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# Conservation in divergent solanaceous species of the unique gene structure and enzyme activity of a gametophytically-expressed flavonol 3-*O*-galactosyltransferase[1]

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**Abstract**

Flavonol 3-*O*-galactosyltransferase (F3GalTase) is a pollen-specific enzyme which glycosylates the flavonols required for germination in petunia. The highly restricted tissue-specific expression and substrate usage make F3GalTase unique among all other flavonoid glycosyltransferases (GTs) described to date, including the well characterized *Bronze*1 (*Bz*1) gene of maize. RFLP mapping, DNA gel blot, and sequence analyses showed that the single copy *F3galtase* gene has a different genomic organization than *Bz*1. Within the promoter of *F3galtase* are potential regulatory motifs which may confer pollen-specific gene expression and activation by Myb and bHLH transcription factors. However, we provide evidence that *F3galtase* is not regulated by *An*4, which encodes a Myb factor known to regulate anthocyanin accumulation in petunia anthers. An unexpected feature of the *F3galtase* promoter was the presence of large blocks of chloroplast and mitochondrial DNA. Gel blot analyses of genomic DNA from the progenitors of *Petunia hybrida*, as well as from *Nicotiana tabacum*, indicated that migration of organellar DNA into the *F3galtase* gene was an ancient event that occurred prior to speciation of the Solanaceae. Together with enzyme assays and HPLC analyses of pollen extracts from tobacco, tomato, and potato, these results confirmed that the unique *F3galtase* gene structure, enzyme activity, and pollen-specific flavonol glucosylgalactosides are conserved throughout the Solanaceae.

## Introduction

In petunia pollen, the flavonols required for germination (Mo *et al.*, 1992; Taylor and Jorgensen, 1992) have a unique glycosylation pattern. F3GalTase forms flavonol 3-*O*-galactosides which are subsequently glucosylated by a glucosyltransferase to form the pollen-specific 3-*O*-(2″- *O*-$\beta$-D-glucopyranosyl)-$\beta$-d-galactopyranoside conjugates of kaempferol and quercetin (Vogt and Taylor, 1995; Miller *et al*., 1999). F3GalTase activity is restricted to pollen and is highly specific for flavonols and UDPgalactose. *In vitro* it shows maximum activity with the endogenous aglycone substrates kaempferol and quercetin which are the most efficient inducers of germination in mutant pollen lacking flavonols (Mo *et al.*, 1992; Vogt *et al.*, 1995; Miller *et al*., 1999). Because F3GalTase is the only enzyme in petunia pollen that metabolizes flavonols, it ultimately controls the relative abundance of the aglycone and the glycosides.

In contrast to the specificity of F3GalTase, all other characterized flavonoid GTs are expressed in multiple tissues and accept a variety of flavonoid (e.g. anthocyanidins and flavonols) (A/FGTs) or UDP sugars as substrates. A consequence of this indiscriminant activ-

[1]The nucleotide sequence of the *F3galtase* gene is available in the NCBI GenBank database under the accession number AF 316552.

ity of A/FGTs is that many different flavonoid glycosides can accumulate in multiple tissues. The maize *Bz*1 gene encodes an A/FGT which converts both anthocyanidins and flavonols into their respective 3-*O*-glucosides. Bronze1 activity in maize pollen generates 10 different glycosides of quercetin, isorhamnetin, and kaempferol of which many, if not all, are also present in sporophytic tissues (Ceska and Styles, 1984).

Until this report, Bz1 and its barley orthologue were the only enzymatically characterized A/FGTs whose genomic structure was known (Furtek *et al*., 1988; Ralston *et al*., 1988; Wise *et al*., 1990). Numerous A/FGT cDNAs have been isolated with *Bz*1 and, perhaps as a consequence of the high level of sequence conservation, *Bz*1 has emerged as the paradigm of A/FGT gene structures. However, the distinctive properties of F3GalTase argue that the *Bz*1 model may not be appropriate for all flavonoid GTs. Therefore, to determine whether the highly restricted substrate usage and pollen-specific expression of F3GalTase are reflected in the gene structure, we isolated and characterized the *F3galtase* gene. In addition, we show that the unique enzyme activity and gene structure of *F3galtase* is not restricted to petunia but is pervasive throughout the Solanaceae.

