Plant Physiol. (1994) 106: 485–491

Cloning and Regulation of Flavonol 3-Sulfotransferase in Cell-Suspension Cultures of *Flaveria bidentis*[1]

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Flaveria spp. **accumulate flavonol sulfate esters whose biosynthesis is catalyzed by a number of position-specific flavonol sulfotransferases. Although the accumulation of sulfated flavonols appears to be tissue specific and developmentally regulated and to vary among related species, little is known about the mechanism of regulation controlling the synthesis of these metabolites. In the present work, we report the isolation of a cDNA clone from *Flaveria bidentis* (pBFST3) encoding flavonol 3-sulfotransferase (F3-ST), which catalyzes the first step in the biosynthesis of flavonol polysulfates. This clone (pBFST3) was expressed in *Escherichia coli* and produced an F3-ST with high affinity for the flavonol aglycones, quercetin, and its 7-methyl derivative, rhamnetin. In addition, the synthetic auxin 2,4-dichlorophenoxyacetic acid was shown to induce F3-ST enzyme activity and F3-ST mRNA transcript levels in cell cultures of *F. bidentis*. The F3-ST mRNA levels increased within the first 3 h, reaching a maximum after 24 h of treatment, and remained elevated for up to 48 h. Treatments with either quercetin 3-sulfate or quercetin 3,7,4′-trisulfate reduced F3-ST enzyme activity in cell cultures but had no effect on the transcript levels. These results are discussed in relation to the putative role of flavonoid conjugates in the regulation of auxin transport.**

Flavonoid sulfates are common in plants (Harborne, 1975), especially in the Asteraceae. The most commonly found compounds are mono- and disulfate esters of flavones and flavonols or their methyl ethers and, less commonly, of their glycosylated derivatives. Tri- and tetrasulfated esters have also been identified, although these compounds have a more limited taxonomic distribution (Barron et al., 1988). Recently, a number of position-specific flavonol STs have been characterized from *Flaveria chloraefolia* (Varin and Ibrahim, 1989) and *Flaveria bidentis* (Varin and Ibrahim, 1991). Different enzymes exhibited strict specificity for position 3 of flavonol aglycones (F3-ST), position 3′ or 4′ of flavonol 3,3′ or 3,4′-disulfates (F3′ or F4′-ST), and position 7 of flavonol 3,3′- or 3,4′-disulfates, thus establishing an enzymatic sequence for the formation of flavonol polysulfates (Varin, 1992).

F. bidentis (Asteraceae) accumulates flavonol mono- to tetrasulfate esters (Barron et al., 1986) and exhibits several ST activities (Varin et al., 1987a). F3-ST activity of *F. bidentis* has recently been shown to be regulated with respect to plant development, being highest in the shoot tips and the first pair of expanded leaves and lowest in mature leaves (Hannoufa et al., 1991). In contrast, cell-suspension cultures of *F. bidentis* exhibit very low constitutive levels of F3-ST activity and do not accumulate any flavonol sulfate (Bleichert et al., 1989).

Studies by Jacobs and Rubery (1988) showed that exogenous treatments of etiolated *Curcubita pepo* hypocotyls with either quercetin or rhamnetin inhibit auxin transport in tissue sections. Transport is inhibited by decreased auxin efflux at the basal end of stem cells and is mediated by competitive binding of quercetin and rhamnetin to a membrane protein, the NPA receptor. It was later shown that sulfated esters of quercetin are antagonists of quercetin in affecting auxin transport in the microsomal preparations of *C. pepo* (Faulkner and Rubery, 1992). Quercetin 3-sulfate has also been shown to bind to the NPA receptor and to de-repress the auxin efflux inhibition caused by quercetin. Because flavonol 3-sulfate is a naturally occurring compound in *F. bidentis* and F3-ST is known to be spatially and developmentally regulated in this species (Hannoufa et al., 1991), the effects of the synthetic auxin 2,4-D on the regulation of F3-ST activity and gene expression in cell-suspension cultures of *F. bidentis* were investigated.

In this paper, we report the isolation of a cDNA clone encoding F3-ST in *F. bidentis*. This clone was used to demonstrate that steady-state mRNA levels of the gene encoding F3-ST are regulated by 2,4-D. Neither of the sulfate esters, quercetin 3-sulfate or quercetin 3,7,4′-trisulfate, had any effect on the steady-state mRNA levels of the F3-ST.

MATERIALS AND METHODS

Plant Material

Seeds of *Flaveria bidentis* var *angustifolia* O.K. (Asteraceae) were kindly provided by Dr. H.R. Juliani (University of Cordoba, Argentina). Seeds were germinated in vermiculite on top of potting soil, and plants were further propagated by cuttings.

A callus culture of *F. bidentis* was initiated from leaf disks

[1] This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada and the Department of Higher Education, Government of Québec. S.A. received a graduate fellowship from the Canadian International Development Agency.

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Abbreviations: F3-ST, flavonol 3-sulfotransferase; F3′-ST, position 3′ of flavonol 3-sulfate; F4′-ST, 4′ of flavonol 3-sulfate; NPA, naphthylphthalamic acid; PAL, phenylalanine ammonialyase; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; ST, sulfotransferase.

and maintained on a Murashige-Skoog semisolid medium containing 3% (w/v) Suc, 4.5 μM 2,4-D, and 0.45 μM kinetin (Bleichert et al., 1989). Calli were broken into small pieces and transferred to a liquid medium of the same composition. The suspension culture was maintained in the light at room temperature either in Erlenmeyer flasks agitated on an orbital shaker at 110 rpm or in 1-L nipple flasks rotating centripetally at 4 rpm. Nipple flasks are near spherical with 10 symmetrically spaced cylindrical protrusions, each approximately 2 cm long and wide. This type of flask increases the aeration of rotating cultures and, therefore, promotes culture growth (Steward et al., 1952).

