neomycin phosphotransferase; PAL, phenylalanine ammonia lyase; THT, tyramine hydroxycinnamoyltransferase; TYDC, Tyr/dihydroxyphenylalanine decarboxylase.

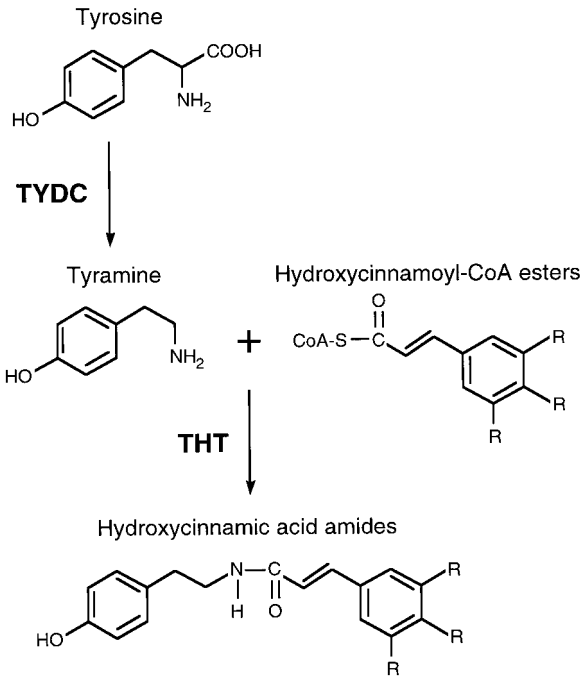


Figure 1. Reactions in the biosynthesis of hydroxycinnamic acid amides that are catalyzed by TYDC and THT.

isolated from tobacco leaves (Negrel and Martin, 1984) and has been purified to homogeneity from potato (Hohlfeld et al., 1995, 1996), tobacco (Negrel and Javelle, 1997), and opium poppy (Yu and Facchini, 1999).

The use of transgenic plants with altered levels of a specific enzyme is a powerful technique with which to study metabolic regulation and to refine our understanding of the physiological roles for secondary metabolic pathways. For example, the co-suppression of PAL activity in transgenic tobacco demonstrated that this enzyme is a rate-determining step in the biosynthesis of phenylpropanoid derivatives, including lignin, and showed that phenolic metabolites are crucial for the resistance of plants to pathogens (Bate et al., 1994; Maher et al., 1994). Introduction of a foreign *TDC* (Trp decarboxylase) (EC 4.1.1.25) gene into canola (*Brassica napus*) resulted in the redirection of Trp into tryptamine rather than into indole glucosinolates (Chavadej et al., 1994). In contrast, expression of the same *TDC* gene in transgenic potato resulted in altered aromatic amino acid biosynthesis and increased susceptibility of the plants to pathogen infestation (Yao et al., 1995). In the present study, we tested the hypothesis that an increase in TYDC activity in canola transformed with chimeric *TYDC* transgenes would increase the incorporation of tyramine and/or hydroxycinnamic acid amides into cell walls and result in a corresponding decrease in cell wall digestibility.

MATERIALS AND METHODS

Growth and Transformation of Canola

Two TYDC cDNAs from opium poppy (*Papaver somniferum* cv Marianne) were placed under the transcriptional control of the CaMV *35S* promoter. pBI35S::TYDC1 was constructed by replacement of *GUS* between the *Kpn*I and *Sac*I sites of pBI122 with a *Kpn*I/*Sac*I fragment from pBluescript (Stratagene) containing the TYDC1 cDNA (Facchini and De Luca, 1994). pBI35S::TYDC2 was constructed by replacement of *GUS* between the *Xba*I and *Sac*I sites of pBI122 with a *Xba*I/*Sac*I fragment from pBluescript containing the TYDC2 cDNA (Facchini and De Luca, 1994). pBI122 is a modified version of pBI121 (CLONTECH, Palo Alto, CA) containing the restriction sites *Apa*I, *Xho*I, *and Kpn*I in an adapter fragment that was inserted into the *Sma*I site between the *35S* promoter and *GUS*. pBI122 also harbors the *NPT II* gene for kanamycin resistance under the control of the constitutive *NOS* (nopaline synthase) promoter. Plasmids were sequenced through the *35S* promoter-*TYDC* junction to verify construct assembly.

pBI35S::TYDC1 and pBI35S::TYDC2 were mobilized in the disarmed *Agrobacterium tumefaciens* strain LB4404 by direct DNA transfer (An, 1987) and used to transform canola (*Brassica napus* cv Westar) by the cotyledonary petiole method (Moloney et al., 1989). Plants were maintained in a growth chamber at a PPFD of 400 μE m^{-2} s^{-1} and a light/dark regime of 16 h (21°C)/8 h (15°C). Regenerated plants were tested for integration of chimeric *TYDC* and *NPT II* genes into the canola genome, TYDC and NPT II enzyme activities, and the presence of TYDC mRNAs.

Nucleic Acid Isolation and Analysis

Genomic DNA was extracted by grinding 100 mg of leaf tissue in 400 μL of 200 mM Tris-HCl, pH 7.8, 250 mM NaCl, 0.5% SDS, and 25 mM EDTA. Debris were removed by centrifugation, and DNA was precipitated with an equal volume of isopropanol and recovered by centrifugation. The pellet was rinsed in 70% ethanol, dried, and redissolved in water. Fragments of *TYDC1* and *TYDC2* were amplified by 30 PCR cycles from 100 ng of genomic DNA using a primer-annealing temperature of 55°C and specific oligonucleotides designed from published sequences (TYDC1 sense primer, AGGGACTACTTGTGAAGCCA; TYDC1 antisense primer, ACTGATTCAAGCAATTTCGC; TYDC2 sense primer, ACTTCTTAGCTGATTATTAT; TYDC2 antisense primer, ACGGCATGAGTCATGTAAAC; Facchini and De Luca, 1994). PCR products were analyzed on 1% agarose gels containing ethidium bromide.

Total RNA was isolated according to the method of Logemann et al. (1987), and 15 μg was fractionated onto 1.0% formaldehyde agarose gels before transfer to nitrocellulose membranes (Sambrook et al., 1989). RNA blots were hybridized to random-primer ^{32}P-labeled (Feinberg and Vogelstein, 1984) full-length TYDC1 or TYDC2 cDNAs at 65°C in 0.25 M sodium phosphate, pH 8.0, 7% SDS, 1% BSA, and 1 mM EDTA. Blots were washed at 55°C, twice with 2× SSC containing 0.1% SDS and twice with 0.2× SSC containing 0.1% SDS (Sambrook et al., 1989) (1× SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). RNA blots were autoradiographed with an intensifying screen on Kodak X-OMAT film at −80°C.