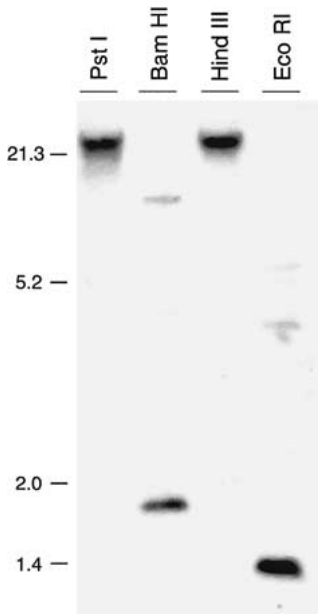


*Figure 1.* Gel blot analysis of petunia genomic DNA hybridized with a fragment of the Sal I/Bgl II insert of the F3GalTase cDNA clone. Genomic DNA (15:g) was digested with the indicated restriction endonucleases and then size-fractionated through 0.75% agarose. After transfer onto a nylon membrane, the immobilized DNA was hybridized with the 683 bp [$^{32}$P]dCTP-labeled *Sal*I/*Bgl*II insert of the F3GalTase cDNA. The migration of size standards is shown (in kb) on the left side of the figure.

## Materials and methods

### *Plant material*

The *Petunia hybrida* (V26, V23, and R51) plants used in this study have been previously described (Taylor and Jorgensen, 1992; Strommer *et al*., 2000). *Petunia hybrida* V10 and W78 and *Nicotiana alata* plants were from our stock. *Petunia parodii*, *P. axillaris*, and *P. integrifolia* were kind gifts of Anton Gerats, University of Gent. Anthers from *Nicotiana tabacum* cv. Xanthi, *Solanum lycopersicum*, and *Solanum tuberosum* were obtained from plants grown at the Washington State University greenhouse facility.

### *Determination of* F3galtase *copy number*

V26 petunia leaf genomic DNA was digested with *Eco*RI, *Hin*dIII, *Bam*HI, or *Pst*I, and the resulting restriction fragments were size-fractionated through 0.75% agarose and transferred onto a nylon membrane according to standard protocols. The resulting blot was hybridized with a [$^{32}$P]dCTP-labeled 683 bp *Sal*I/*Bgl*II restriction fragment of the pF3GalTase1 cDNA (Miller *et al*., 1999) for 48 h, washed twice in 0.2 × SSPE at 60 °C, and exposed to X-ray film for five days at −80 °C.

### *Chromosomal mapping*

Two families of V23/R51 hybrids previously used to map a number of cloned genes (Strommer *et al*., 2000) provided DNA for RFLP mapping of the *F3galtase* gene. The [$^{32}$P]dCTP-labeled 683 bp *Sal*I/*Bgl*II restriction fragment was hybridized to *Xba*I-digested DNA from 21 plants using the methods described in Strommer *et al*. (2000).

### *Isolation of the* F3galtase *genomic clone*

A genomic library (a kind gift from C. Napoli), made from DNA of the V26 inbred line of *Petunia hybrida* and packaged in the λDASH phage DNA vector (Stratagene), was screened using the [$^{32}$P]dCTP-labeled 683 bp *Sal*I/*Bgl*II restriction fragment of the F3GalTase cDNA as a hybridization probe. Positive plaques were purified through three rounds of plating, filter transfers, and hybridization to the

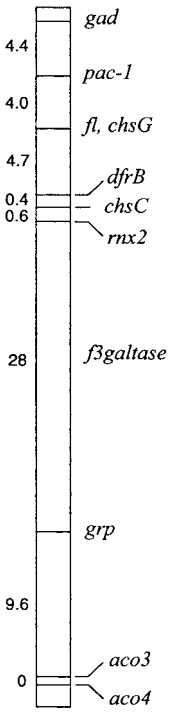


*Figure 2.* Map of *Petunia hybrida* chromosome II showing the *F3galtase* locus.