Chemicals

2,4-D was purchased from Sigma. [^{35}S]PAPS (1.57 Ci/mmol) was purchased from New England Nuclear, and quercetin 3-sulfate came from Sarsynthèse (Merignac, France). Tetrabutylammonium dihydrogen phosphate was obtained from Aldrich Chemical Co. (Milwaukee, WI), and the immunodetection kit was purchased from Bio-Rad (Mississauga, ON). UniZap cDNA synthesis kit was obtained from Stratagene (La Jolla, CA) and Sequenase version 1 sequencing kit was from United States Biochemical (Cleveland, OH). All other chemicals were of analytical grade.

cDNA Cloning of F3-ST

Total RNA was isolated from the shoot tips of *F. bidentis* (Logemann et al., 1987), and poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). A cDNA library was constructed in λUniZap II according to the manufacturer's instructions (Stratagene). Approximately 6.0×10^5 plaques were screened using polyclonal antibodies that were raised against the F3-ST of *Flaveria chloraefolia* (Varin and Ibrahim, 1992). The plasmid was rescued from positive clones by in vivo excision according to the manufacturer's instructions (Stratagene). Clones were further screened by western blot analysis of *Escherichia coli* lysates from positive clones and subsequently by screening for F3-ST activity as described below. A single clone, pBFST3, which expressed F3-ST activity, was chosen for DNA sequencing. A series of nested deletions of this clone was created using exonuclease III and mung bean nuclease (Sambrook et al., 1989). DNA sequences of both strands were determined by the dideoxy chain termination method (Sanger et al., 1977).

Treatment of Plant Cell Cultures

Cell-suspension cultures (10 d old) were treated with varying concentrations of 2,4-D or with the flavonols quercetin, quercetin 3-sulfate, or quercetin 3,7,4′-trisulfate under sterile conditions. Triplicate samples were harvested at the indicated intervals by filtration through Whatman No. 1 filter paper and rinsed extensively with Murashige-Skoog medium to remove the residual 2,4-D and flavonols. The harvested cells were weighed, frozen in liquid nitrogen, and then stored at −70°C.

To determine the induction kinetics of F3-ST activity by 2,4-D, 10-d-old nipple-flask cultures were exposed to 25 μM 2,4-D, and 20-mL samples were taken at 3-h intervals and used for the determination of F3-ST activity. Additional experiments were conducted using actinomycin D, which was added to the Erlenmeyer flask cultures at 0.1 mM final concentration, 3 h prior to treatment with 2,4-D. Control experiments were performed in which 2,4-D was substituted by water. The effect of flavonols on F3-ST activity was determined by transferring 25 mL of 10-d-old cell-suspension cultures to 250 mL of Murashige-Skoog medium containing either quercetin, quercetin 3-sulfate, or quercetin 3,7,4′-trisulfate at a final concentration of 3 μM. Samples were taken daily and assayed for ST activity.

Preparation of Protein Extracts

E. coli Lysates

Overnight bacterial cultures (150 μL), grown in Luria broth culture medium with tetracycline (100 μg mL^{-1}) and ampicillin (50 μg mL^{-1}), were used to inoculate 3 mL of the same medium. Isopropyl β-D-thiogalactopyranoside was added after 1 h to a final concentration of 1 mM. After 2 h the culture was centrifuged (10,000*g*), and the cell pellets were resuspended in 1 mL of the enzyme assay buffer (50 mM Tris-HCl, pH 7.5, 14 mM 2-mercaptoethanol) and then lysed by sonication. The lysate was cleared by centrifugation (10,000*g*), and the supernatant was desalted by passage through a PD-10 column (Pharmacia). The desalted protein preparations were used as the enzyme source for ST assay.

Cell-Free Extracts from Cell Cultures

Frozen tissues were thawed and ground in 2 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol and Polyclar (Polyclar:cells, 1:10 [w/w]). The homogenate was sonicated and centrifuged, and the supernatant was desalted before being used as the enzyme source.

ST Assay

Flavonol ST activity was measured according to the method of Varin et al. (1987b). The reaction mixture contained 1 μM of the flavonol substrate, 1 μM [^{35}S]PAPS (0.1 μCi), and up to 60 μg of protein in a total volume of 100 μL. The sulfated reaction product was extracted in 0.1% (w/v) tetrabutylammonium dihydrogen phosphate-ethyl acetate and counted for radioactivity in a toluene-based scintillation fluid. It was identified by co-chromatography with reference compounds and autoradiography on x-ray film.

Protein Determination

Protein concentrations were determined according to the method of Bradford (1976) using the Bio-Rad reagent and BSA as the protein standard.