TYDC, THT, and NPT II Assays

For TYDC and THT assays, plant tissues were frozen under liquid nitrogen and ground to a fine powder with a mortar and pestle. Powdered tissues were extracted with 200 mM Tris-HCl, pH 7.8, debris was removed by centrifugation, and the supernatant was desalted using a PD-10 column (Pharmacia). TYDC activity was assayed by measuring the release of $^{14}CO_2$ from L-[*carboxyl*-^{14}C]Tyr (Facchini and De Luca, 1994). The TYDC assay contained 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 25 μM pyridoxal-1-phosphate, 0.1 μCi (specific activity = 55 mCi mmol^{-1}) [^{14}C]Tyr, and 500 μL of protein extract (total volume = 1 mL) in an airtight vial. Reactions were incubated for 60 min at 35°C with constant agitation. Enzymatically liberated $^{14}CO_2$ was trapped on quaternary ammonium-saturated GF/D filters suspended above the reaction solution. Reactions were stopped by the addition of 0.2 N HCl and agitated for an additional 1 h before scintillation counts from GF/D filters were determined.

4-Coumaroyl-CoA for the THT assay was enzymatically synthesized using total protein extract from *Escherichia coli* harboring pQE19, which expresses a recombinant tobacco 4-coumarate:coenzyme A ligase (4CL) (Lee and Douglas, 1996). The synthesis reaction consisted of 0.1 mM CoA, 0.2 mM 4-coumaric acid (Sigma), 2.5 mM ATP, 1 mM DTT, and 300 mg of total bacterial protein extract (Meng and Campbell, 1997). After 1 h of incubation, the synthesized 4-coumaroyl-CoA was purified using a Sep-Pak C_{18} column (Waters). The 4-coumaroyl-CoA was concentrated, and its identity and purity were confirmed by TLC and comparison of the UV spectrum with that of an authentic standard. THT activity was measured as described previously (Yu and Facchini, 1999). Ninety microliters of desalted enzyme extract in 50 mM Tris-HCl, pH 7.8, was incubated for 1 h with 0.5 μCi of [8-^{14}C]tyramine and 100 nmol of 4-coumaroyl-CoA. Reactions were stopped by the addition of 1.0 M HCl, and 20 μL was applied to a silica gel 60 F_{254} TLC plate that was subsequently developed in chloroform:methanol (5:4). The developed TLC plate was autoradiographed for 12 h. Radiolabeled spots corresponding to 4-coumaroyltyramine (R_F = 0.82) were scraped off the plate and radioactivity was quantified by liquid scintillation counting.

A dot-blot assay was used to determine the level of NPT II activity (Radke et al., 1988). Leaf tissue (100 mg) was extracted in 100 μL of 50 mM sodium phosphate, pH 7.0, 14 mM β-mercaptoethanol, 10 mM EDTA, 0.1% Sarcosyl, and 0.1% Triton X-100. The soluble protein extract was incubated in 15 mM Tris-maleate, pH 7.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, and 0.5 mM DTT with 10 μCi of [γ-^{32}P]ATP (specific activity, 3000 Ci mmol^{-1}) in the presence or absence of 0.1 mg mL^{-1} kanamycin at 37°C for 1 h. Radiolabeled products were immobilized on P-81 paper (Whatman, Maidstone, UK), which was then washed at 65°C for 1 h in 10 mM sodium phosphate, pH 7.0, containing 1.0% SDS. The P-81 paper was dried and autoradiographed for 24 h. The total protein concentration of plant extracts was determined by the method of Bradford (1976).

Extraction and Analysis of Amino Acids

Leaves (1 g) were freeze-dried and ground in 100% methanol (10:1 [v/w]). The homogenate was incubated at 60°C for 30 min and then centrifuged for 15 min at 12,000*g*. The supernatant was collected, and the pellet was extracted once more with 50% methanol. The combined extracts were reduced 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 mL^{-1}) (Varian, Sugarland, TX) and incubated at room temperature for 10 min to generate fluorescent amino acid derivatives. After extraction of the free fluorescent dye in 70 μL of pentane ethyl acetate (80:20), 20 μL of the aqueous phase containing the amino acid derivatives was subjected to HPLC (Amino Tag column and Fluorichrome detector, Varian). Each amino acid was quantified as a percentage of total amino acids.

Alkaline Hydrolysis and Analysis of Cell Walls

Leaves (1 g) were ground in 100% methanol (1:1 [w/v]). The homogenate was incubated at 60°C for 30 min and then centrifuged for 15 min at 12,000*g*. The pellet was extracted two more times with 50% methanol, ensuring that no soluble aromatic compounds were present. The pellet was then hydrolyzed in 1.0 M NaOH for 4 h at 37°C. Insoluble debris were removed by centrifugation. The supernatant was acidified (pH 2.0) with 6.0 M HCl and extracted three times with equal volumes of ethyl acetate. The pooled ethyl acetate fractions were reduced to dryness and the residue was recovered in methanol. Extracted samples were applied to a silica gel 60 F_{254} TLC plate, which was developed in chloroform:methanol (5:4). Authentic standards displayed the following R_F values: tyramine, 0.23; and 4-coumaroyltyramine, 0.82.

Tyramine in hydrolyzed cell walls extracts was quantified by HPLC on a liquid chromatography system (BioSys 500, Beckman) and a photodiode array detector (System Gold 168, Beckman) using a C_{18} reverse phase column (4.6 × 250 mm; Ultrasphere, Beckman) at 1200 psi with an isocratic gradient of methanol:water (8:2) containing 0.1% triethylamine and a flow rate of 0.5 mL min^{-1}. The tyramine peak was identified from its UV spectrum and by comparison of its retention time with that of an authentic standard.