F3GalTase probe. Phage DNA was isolated from positive plaques using the Wizard Lambda Preps DNA isolation kit (Promega). The *TaqPlus Long* PCR System (Stratagene) was used to isolate a DNA fragment containing the *F3galtase* gene from the entire λ phage insert. A 100 $\mu$l amplification reaction contained 20 mM Tris-HCl pH 9.2, 60 mm KCl, 2 mM $MgCl_2$, 50 pmol of a T3 promoter primer (5′-CGAAATTAACCCTCACTAAAGG-3′), 50 pmol of primer 10 (Miller *et al*., 1999), 6 units of *TaqPlus Long* polymerase mixture, and 50 ng of the purified phage DNA. The cycling conditions were as follows: initial denaturation for 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 20 min at 72 °C. The resulting amplicon (about 12 kb) was gel-purified, ligated into the pCR-XL-TOPO plasmid (Invitrogen), and transformed into TOP10 *E. coli* cells according to the manufacturer's instructions. This plasmid (p*F3galtase*-gen1) was used as a template for sequencing using a Perkin-Elmer automated ABI DyeDeoxy system.

*Start of transcription*

The transcription start site of the *F3galtase* gene was determined by primer extension analysis as previously described (McKnight *et al*., 1981). Primer extension products were synthesized using Thermoscript (GibcoBRL) reverse transcriptase at 45 °C, 50 $\mu$g of total RNA from stage 6 V26 petunia pollen, and a [$^{32}$P]-dATP end-labeled anti-sense primer (5′-TAAATGTCAC-GTTAGGTAATGC-3′). DNA sequencing was performed with the same primer and the p*F3galtase*-gen1 plasmid as a template. The end-labeling and sequencing reactions were performed with the *fmol* DNA Cycle Sequencing System (Promega) according to the manufacturer's instructions.

F3galtase *Ct, Mt, and coding sequences in* Petunia hybrida *progenitors and* Nicotiana

*P. parodii*, *P. axillaris, P. integrifolia*, *P. hybrida*, and *N. tabacum* leaf DNA was digested with *Eco*RI/*Bgl*I, size-fractionated through 0.9% agarose, and transferred onto a nylon membrane. The immobilized DNA was hybridized with the following [$^{32}$P]dCTP-labeled DNA fragments derived from either PCR amplification of a specific region, or restriction endonuclease digestion, of the p*F3galtase*-gen1 plasmid: (1) a 445 bp *Hin*dIII/*Bgl*II fragment containing the mitochondrial II insertion sequence (Mt probe); (2) a 365 bp PCR-generated amplicon containing the chloroplast insertion sequence (Ct probe); and (3) a 680 bp *Eco*RI/*Bam*HI fragment containing 434 bp of the gene downstream of the transcription start site and 246 bp upstream of the transcription start site (coding sequence probe). Segments of the *F3galtase* gene from which the Mt, Ct, and coding sequence probes were derived are shown in Figure 5. The immobilized DNA was hybridized with one of the three probes for 24 to 48 h, washed with either 0.2 × or 2.0 × SSPE at 60–65 °C and then exposed to X-ray film. Hybridization signals were quantified as previously described (Miller *et al.*, 1999) with a glyceraldehyde 3-phosphate dehydrogenase (GAP) cDNA.

*Glycosyltransferase enzyme assays and HPLC analysis of pollen flavonoids*

Stage 6–9 flowers were collected from V26, V10, and W78 plants. The corolla tissue was frozen, macerated in a mortar and pestle under liquid nitrogen, mixed with five volumes of extraction buffer (0.1 m Tris-HCl pH 7.5, 1 mm DTT, 0.5 mm PMSF), and centrifuged at 12 000 × *g* for 5 min (4 °C). The supernatant was decanted and used for enzyme activity analyses.