Northern Blot Analysis

Total RNA was isolated according to the method of Logemann et al. (1987) and quantitated by UV spectrophotometry. Equal amounts (5 or 10 μg) of total RNA were fractionated on a 1% agarose formaldehyde gel. The gel was then stained with ethidium bromide and blotted onto Hybond N membrane (Amersham, Arlington Heights, IL). Prehybrid-

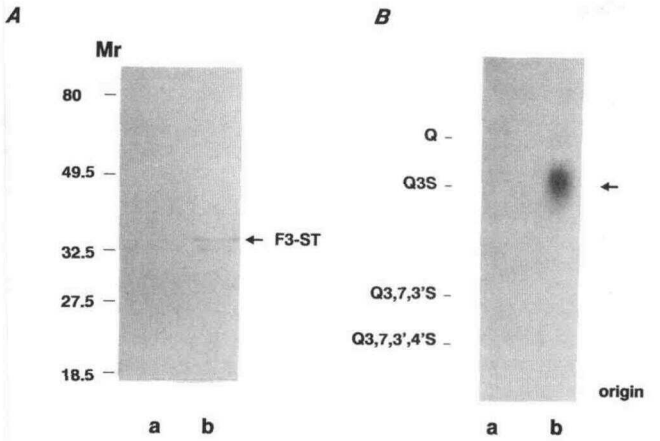

Figure 1. A, Western blot analysis of lysates from isopropyl β-D-thiogalactopyranoside-induced *E. coli*. Lane a, Control containing pBluescript SK⁻; lane b, pBFST3. Blot was probed with a polyclonal antibody raised against *F. chloraefolia* F3-ST. The arrow indicates an immunoreactive band corresponding to F3-ST. B, Autoradiogram of the chromatographed enzyme reaction products from the lysate of *E. coli* control cultures after incubation with quercetin as substrate and PAPS as co-substrate (lane a) and that expressing the cDNA clone pBFST3 (lane b). The relative migration of reference compounds are marked to the left: Q, quercetin; Q3S, quercetin 3-sulfate; Q3,7,3'S, quercetin 3,7,3'-trisulfate; Q3,7,3'4'S, quercetin 3,7,3',4'-tetrasulfate. Labeled spot in lane b co-migrates with quercetin 3-sulfate.

izations and hybridizations were performed as described by Gulick and Dvorak (1990). The F3-ST probe was synthesized with the random primer-labeling method of Amersham. The blots were then washed three times with a mixture containing 0.1× SSC, 0.1% SDS, and 0.1% sodium PPi for 20 min at 55°C. Equal sample loading was confirmed by probing blots with an rRNA control probe. The blots were then exposed to Kodak XAR film with an intensifying screen at −80°C.

Western Blot Analysis

E. coli Lysates

Protein extracts (20 μg) were fractionated by SDS-PAGE using 12% acrylamide gels. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes using a semidry electrotransfer apparatus according to the manufacturer's instructions (Bio-Rad). The blot was developed with anti-F3-ST immune serum (1:2000) and nonimmune serum (1:2000) as primary antibody and then with alkaline phosphatase-conjugated anti-rabbit IgG antibody as the secondary antibody.

Cell-Free Extracts from Cell Cultures

Samples were ground in liquid nitrogen to a fine powder and extracted with SDS sample buffer (1:2 [w/v]). About 20 μg of protein extracts were fractionated under the same conditions as described above. Equal sample loading was confirmed by Coomassie blue staining. The blot was developed with anti-F3-ST immune serum (1:500).

RESULTS

Characterization of F3-ST cDNA Clone

A clone, designated pBFST3, was identified by antibody screening of a *F. bidentis* cDNA library and subsequent western blot analysis of the *E. coli* lysate of a strain carrying the plasmid. The antibody reacted with a protein band from the lysate with an estimated molecular mass of 37 kD (Fig. 1A, lane b); this is about 5 kD smaller than the predicted molecular mass of the cloned gene product, which includes 59 amino acid residues encoded by the vector and the 5′ untranslated region of the cDNA. This suggests that translation of the gene product in the *E. coli* expression system may initiate from the ATG corresponding to the first amino acid of the native coding sequence.

The identity of the clone, as the F3-ST, was further confirmed by measuring the F3-ST activity in the *E. coli* lysate. The level of F3-ST activity in *E. coli* transformed with pBFST3 was between 20 and 60 pkat mg^{-1}. The F3-ST exhibited two pH optima, 6.5 in bis-Tris and 8.5 in phosphate buffers. The cloned F3-ST showed substrate preference for quercetin and its 7-methyl derivative, rhamnetin (Table I). The reaction product, when quercetin was used as substrate, co-chromatographed with authentic quercetin 3-sulfate (Fig. 1B, lane b). Control bacterial cultures, transformed with pBluescript SK⁻, had no detectable ST activity. The K_m values of the cloned F3-ST for the flavonol substrate quercetin and the sulfate donor PAPS were 0.3 and 0.4 μM, respectively. These K_m values are of the same order of magnitude as those previously reported for the *F. chloraefolia* F3-ST (Varin and Ibrahim, 1992).

Sequence Analysis of pBFST3

The cDNA insert of pBFST3 is 1211 bp, excluding the poly(A) tail. It has an open reading frame beginning at nucleotide 55 and terminating at nucleotide 992, which encodes a putative protein of 312 amino acids with a predicted molecular mass of 36 kD (Fig. 2).

The *F. bidentis* F3-ST (pBFST3) nucleotide sequence has 92.5% identity with the *F. chloraefolia* F3-ST clone (pFST3) and 73.4% identity with the *F. chloraefolia* F4′-ST clone (pFST4′) (Varin et al., 1992). The deduced amino acid sequence of pBFST3 shows 95.5 and 71.2% identity with those of pFST3 and pFST4′, respectively (Fig. 3).