Cell Wall Digestibility Measurement

Cell wall digestibility was determined by measuring the number of protoplasts released after digestion of leaf tissue with hydrolytic enzymes (Brisson et al., 1994; Yao et al., 1995). Leaf sections (5 mm) were placed in a Petri dish containing 10 mL of plasmolysis solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, and 500 mM mannitol). After 2 h of incubation, the plasmolysis solution was replaced with enzymatic solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, 500 mM mannitol, 25 mg mL^{-1} cellulase, and 3 mg mL^{-1} pectinase). After 3 h of incubation, the enzyme solution

was removed, and 10 mL of high-density solution (50 mM Hepes, pH 5.5, 50 mM $CaCl_2$, and 500 mM Suc) was added to allow the protoplasts to float. The high-density solution was centrifuged at 3000*g* for 5 min, and the top 9 mL was removed. Protoplasts in the remaining 1 mL were counted in a hemocytometer.

Histochemical Analysis of Cell Walls

Cross-sections of leaves (approximately 500 μm thick) were prepared by hand-sectioning, and aromatic compounds were monitored by UV autofluorescence using a microscope (Aristoplan, Wild-Leitz, Wetzlar, Germany).

RESULTS

TYDC and THT Activities in Wild-Type Canola

The basal levels of TYDC and THT activity in various organs of wild-type canola were determined. The highest levels of TYDC activity were detected in roots (Fig. 2A). TYDC activity in young leaves was approximately 17% of that in roots but was considerably lower in mature leaves, stems, and flower buds. THT activity was abundant in roots but was highest in mature leaves (Fig. 2B). Substantial THT activity was detected in flower buds but was found at lower levels in stems and young leaves.

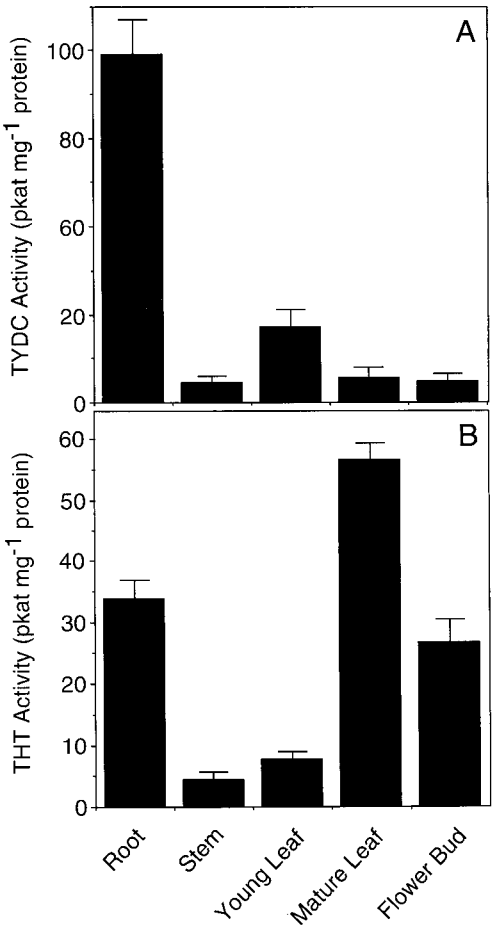


Figure 2. TYDC and THT activities in wild-type canola. Bars represent the means ± SE of three experiments.

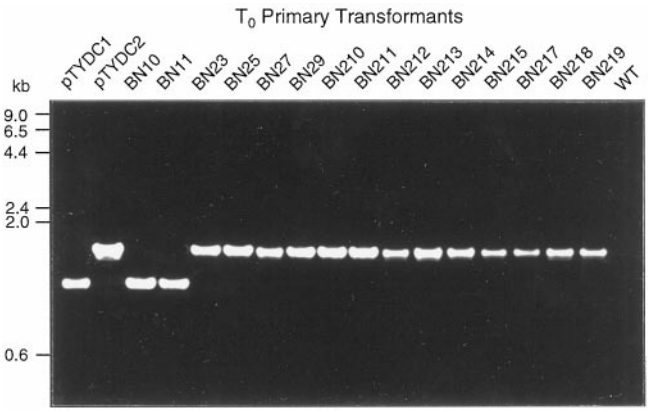


Figure 3. PCR assay demonstrating the integration of chimeric opium poppy *TYDC1* and *TYDC2* transgenes into the canola genome. Genomic DNA from two canola lines transformed with *35S::TYDC1* (BN10 and BN11) and 13 canola lines transformed with *35S::TYDC2* (BN23 through BN219) were used as templates for PCR with either *TYDC1*- or *TYDC2*-specific primers. Cloned opium poppy TYDC1 (pTYDC1) and TYDC2 (pTYDC2) cDNAs were used as positive control templates, whereas extracted wild-type canola genomic DNA (WT) was used as a negative control template.

Transformation of Canola with Chimeric *TYDC* Genes

Approximately 2000 canola cotyledonary petioles were treated with *A. tumefaciens* harboring either pBI35S::TYDC1 or pBI35S::TYDC2. Two putative *35S::TYDC1* (BN10 and BN11) and 13 putative *35S::TYDC2* (BN23 through BN219) transgenic plants were regenerated under kanamycin selection (Fig. 3). Regeneration efficiencies were 0.2% for *35S::TYDC1* and 1.3% for *35S::TYDC2*. As a control, canola cotyledonary petioles were treated with *A. tumefaciens* harboring pBI122 and a regeneration efficiency of 18% was obtained. In comparison, the regeneration efficiency for canola cotyledonary petioles treated with *A. tumefaciens* harboring pCGN 783 was reported at 83%, and the transformation efficiency was estimated at 55% (Moloney et al., 1989). Furthermore, 85 putative transgenic canola plants were regenerated after treatment of stem segments with *A. tumefaciens* harboring a chimeric *TDC* gene in pBI 121, of which 11 were confirmed as transgenic (Chavadej et al., 1994).

Regenerated kanamycin-resistant plants were allowed to flower and set seed. Each T_0 plant was tested for the presence of the chimeric *TYDC1* or *TYDC2* transgenes. PCR results suggested that all regenerated plants were transgenic (Fig. 3). No PCR products were amplified with either *TYDC1*- or *TYDC2*-specific primers using wild-type genomic DNA as a template. Further evidence for the transformation of regenerated plants was obtained by direct assay for NPT II activity. Plants transformed with *NOS::NPT II* are resistant to kanamycin because NPT II phosphorylates and, consequently, detoxifies the antibiotic. The dot-blot assay shown in Figure 4 illustrates the relative levels of NPT II activity in regenerated T_0 plants.