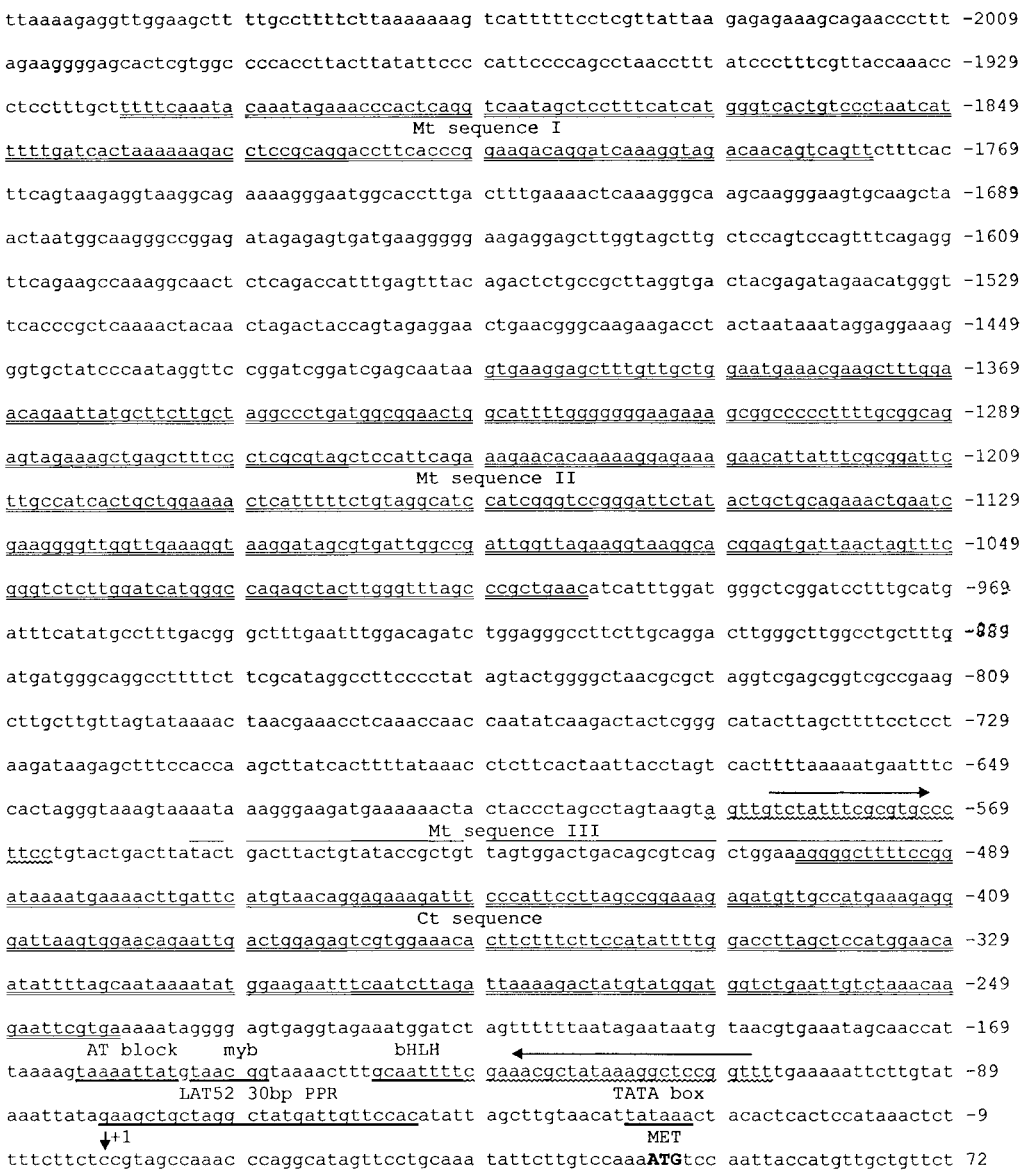


*Figure 3.* Nucleotide sequence of the *F3galtase* genomic clone 5′-ward of the ATG translation initiation codon. Regions of the nucleotide sequence homologous to organellar DNA is double-underlined and designated Mt for mitochondria and Ct for chloroplast. The transcription initiation nucleotide is designated as +1 and the translation initiation codon is in bold. Other putative regulatory sequences are labeled with a single bold underline and include the TATA box, potential bHLH and Myb binding sites, an AT block, and a 28 bp sequence with similarity to the LAT52 30 bp proximal promoter region. Primer sequences for PCR amplification of the Ct probe are designated with a wavy underline and have arrows pointing in the direction of amplification.

Pollen was extruded from anthers and a crude homogenate was obtained (Vogt and Taylor, 1995) in extraction buffer +1% Triton X100 for use in F3GalTase enzyme assays. The extraction of F3GalTase activity from pollen is enhanced by 1% Triton X100 and its activity is not reduced. Because the effects of Triton X100 on A/FGT activity has not been determined, extracts for this enzyme were prepared in the absence of detergent to avoid the potential inactivation of activity.