F3-ST Activity during Growth of *F. bidentis* Cell Cultures

Growth of *F. bidentis* cell culture was monitored by determining the fresh weight and protein content during a period

Table I. *Substrate specificity of F3-ST cloned in E. coli*

Substrate	Relative Activity[a,b]
Quercetin	100
Rhamnetin	75
Isorhamnetin	10

[a] F3-ST activity with quercetin as substrate is 50 pkat mg^{-1}. [b] Naringenin, luteolin, kaempferol, tamarixetin, quercetin 3-sulfate, kaempferol 3-sulfate, and tamarixetin 3-sulfate did not act as sulfate acceptors.

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      TCTCGATCTATCACAAAAGTATTGAACTTCCTATTTGTATCATTTCGATCGATT
      ATG GAG GAT ATT ATC AAA ACA CTT CCA CAA CAC ACT TGT AGC
1     M  E  D  I  I  K  T  L  P  Q  H  T  C  S
      TTT TTG AAA CAA AGG TTC ACC TTG TAC AAG TAT CAA GAC GTG
15    F  L  K  Q  R  F  T  L  Y  K  Y  Q  D  V
      TGG AAT CAT CAA GAG TTT CTT GAA GGA CGA ATG TTA TCT GAA
29    W  N  H  Q  E  F  L  E  G  R  M  L  S  E
      CAA ACC TTC AAG GCA CAT CCC AAT GAT GTA TTT CTC GCT AGT
43    Q  T  F  K  A  H  P  N  D  V  F  L  A  S
      TAT CCC AAA AGT GGC ACG ACA TGG CTA AAA GCC TTG GCA TTT
57    Y  P  K  S  G  T  T  W  L  K  A  L  A  F
      GCC ATC ATA ACG CGA GAA AAG TTT GAT GAT TCC ACA AGT CCT
71    A  I  I  T  R  E  K  F  D  D  S  T  S  P
      TTG CTC ACA ACC ATG CCT CAT GAT TGC ATT CCT CTC CTA GAG
85    L  L  T  T  M  P  H  D  C  I  P  L  L  E
      AAA GAC CTT GAA AAA ATT CAA GAA AAC CAA AGG AAC TCA CTC
99    K  D  L  E  K  I  Q  E  N  Q  R  N  S  L
      TAC ACA CCC ATC TCG ACA CAC TTT CAT TAC AAA TCC CTA CCT
113   Y  T  P  I  S  T  H  F  H  Y  K  S  L  P
      GAG TCA GCC CGG ACA TCA AAC TGC AAG ATA GTT TAC ATA TAC
127   E  S  A  R  T  S  N  C  K  I  V  Y  I  Y
      CGG AAT ATG AAA GAT GTC ATT GTT TCT TAT TAC CAT TTC CTG
141   R  N  M  K  D  V  I  V  S  Y  Y  H  F  L
      AGA CAG ATA GTT AAA CTA TCT GTG GAA GAG GCC CCA TTT GAG
155   R  Q  I  V  K  L  S  V  E  E  A  P  F  E
      GAG GCA GTT GAT GAG TTT TGT CAA GGT ATT TCA AGT TGT GGA
169   E  A  V  D  E  F  C  Q  G  I  S  S  C  G
      CCA TAT TGG GAA CAC ATC TTG GGA TAC TGG AAA GCA AGC TTG
183   P  Y  W  E  H  I  L  G  Y  W  K  A  S  L
      GAG AAG CCA GAG ATA TTT CTT TTC TTG AAA TAC GAA GAC ATG
197   E  K  P  E  I  F  L  F  L  K  Y  E  D  M
      AAA AAA GAT CCG GTA CCA AGT GTG AAG AAA CTT GCA GAT TTC
211   K  K  D  P  V  P  S  V  K  K  L  A  D  F
      ATT GGG CAT CCC TTT ACA CCC AAA GAA GAG GAA GCG GGT GTG
225   I  G  H  P  F  T  P  K  E  E  E  A  G  V
      ATT GAA AAT ATT ATA AAG TTA TGT AGT TTT GAG AAA TTA AGC
239   I  E  N  I  I  K  L  C  S  F  E  K  L  S
      AGC TTA GAA GTA AAC AAA AGT GGA ATG CAT CGT CCT GAA GAA
253   S  L  E  V  N  K  S  G  M  H  R  P  E  E
      GCT CAT TCC ATT GAA AAC CGA CTT TAC TTT AGA AAG GGT AAA
267   A  H  S  I  E  N  R  L  Y  F  R  K  G  K
      GAT GGA GAT TGG AAG AAC TAC TTT ACT GAT GAG ATG ATT GAG
281   D  G  D  W  K  N  Y  F  T  D  E  M  I  E
      AAA ATA GAC AAA TTG ATC GAT GAA AAA TTA GGT GCC ACT GGT
295   K  I  D  K  L  I  D  E  K  L  G  A  T  G
      TTA GTT CTA AAA TAAAAGGCTCAAGATCATACTTTATTTGAGAGCAAAACA
309   L  V  L  K  •
ACTCGATTTCAAACTATCTTTCATTATTCTTGTTTTAAATAATTGAGGGCTAGGT
CTCTTTTCAATTGTTTTCTTCATTATGTAATTTAATTTGGCCTTATGTATTTTG
TTGTATGTCTTATTAATAATGATTATTTTCTTTCTTGGAAGATTTGTTAAACCAA
ATTGATAACTTTTGACCAAAAAAAAAAAAAAAAAAAAA
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Figure 2. The nucleotide and amino acid sequences of pBFST3.

of 14 d (Fig. 4). Cultures grown in nipple flasks showed a sigmoidal growth curve, and cells reached the stationary phase after 10 d. The activity of F3-ST activity reached a maximum 2 d after subculture and gradually decreased up to d 14.