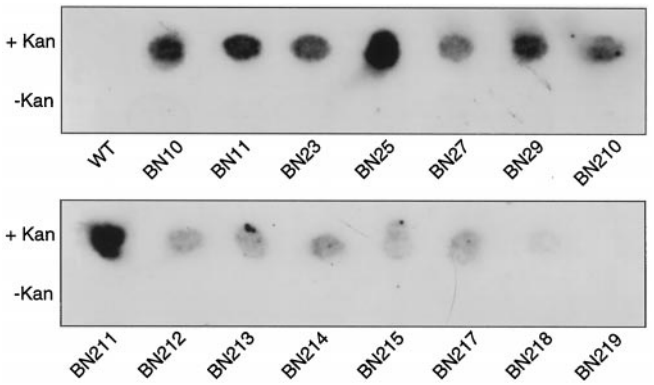


Figure 4. NPT II dot-blot assay of leaf extracts from putative T_0 transgenic canola plants. Labeling reactions were performed with [γ-^{32}P]ATP and plant extracts in the presence (+Kan) or absence (−Kan) of kanamycin. The negative control was wild-type (WT) canola. BN10 and BN11 were transformed with *35S::TYDC1*, whereas all other plants were transformed with *35S::TYDC2*.

No radioactivity was immobilized on the P-81 paper in the absence of kanamycin. NPT II activity was not detected in wild-type plants but was detected in 14 putative transformants (Fig. 4). NPT II activity in T_0 plants confirms that transgenes were inserted into transcriptionally active genomic regions.

TYDC Activity in Consecutive Generations of Transgenic Canola

TYDC activity levels in young leaves of T_0 plants are shown in Figure 5. Despite detectable NPT II activity in all but one primary transformant, most T_0 plants showed suppressed levels of wild-type TYDC activity. In BN10 and BN213, TYDC activity was similar to that in wild-type plants, but in BN11, BN212, and BN214, it was less than 20% of the wild-type level. The mean TYDC activity of all primary transformants was approximately 50% of that in wild-type plants. TYDC1 and TYDC2 mRNAs were not detected in T_0 plants (data not shown), suggesting that a *trans*-silencing mechanism might be responsible for the suppressed *35S::TYDC* expression and endogenous TYDC activity (Matzke and Matzke, 1995). Although a canola TYDC cDNA was not available to measure endogenous TYDC mRNA levels, the apparent homology among known TYDC genes across species is sufficiently low (Facchini and De Luca, 1994) to ensure that opium poppy

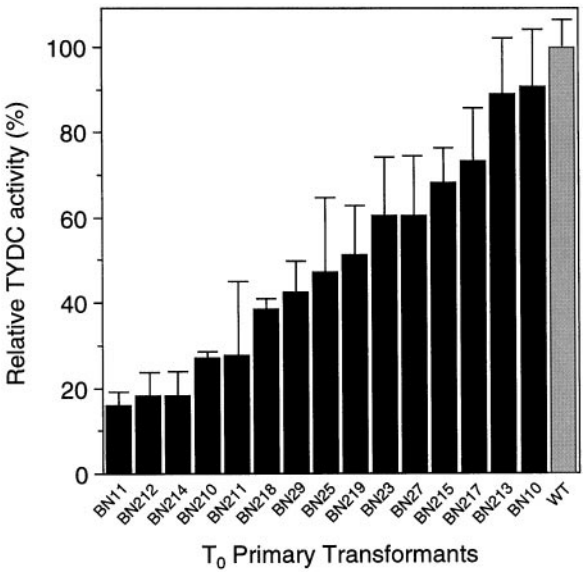


Figure 5. Relative TYDC activity in young leaves of T_0 canola transformed with *35S::TYDC1* (BN10 and BN11) and *35S::TYDC2* (BN23 through BN219). The relative TYDC activity in wild-type (WT) leaves is shown for comparison. Bars represent the means ± SE of three experiments. TYDC specific activity in wild-type leaves was approximately 20 pkat mg^{-1} protein.

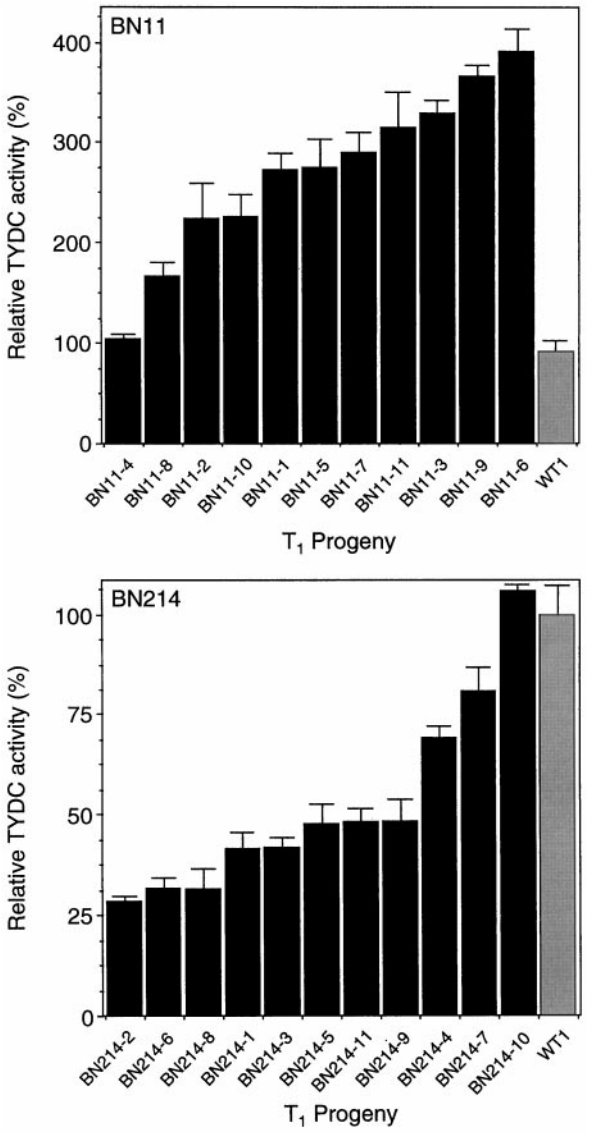


Figure 6. Relative TYDC activity in young leaves of the T_1 progeny of BN11 (*35S::TYDC1*) and BN214 (*35S::TYDC1*) canola lines. The relative TYDC activity in wild-type (WT) leaves is shown for comparison. Bars represent the means ± SE of three experiments. TYDC specific activity in wild-type leaves was approximately 20 pkat mg^{-1} protein.

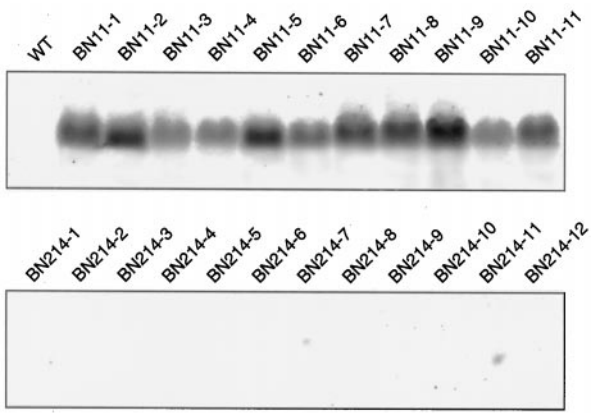


Figure 7. Gel-blot analysis of RNA from young leaves of the T_1 progeny of BN11 (*35S::TYDC1*) and BN214 (*35S::TYDC1*) canola lines. RNA extracted from young leaves of wild-type (WT) canola plants was used as a control. Total RNA was extracted and 15 μg was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with ^{32}P-labeled *TYDC1*- or *TYDC2*-specific probes. Gels were stained with ethidium bromide before blotting to ensure equal loading.

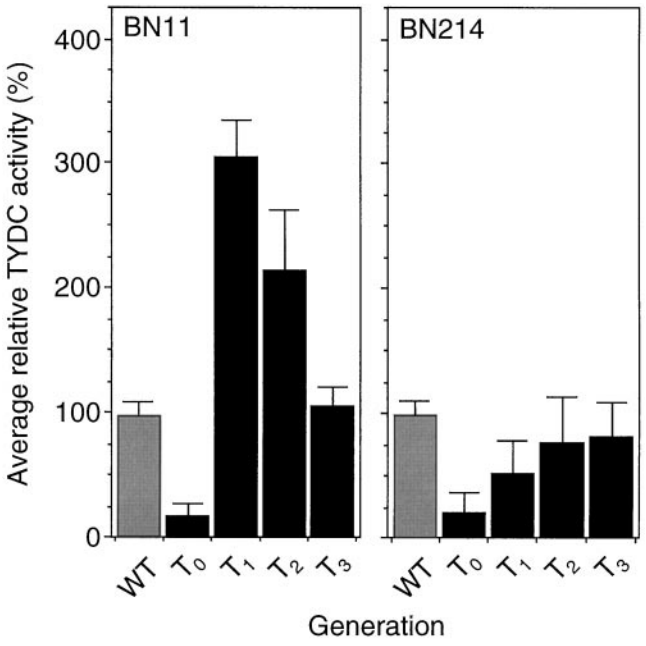


Figure 8. Relative mean TYDC activity in young leaves of successive self-pollinated generations (T_1, T_2, and T_3 plants) derived from BN11 (*35S::TYDC1*) and BN214 (*35S::TYDC1*) canola lines. The relative TYDC activity in wild-type (WT) canola leaves is shown for comparison. Bars represent the means ± SE. TYDC specific activity in wild-type leaves was equal to approximately 20 pkat mg^{-1} protein.

TYDC probes did not hybridize with canola TYDC mRNAs under high-stringency conditions. Although no definite conclusions about the silencing of endogenous canola TYDC genes can be drawn, our data clearly show the specific silencing of *35S::TYDC* transgenes.

Two transgenic canola lines were selected to analyze the inheritance of TYDC suppression. BN11 (*35S::TYDC1*) and BN214 (*35S::TYDC2*) showed the most severely suppressed TYDC activity among T_0 plants (Fig. 5) but exhibited high levels of NPT II activity (Fig. 4). All BN11 and BN214 T_1 plants tested positive for the presence of *TYDC* transgenes by PCR analysis. This departure from the expected segregation ratio for a hemizygous single-copy gene suggests that *TYDC* transgenes were present in multiple copies in T_0 plants. TYDC activity in the T_1 progeny of BN214 was suppressed relative to wild-type plants (Fig. 6) and was within a range similar to that displayed by T_0 plants transformed with *35S::TYDC2* (Fig. 5). Among 11 tested BN214 T_1 plants, the mean TYDC activity was approximately 55% of the wild-type level. In contrast, TYDC activity increased in BN11 T_1 progeny relative to the wild-type level (Fig. 6). The mean TYDC activity among BN11 T_1 plants was 3-fold higher than the wild-type level and was 15-fold higher than the BN11 T_0 parent.

TYDC1 mRNAs were detected in young leaves of all T_1 progeny of BN11 (Fig. 7). In contrast, TYDC2 mRNAs were not detected in any BN214 T_1 progeny, and homologous TYDC mRNAs were not detected in wild-type leaves (Fig. 7). These data show that silencing of *35S::TYDC1* was overcome in BN11 T_1 plants, but *35S::TYDC2* silencing was maintained in BN214 T_1 plants. The T_1 progeny of two other T_0 plants, BN10 (*35S::TYDC1*) and BN212 (*35S::TYDC2*), were tested to verify the reproducibility of transgene inheritance and expression. TYDC1 mRNAs were detected in all BN10 T_1 progeny, which also exhibited higher levels of TYDC activity compared with wild-type plants (data not shown). In contrast, all BN212 T_1 progeny showed suppressed levels of TYDC activity compared with wild-type plants, and TYDC1 mRNAs were not detected (data not shown). Thus, the reversion of transgene suppression from the T_0 to T_1 generations in plants transformed with *35S::TYDC1* (BN10 and BN11), and the continued transgene silencing in T_1 plants transformed with *35S::TYDC2* (BN212 and BN214), occurred in independent transgenic lines.