Effects of 2,4-D on F3-ST Activity

Preliminary experiments to study the effect of 2,4-D on F3-ST activity were carried out using batch cultures in Erlen-

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pBFST3          MEDIIKTLPQHTCSFLKQRFTLYKYQDVWNHQEFLEGRML
pFST3           ----------H-------K-A----------I-
pFST4'    METTKTQFES-AEM--K-------S--G-I-------F-GL-NNI--AI-

pBFST3          SEQTFKAHPNDVFLASYPKSGTTWLKALAFAIITREKFDDSTSPLLTTMP
pFST3           ---K----------------------W*IC-------------------
pFST4'    AQ-S---R-D----C--------------Y--V------EF------NI-

pBFST3          HDCIPLLEKDLEKIQENQRNSLYTPISTHFPHYKSLPESARTSNCKIVYIY
pFST3           ----------------------------------------------------
pFST4'    -N---YI----K--V---N--CF--MA--MP-HV--K-ILAL---M----

pBFST3          RNMKDVIVSYYHFLRQIVKLSVEEAPFEEAVDEFCQGISSCGPYWEHILG
pFST3           ----------------------------F-------------------K-
pFST4'    --I------F---G-E-T--PL-D------F---YH---QF----D-L--

pBFST3          YWKASLEKPEIFLFLKYEDMKKDPVPSVKKLADFIGHPFTPKEEEAGVIE
pFST3           ----------------------------------------------------
pFST4'    -------R--VI-------V----TSN--R--E---Y---FE--KE----

pBFST3          NIIKLCSFEKLSSLEVNKSGMHRPEEAHSIENRLYFRKGKDGDWKNYFTD
pFST3           D-V-----------------------------------------------
pFST4'    S--------N--N-------NSK**GFLP---------A----------