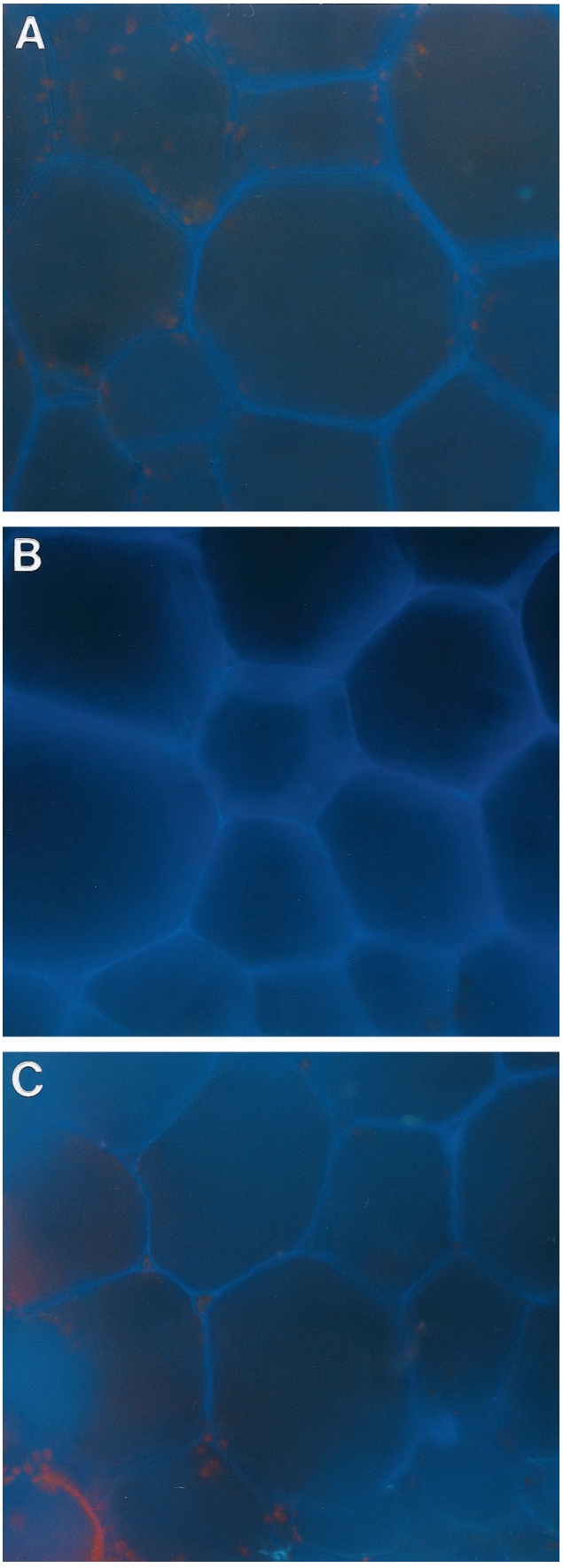
TYDC activity did not increase above wild-type levels through four successive generations of the BN214 line (Fig. 8). Mean TYDC activity among BN214 progeny increased from approximately 50% of wild-type levels in T_1 plants to near-wild-type levels in T_3 plants. TYDC mRNAs were not detected in the T_2 or T_3 progeny of BN214 (data not

Table I. *Analysis of aromatic amino acids in young leaves and cell wall-bound tyramine levels in mature leaves from wild-type and transgenic canola plants*

Values represent the means ± SE of four independent measurements.

Plant	Amino Acid		Tyramine
	Tyr	Phe	
	% total		*μg g^{-1} fresh wt*
Wild type	18.3 ± 5.6	2.0 ± 0.6	7.2 ± 4.5
BN11 (*35S:: TYDC1*)[a]	12.0 ± 3.4	2.7 ± 0.8	14.7 ± 3.6
BN214 (*35S:: TYDC2*)[b]	24.9 ± 5.8	1.8 ± 0.4	5.9 ± 4.4

[a] Plants tested were T_1 progeny that showed the highest TYDC activity (BN11-6 and BN11-9). [b] Plants tested were T_1 progeny that showed the lowest TYDC activity (BN214-2 and BN214-6).



shown). Mean TYDC activity among BN11 progeny decreased from maximum levels found in T_1 plants to nearly wild-type levels in T_3 plants (Fig. 8). TYDC mRNA levels also decreased in T_2 plants to less than 20% of those found in T_1 plants and, ultimately, to undetectable levels in T_3 plants (data not shown). All T_2 and T_3 plants derived from BN11 and BN214 lines tested positive for the presence of *TYDC* transgenes by PCR analysis. The failure to recover the parental genotype through three generations is consistent with the suggested multiple insertion of *TYDC* transgenes in T_0 plants.

Aromatic Amino Acid and Cell Wall-Bound Amine Analysis

In two T_1 plants, BN11-6 and BN11-9 (*35S::TYDC1*), TYDC activity in young leaves was 3- to 4-fold higher than wild-type levels (Fig. 6). In two other T_1 plants, BN214-2 and BN214-6 (*35S::TYDC2*), TYDC activity in young leaves was only 30% of wild-type levels (Fig. 6). Internal cellular pools of amino acids were measured in young leaves of these T_1 plants and compared with wild-type plants. Although the concentration of most amino acids did not vary (data not shown), Tyr pools were somewhat lower and higher in the tested T_1 progeny of BN11 and BN214, respectively, relative to wild-type controls (Table I). In contrast, Phe pools were not significantly different in the tested T_1 progeny of BN11 and BN214 compared with wild-type plants (Table I). Differences in TYDC activity and amino acid levels between wild-type and transgenic plants were much less apparent in mature leaves.

Neither tyramine nor hydroxycinnamic acid amides of tyramine were detected in methanol extracts from young or mature leaves, stems, or roots of wild-type or transgenic plants. However, cell wall-bound tyramine levels increased 2-fold in mature BN11-6 and BN11-9 leaves compared with wild-type plants (Table I). In contrast, cell wall-bound tyramine extracted from mature BN214-2 and BN214-6 leaves was similar to wild-type levels (Table I). No difference in cell wall-bound tyramine was detected in young leaves from wild-type and transgenic plants.

The difference in cell wall-bound tyramine levels between some transgenic and wild-type plants prompted a microscopic examination of UV autofluorescence in corresponding leaf sections. This method has been used for the detection of cell wall-bound phenolic amides (Clarke, 1982), ferulic acid (Nicholson, 1992; Kato et al., 1994), and lignin and suberin (Monties, 1989; Schmutz et al., 1993). As shown in Figure 9, the autofluorescence intensity of mesophyll cells from mature BN11-9 leaves (Fig. 9B) was stronger than that from mature, wild-type leaves (Fig. 9A). In contrast, the autofluorescence intensity of mesophyll cells from mature BN214-6 leaves (Fig. 9C) was weaker than that from mature wild-type leaves (Fig. 9A). Relative autofluorescence intensity was consistent in plants that showed

Figure 9. UV autofluorescence microscopy of leaf mesophyll cross-sections from wild-type (A) and transgenic (B and C) canola plants. B, BN11-9 leaf; C, BN214-6 leaf. Tissues were photographed immediately after hand sectioning. Magnification, ×150.