pBFST3          EMIEKIDKLIDEKLGATGLVLK   312
pFST3           --TQ----------------    311
pFST4'    --T----------S-------   320
```

Figure 3. The amino acid sequence alignment of *F. bidentis* F3-ST (pBFST3), *F. chloraefolia* F3-ST (pFST3), and *F. chloraefolia* F4′-ST (pFST4′). The pBFST3 shows 95.5 and 71.2% identity with pFST3 and pFST4′, respectively. Dashes indicate identical amino acids and asterisks indicate gaps in the aligned sequence.

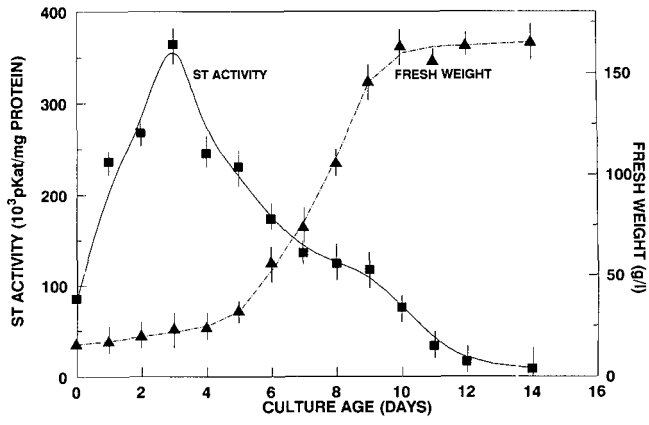

Figure 4. F3-ST activity in relation to age of *F. bidentis* cell cultures.

meyer flasks. The activity of F3-ST in the cell cultures increased with an increasing concentration of 2,4-D, reaching a maximum at 25 μM, and declined at higher auxin levels (Fig. 5A). Cell cultures grown in nipple flasks and treated with 25 μM 2,4-D also showed a rapid increase of F3-ST activity within the first 3 h and reached a maximum at 24 h, as compared with the control cultures (Fig. 5B).

To determine whether the increase of F3-ST activity in response to 2,4-D was a consequence of an increased level of gene expression, two approaches were used. The first approach was to examine the level of F3-ST mRNA transcripts in response to exposure to 2,4-D. Northern blot analysis was performed using pBFST3 as a probe. A transcript of 1300 bp was detected by this probe; its level increased within 3 h of exposure of the cultured cells to 2,4-D, reached a maximum of about 10-fold increase 24 h after treatment, and then showed a subsequent slower decrease (Fig. 5C).

In another experiment, batch cultures (grown in 250-mL Erlenmeyer flasks) were pretreated with the RNA polymerase inhibitor actinomycin D (0.1 mM) 3 h prior to the addition of 2,4-D. F3-ST activity observed under these conditions was lower by about 50% 15 h after, and by about 70% 24 h after, the addition of auxin as compared with controls containing no actinomycin D (Fig. 6). The latter exhibited maximum enzyme activity 12 h after addition of 2,4-D, unlike the 24-h maximum observed in cultures grown in 1-L nipple flasks (Fig. 5B).

Effect of Flavonols on F3-ST Activity

The effects of quercetin and its sulfated derivatives (quercetin 3-sulfate and quercetin 3,7,4′-trisulfate) on enzyme activity were determined during culture growth. Quercetin sulfate levels of 3 mM were high relative to the K_i for the enzyme (0.25 μM; Varin and Ibrahim, 1992) but within the range of concentrations of these compounds found in *F. bidentis*. Values for endogenous sulfated flavonoids vary from 30 mM in the apical bud to 1 mM in the third leaf (Hannoufa et al., 1991). Whereas quercetin had no detectable effect on the activity profile of F3-ST, both flavonol sulfate esters reduced enzyme activity to approximately 50% of those levels observed with the control cultures (Fig. 7A). The decline in

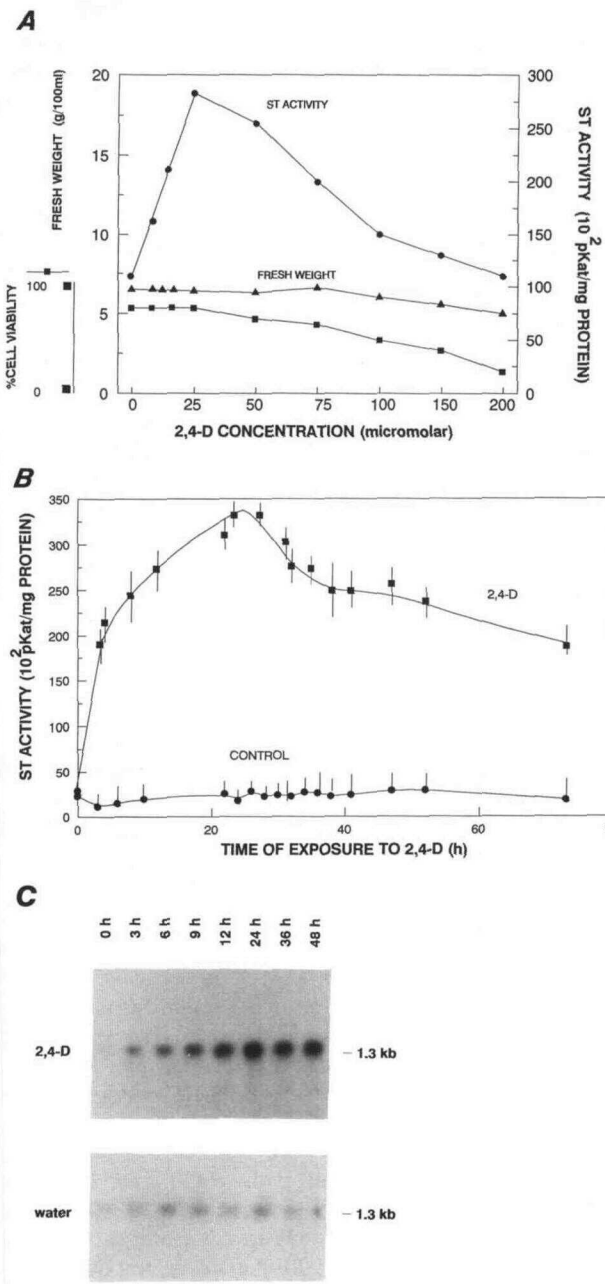

Figure 5. A, Dose-response curve. Ten-day-old cultures, grown in 250-mL Erlenmeyer flasks, were treated with the indicated concentrations of 2,4-D and harvested 24 h after treatment, and cell-free extracts were assayed for F3-ST activity. B, Time-response curve. Ten-day-old cultures, grown in 1-L nipple flasks, were treated with 25 μM 2,4-D; F3-ST activity was monitored for 72 h. C, Northern blot analysis of BF3ST gene product from cell cultures. Ten-day-old cultures, grown in 1-L nipple flasks, were treated with 2,4-D or were untreated (2,4-D substituted by water). Lanes contain 5 μg of total RNA taken at the indicated times. Blots were probed with cDNA clone, pBFST3, labeled with ^{32}P by random primer method (Amersham).

F3-ST activity in response to either quercetin 3-sulfate or quercetin 3,4,7′-trisulfate does not seem to be a consequence of a change in the F3-ST gene expression, because the levels of the enzyme-specific transcript were relatively stable during the treatment period (Fig. 7B). However, more frequent sampling might reveal fluctuation, especially prior to d 3.

DISCUSSION

In this paper, we describe the isolation, characterization, and expression of a cDNA clone encoding the F3-ST from *F. bidentis* (pBFST3). Furthermore, the effects of 2,4-D as well as both the enzyme substrate, quercetin, and the sulfated products, quercetin 3-sulfate and quercetin 3,7,4′-trisulfate, on the activity of the F3-ST in *F. bidentis* were investigated.