Table II. *Release of protoplasts from wild-type and transgenic mature canola leaves*

Values represent the means ± SE from three independent experiments.

Plant	Protoplasts Released	% Wild Type
	$\times 10^5$ g^{-1} *fresh wt*	
Wild type	10.3 ± 1.4	—
BN11 (*35S::TYDC1*)[a]	4.3 ± 0.5	42
BN214 (*35S::TYDC2*)[b]	19.3 ± 2.3	187

[a] Plants tested were T_1 progeny that showed the highest TYDC activity (BN11-6 and BN11-9). [b] Plants tested were T_1 progeny that showed the lowest TYDC activity (BN214-2 and BN214-6).

similar levels of TYDC activity. No difference in autofluorescence intensity was detected in young leaves from wild-type or transgenic plants.

Cell Wall Digestibility

Oxidative crosslinking of hydroxycinnamic acid amides of tyramine in the cell wall has been suggested to increase cell wall strength and provide a barrier against microbial penetration (Clarke, 1982; Negrel et al., 1993a). The cell wall digestibility of leaves from wild-type and transgenic plants was tested by measuring the number of protoplasts released after incubation with cellulase and pectinase (Brisson et al., 1994; Yao et al., 1995). This approach assumes that the efficiency of protoplast release is directly proportional to cell wall digestibility. No significant difference in protoplast release efficiency was found in young leaves from wild-type or transgenic plants (data not shown). However, BN11-6 and BN11-9 leaf sections released an average of only 42% of the protoplasts released by wild-type leaf sections (Table II). In contrast, BN214-2 and BN214-6 leaf sections released an average of 87% more protoplasts than wild-type canola leaf sections (Table II).

Data presented in Table II suggest a correlation between TYDC activity and cell wall digestibility in select T_1 (i.e. those with the highest and lowest levels of TYDC activity) and wild-type plants. To further test this relationship, protoplast release efficiency was directly compared with relative TYDC activity in mature leaves from randomly selected T_2 and T_3 progeny of the BN11 and BN214 transgenic lines (Fig. 10). The mean TYDC activity approached wild-type levels in successive progeny of both lines (Fig. 8), but individual T_2 and T_3 plants displayed a wide range of relative TYDC activity. As shown in Figure 10, high levels of TYDC activity correlated with reduced protoplast release efficiency, whereas low levels of TYDC activity relative to wild-type plants resulted in an increased recovery of protoplasts.

DISCUSSION

Canola was transformed with chimeric transgenes under the transcriptional control of the CaMV 35*S* promoter and encoding two TYDC isoforms from opium poppy. The transgenic plants were used to test the hypothesis that higher levels of TYDC activity will promote the conversion of the cellular Tyr pool to tyramine, which in turn will increase cell wall-bound hydroxycinnamic acid amide formation. Elevated amide levels would be expected to decrease cell wall susceptibility to digestion by hydrolytic enzymes.

Increased TYDC activity in transgenic plants is potentially harmful because of the phytotoxicity of hydroxyphenylethylamines such as tyramine (Negrel et al., 1993b). Detoxification of tyramine was reported in cultured tobacco cells only under conditions that favored THT induction. The exclusive recovery of T_0 plants with suppressed TYDC activity relative to wild-type plants (Fig. 5) suggests that expression of *35S::TYDC* transgenes inhibited the plant-regeneration process. Moreover, the low transformation efficiency is consistent with the suggestion that only T_0 plants that exhibited specific *trans*-silencing of *35S::TYDC1* and *35S::TYDC2*, but not *NOS::NPT II*, were recovered. In contrast, tobacco transformed with *35S::TDC* (Songstad et al., 1990) was reported to accumulate both soluble tryptamine and tyramine because of a proposed modification in the substrate specificity of Trp decarboxylase in tobacco (Songstad et al., 1991). Similarly, canola (Chavadej et al., 1994) and potato (Yao et al., 1995) transformed with *35S::TDC* accumulated high levels of tryptamine without any apparent adverse effects; therefore, we cannot rule out the possibility that the transformation efficiency was low for unknown reasons.

In the BN10 and BN11 lines, *35S::TYDC1* was active in T_1 plants, whereas *35S::TYDC2* remained silent in all transgenic progeny of the BN212 and BN214 lines. The consistent results obtained for independent lines transformed with each construct suggests that transgene expression was related to the specific nucleotide sequences of *TYDC1* and *TYDC2*. Expression of *35S::TYDC* transgenes might have

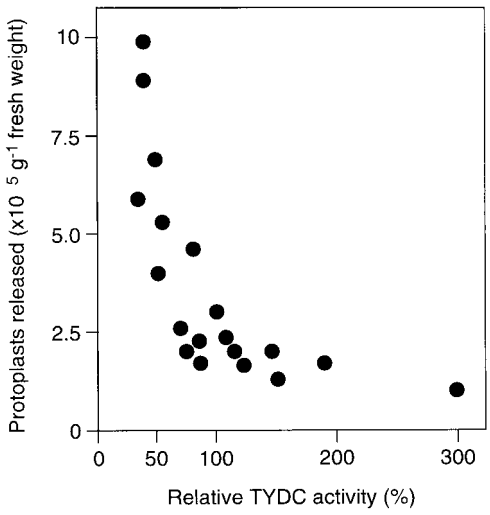


Figure 10. Comparison of relative TYDC activity with protoplast release efficiency in mature leaves of individual T_2 and T_3 progeny of BN11 (*35S::TYDC1*) and BN214 (*35S::TYDC2*) transgenic canola lines. TYDC activity and protoplast release efficiency were measured in the same leaf. Wild-type TYDC activity (100%) was equal to approximately 10 pkat mg^{-1} protein.

been affected by homologous *TYDC* genes in canola (Matzke and Matzke, 1995). *TYDC* genes from opium poppy (Facchini and De Luca, 1994) and parsley (Kawalleck et al., 1993) share >60% nucleotide identity. Similar homology between *TYDC* genes from opium poppy and canola is probable. Reactivation of a silenced transgene after passage through successive generations has been reported previously. For example, transformation of tobacco with the bean *PAL2* gene resulted in transgenic plants with severely reduced PAL activity (Elkind et al., 1990). The sense suppression of PAL in T_0 plants was progressively overcome in pedigrees of homozygous progeny (Bate et al., 1994). A β-1,3-glucanase transgene (*gn1*) in tobacco driven by the CaMV 35*S* promoter was silent during seed germination and vegetative growth but was activated in meiotically derived seed tissues (Castresana and Balandin, 1997). Expression of *gn1* was not reactivated in plantlets regenerated mitotically from leaf explants of *gn1*-suppressed plants.