Sequence analysis of the pBFST3 clone revealed high sequence similarity with the F3-ST and moderate levels of similarity to the F4′-ST genes from *F. chloraefolia* at both the nucleotide and amino acid levels (Varin et al., 1992), although the enzyme exhibited lowest affinity to kaempferol. The K_m values for both quercetin and PAPS, as well as the pH optima of *F. bidentis* F3-ST expressed in *E. coli*, were quite similar to those reported for the *F. chloraefolia* F3-ST (Varin and Ibrahim, 1992). However, the substrate specificity of the protein encoded by the cloned *F. bidentis* F3-ST differed from that of *F. chloraefolia* F3-ST in that the former enzyme accepted quercetin, rhamnetin, and isorhamnetin, but not kaempferol, as substrates. The latter substrates were previously reported to be sulfated by cell-free extracts of both *F. bidentis* and *F. chloraefolia* (Varin et al., 1987a). In addition, the relative sulfate acceptor ability of rhamnetin and isorhamnetin in that study also differed from that reported here. However, the two studies are not strictly comparable because the former study measured ST activity in cell-free plant extracts, whereas the present study used a cloned F3-ST that was expressed in *E. coli* and used the desalted bacterial cell lysate. It may be that either the *E. coli* expression system is affecting the substrate specificity of the cloned enzyme or that the kaempferol-sulfating activity observed in cell-free extracts is due to a still unidentified enzyme.

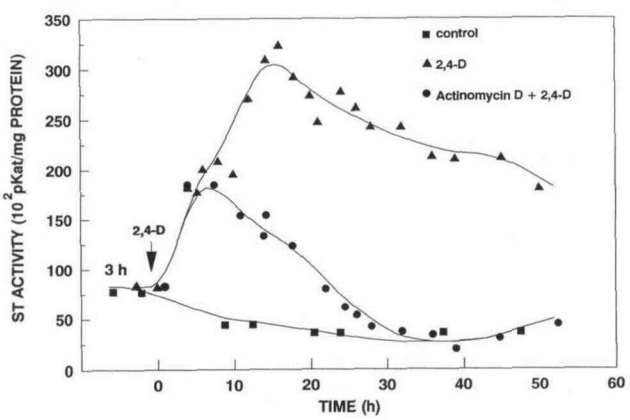

Figure 6. F3-ST induction kinetics in response to 2,4-D and actinomycin D treatments. Cell cultures grown in 250-mL Erlenmeyer flasks were either not treated or were treated with actinomycin D for 3 h prior to treatment with 25 μM 2,4-D.

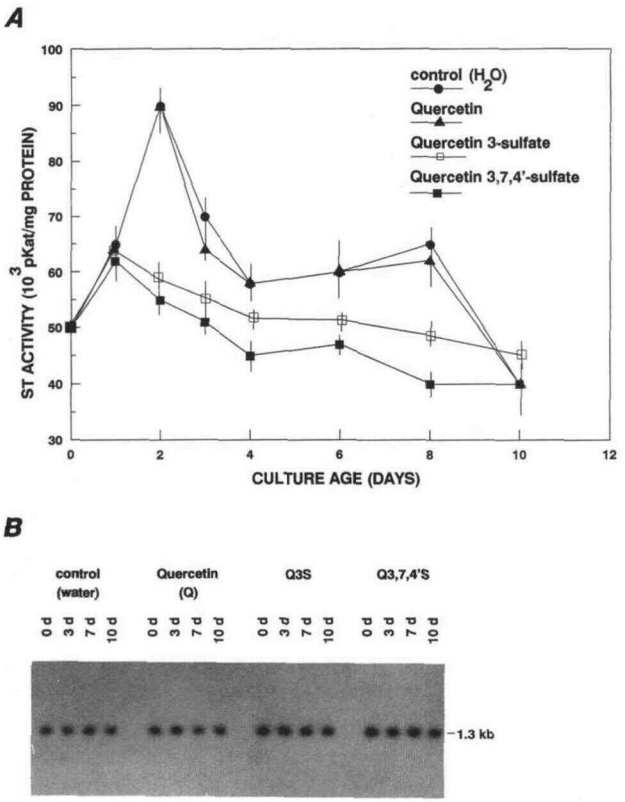

Figure 7. The effect of different flavonols on F3-ST activity. A, Ten-day-old cultures from 250-mL Erlenmeyer flasks were transferred to media containing 3 μM of quercetin (▲), quercetin 3-sulfate (□), or quercetin 3,7,4′-trisulfate (■) as compared to control (●). These were subsequently sampled and assayed for F3-ST activity. Bars indicate SD calculated from triplicate experiments. B, Northern blot analysis of cell cultures following treatment with quercetin (Q), quercetin 3-sulfate (Q3S), and quercetin 3,7,4′-trisulfate (Q3,7,4′S). A control treatment substituted water for the flavonols. Samples were collected on d 0, 3, 7, and 10. Each lane was loaded with 10 μg of total RNA. Blots were probed with pBFST3. Each treatment should be compared to its own 0 d control, because the darkening from left to right on the blot is likely an artifact. The blot was exposed to Kodak XAR film 5 times longer than the northern blots shown in Figure 5C.

Study of the regulation of F3-ST expression in *F. bidentis* cell cultures showed that the F3-ST activity increased 2 to 3 d after subculture, although a corresponding accumulation of flavonol sulfate esters was not detected. The transient increase in enzyme activity is probably due to the transfer of cultured cells to fresh media (Fig. 4) by the so-called "dilution effect," which is characterized by a short lag period (Kuhn et al., 1984). These results are similar to those reported for the early enzymes of the phenylpropanoid pathway, especially PAL, in both parsley and soybean cell cultures (Hahlbrock et al., 1980).

In this study, *F. bidentis* cell cultures grown in nipple flasks and treated with 2,4-D showed increases in F3-ST activity. Northern blot analysis revealed that the increase in F3-ST activity was concomitant with an increase in F3-ST transcript levels. Both the enzyme activity and mRNA transcript levels increased within 3 h of treatment with 2,4-D. F3-ST activity reached a maximum at 24 h and declined gradually up to 48 h, although it remained severalfold higher than control levels throughout this time course. Transcript levels reached a maximum 24 h after treatment and then declined slightly (Fig. 5C). When cell cultures were grown in Erlenmeyer flasks, F3-ST activity reached a maximum somewhat earlier in response to 2,4-D treatment (Fig. 6).

It is possible that part of the signal on the northern blots (Figs. 5D and 7B) could be due to the cross-hybridization of the pBFST3 probe with mRNAs of other, as yet uncloned, STs. However, this source of error seems unlikely; intact tissues of *F. bidentis* contain position-specific ST activities other than the F3-ST (Varin and Ibrahim, 1991). No such activity was detected in *F. bidentis* cell cultures, including those treated with 2,4-D. The comparison of DNA sequence between STs with different position-specific activities isolated from the same species showed only moderate similarities. The pFST3 and pFST4′ from *F. chloraefolia* have 76% overall sequence similarity, and two clones from *F. bidentis*, pBFST3 and pBFSTX (a flavonol ST-like cDNA clone with as yet uncharacterized enzymatic activity), have 74% overall sequence similarity. The 200-bp internal region with the highest sequence similarity between pBFST3 and pBFSTX are 78.5% identical. If similar levels of sequence identity exist between STs with different position-specific activities, cross-hybridization of the pBFST3 probe to other flavonol STs would not be expected under the stringency conditions that were used. Hybridization of pBFSTX to northern blots of total RNA of 2,4-D-treated cell cultures gave signals less than 10% of the signal from pBFST3 (data not shown).