Reactivation of *35S::TYDC1* in canola might also require passage through meiosis. The expression of *NOS::NPT II* in T_0 plants shows that T-DNA insertions occurred in transcriptionally active genomic regions (Fig. 4). NPT II activity was detected in consecutive generations of all transgenic lines except BN219 (data not shown); thus, the silencing of *35S::TYDC1* in T_0 plants, its reactivation in T_1 plants, and its subsequent and gradual re-suppression in T_2 and T_3 plants are related to the *TYDC* transgene. Canola might be prone to silencing aromatic amino acid decarboxylase transgenes. Expression of a *35S::TDC* transgene in canola, which was not initially silenced in T_0 plants (Chavadej et al., 1994), also became completely suppressed over four successive generations (V. De Luca, personal communication). Such meiotically heritable alterations in transgene activity have been demonstrated for *trans*-silencing mechanisms mediated by reading frame (Matzke and Matzke, 1995) and promoter homology (Park et al., 1996). TYDC1 mRNA levels in BN11 T_1 progeny did not always translate into a proportional increase in TYDC activity (Figs. 6 and 7), suggesting that posttranslational regulation of heterologous TYDC activity might have also occurred in plants that expressed *35S::TYDC1*.

The increase in TYDC activity in young leaves of BN11-6 and BN11-9 T_1 plants resulted in decreased cellular Tyr pools and increased cell wall-bound tyramine in corresponding mature leaves compared with wild-type plants (Table I). In contrast, the increased Tyr pools in young leaves of BN214-2 and BN214-6 plants are consistent with the reduced TYDC activity relative to wild-type levels. TYDC activity levels were not much different in mature leaves of transgenic plants, so little difference was found between the cellular Tyr pools of mature leaves. In contrast, increased cell wall-bound tyramine levels were only detected in mature leaves that also showed high THT activity. A temporal discrepancy between the optimum expression of *TYDC* transgenes and the maximum insolubilization of tyramine was apparent; therefore, TYDC activity was altered most abundantly in young leaves, but cell wall modifications (i.e. tyramine levels and digestibility) were not detectable until leaves matured. This discrepancy might be related to the transient nature of heterologous TYDC activity in canola and the low endogenous THT activity in young leaves.

Our data provide in vivo evidence that the formation of tyramine represents a sink for Tyr in canola and that TYDC activity appears to determine, at least in part, the level of cell wall-bound tyramine in mature leaves. This conclusion is supported by the kinetic properties of THT from various species. THT follows Michalis-Menton kinetics in the presence of low concentrations of hydroxycinnamoyl-CoA derivatives but exhibits negative cooperativity at concentrations above 2 to 3 μM, resulting in a decrease in the affinity for tyramine (Hohlfeld et al., 1995; Negrel and Javelle, 1997; Yu and Facchini, 1999). This negative cooperativity has been proposed as a physiological mechanism involved in the regulation of amide biosynthesis (Negrel and Javelle, 1997). The cellular concentrations of hydroxycinnamoyl-CoA derivatives are probably much lower than the level of tyramine (Hahlbrock and Scheel, 1989). Negative cooperativity implies that an increase in the cellular tyramine pool will lead to an increase in amide formation, even at constant levels of hydroxycinnamoyl-CoA derivatives (Negrel and Javelle, 1998). An increase in the cellular pool of hydroxycinnamoyl-CoA esters would result in increased amide formation only if tyramine levels also increase; thus, the level of TYDC activity should play a role in the regulation of cell wall-bound amide biosynthesis.

Tyramine released by alkaline hydrolysis was probably insolubilized in cell walls as hydroxycinnamic acid amides. The harsh treatment required to extract tyramine would also be expected to hydrolyze amide bonds. In addition, most amides are highly cross-linked in the cell wall, preventing their extraction by alkaline hydrolysis. Numerous bond types between cell wall components and tyramine derivatives have been demonstrated (Borg-Olivier and Monties, 1993).

UV autofluorescence examination of leaf sections from transgenic canola confirmed that cell wall modification had occurred (Fig. 9). The stronger autofluorescence of mature mesophyll cell walls from BN11-6 and BN11-9 T_1 plants relative to wild-type leaves suggests an increased content of aromatic residues. In contrast, the weaker autofluorescence of mature mesophyll cell walls from BN214-2 and BN214-6 T_1 plants suggests a reduced incorporation of aromatic compounds. These data are consistent with the relative levels of tyramine solubilized from cell walls of wild-type and transgenic plants (Table I). However, the alkaline-hydrolyzed tyramine might not reflect the entire hydroxycinnamic acid amide content of the cell walls; thus, UV autofluorescence intensity might not always have been proportional to extracted tyramine levels.

A change in the availability of tyramine could be expected to result in an alteration of cell wall strength, because tyramine-derived amides are purported to become oxidatively cross-linked, together with other phenolics, in the cell wall (Clarke, 1982; Negrel and Lherminier, 1987; Matern and Kneusel, 1988; Iiyama et al., 1994). An inverse relationship between the level of TYDC activity and the susceptibility of cell walls to enzymatic hydrolysis was revealed by measuring protoplast release efficiency (Fig.

10). Protoplast recovery from mature leaf sections was reduced by 60% in BN11-6 and BN11-9 T_1 plants, with a mean TYDC activity that was 3- to 4-fold higher than that in wild-type plants (Table II). In contrast, the 50% reduction in mean TYDC activity in BN214-2 and BN214-6 T_1 plants corresponded to an increase in the cell wall digestibility of mature leaves. The similar protoplast release efficiency of young leaf tissue from wild-type and transgenic plants was consistent with our inability to detect differences in cell wall-bound tyramine levels in young leaves with altered TYDC activity.

We have shown that *35S::TYDC* transgenes in canola are subject to transcriptional silencing mechanisms. In transgenic plants with elevated TYDC activity, internal Tyr pools decreased and cell wall-bound tyramine levels increased compared with wild-type plants. Increased TYDC activity also correlated with decreased enzymatic digestibility of cell walls. Overall, our data suggest that *TYDC* and tyramine affect cell wall properties that might have implications in plant-pathogen interactions.

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