Preincubation of the cultured cells with the transcriptional inhibitor actinomycin-D reduced the 2,4-D-dependent induction of F3-ST activity (Fig. 6), as well as the level of immunoreactive protein (data not shown). These results suggest that enzyme induction resulting from exposure to 2,4-D is regulated at the level of gene expression. Northern blot analysis revealed that the level of F3-ST transcripts was increased about 10-fold following exposure to 25 μM 2,4-D, which is in contrast to the reported effect of 2,4-D on the early enzymes of the flavonoid pathway. In fact, 2,4-D has previously been reported to down-regulate PAL, chalcone synthase, and chalcone-flavanone isomerase in carrot tissue cultures (Ozeki et al., 1989, 1990). However, the differences in the reported effects of 2,4-D on gene expression in tissue cultures may be due to species-specific responses or other factors.

Recently, Mavandad et al. (1990) and Loake et al. (1991) reported that the phenylpropanoid pathway intermediates *t*-cinnamic acid and *p*-coumaric acid regulate expression at the level of transcription for PAL, the first enzyme of the phenylpropanoid pathway, and for chalcone synthase, the first committed enzyme in flavonoid biosynthesis. These results suggest that a regulatory mechanism may respond to a high concentration of pathway intermediates and, thus, may control the rate of synthesis of the enzymes involved. In this study, the exposure of *F. bidentis* cultures to either quercetin 3-sulfate or quercetin 3,7,4′-trisulfate decreased F3-ST enzyme activity by approximately 50%, although the decrease might be due partly to inhibition by the product uptake. The

treatment with either of the sulfated compounds had no effect on F3-ST transcript levels. Moreover, quercetin 3-sulfate has been demonstrated to inhibit F3-ST activity noncompetitively in vitro (Varin and Ibrahim, 1992), suggesting that the regulation of this enzyme by the sulfated product is not at the transcriptional level but more likely occurs at the enzymatic level.

It has been reported that flavonol aglycones may act as inhibitors of polar auxin transport and that flavonol conjugates could act as antagonists of this inhibition. Some flavonoid aglycones (quercetin, kaempferol, or apigenin) bind to the NPA receptor and inhibit the eflux of auxin from the basal end of stem cells, thereby inhibiting polar transport and causing intracellular accumulation (Jacobs and Rubery, 1988). In contrast, flavonol conjugates, including quercetin 3-sulfate, have been shown to strongly bind the NPA receptor and block the quercetin-stimulated accumulation of auxin (Faulkner and Rubery, 1992). The fact that F3-ST gene expression is up-regulated by 2,4-D suggests that the polar transport of auxin may be autoregulated at the level of gene expression. In such a model, high levels of auxin would cause increased levels of F3-ST activity and result in the depletion of quercetin and in increased levels of 3-sulfated flavonols. Another point of regulation of auxin transport is that flavonol sulfates inhibit F3-ST activity. Both of these changes would tend to function as a balancing component of the regulation of auxin transport. The high concentration of both sulfated flavonols and ST enzyme activity in shoot tips and their lower concentration in older tissues in this species (Hannoufa et al., 1991) are consistent with the role of a regulator that would stimulate auxin transport from the apical tissues. Although flavonoid aglycones and their conjugated forms are of widespread occurrence in plants, some of them, including sulfated flavonoids, have limited taxonomic distribution. However, in species in which they do occur, such as *Flaveria* spp., sulfated flavonols may be involved in an interactive mechanism for both the positive and negative control of auxin transport. The intracellular localization of both conjugated and unconjugated flavonols at the ultrastructural level is necessary to evaluate this model. This remains an important area for further investigation.

Received December 24, 1993; accepted June 17, 1994.
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