A/FGT activity in both tissue extracts was assayed according to the method of Ford *et al.* (1998) using 3 $\mu$l of either the corolla or pollen extracts and kaempferol as the flavonoid substrate. F3GalTase activity was assayed (Miller *et al.*, 1999) using 3 $\mu$l of the corolla or 1–3 $\mu$l of the pollen extracts and

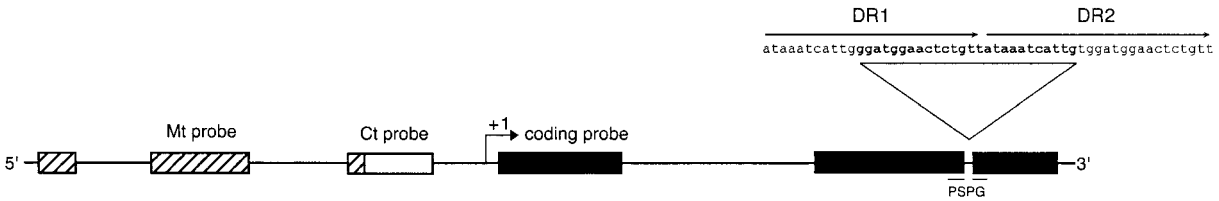


*Figure 4.* Gene structure of *F3galtase*. The *F3galtase* coding sequence is indicated by black rectangles (exons) and thin lines (introns). The transcription start at +1 is indicated with an arrow. The DNA sequences in the promoter region which are homologous to organellar DNA sequences are indicated as hatched rectangles for the three Mt insertion sequences and a white rectangle for the Ct insertion sequence. The direct repeats proximal to intron II are designated as DR1 and DR2 with the intergenic sequence in bold. The relative position of the nucleotide sequences encoding the PSPG box are underlined. Segments of the sequence that were used as nucleotide probes in the experiment described in Figure 5 are indicated as 'Mt probe', 'Ct probe', and 'coding probe'.

kaempferol as the substrate. Pollen flavonoids were analyzed and quantified as previously described (Vogt *et al.*, 1995; Miller *et al.*, 1999).

## Results

### F3galtase *gene copy number and chromosomal mapping*

DNA gel blot analysis was performed on wild-type (V26) petunia genomic DNA digested with a series of restriction enzymes with 6 bp recognition sites. The hybridization probe was a fragment from the 5′ region of the F3GalTase cDNA which has the lowest level of sequence identity with other flavonoid GT genes (Miller *et al.*, 1999). The hybridization pattern presented in Figure 1 is consistent with the presence of a single-copy gene. Restriction fragment length polymorphism (RFLP) map based analysis of a segregating population of petunia hybrids confirmed that the *F3galtase* gene mapped to a single locus in the petunia genome. Of the 21 DNA samples chosen to represent plants exhibiting minimal recombination overall, 18 yielded clear data which allowed placement of the *F3galtase* locus on chromosome II. Among the sub-population of plants used, there was evidence for three crossover events between *rnx2*, which encodes a self-incompatibility RNase (Lee *et al.*, 1992), and *grp*, which encodes a glycine-rich protein (Strommer *et al.*, 2000). The *F3galtase* locus mapped between these two, on the *rnx2* side of one crossover (1/18 recombinants) and the *grp* side of two crossovers (2/18) as shown in Figure 2.

### *Sequence analysis of the* F3galtase *genomic clone*

A λ phage genomic library made from DNA of V26 *Petunia hybrida* was screened with a nucleotide probe comprising the first 683 bp of the F3GalTase cDNA. The entire *F3galtase* gene was contained on a 12 kb clone. A 4.257 kb segment was sequenced and contained nucleotides encoding the entire F3GalTase protein as well as 2.088 kb of sequence upstream of the transcription start site (Figure 3). This 2.088 kb region potentially comprises some or all of the promoter elements and will hereafter be referred to as the promoter.

Figure 4 shows the genomic organization of *F3galtase*. The three exons are 472, 567, and 316 bp. Intron I is 742 bp and is located between nucleotides 517 and 1261, whereas intron II is 26 bp and occurs between nucleotides 1827 and 1853. Intron II is located in the middle of two contiguous, virtually perfect, direct repeats (DR) and is comprised of the 3′ end of DR1 and the 5′ end of DR2 (Figure 4). Thus, precise excision of intron II would yield a single copy of the DR. Of particular importance is the placement of intron II: it falls within the portion of the gene that encodes the highly conserved PSPG box implicated in substrate binding (Vogt and Jones, 2000) and interrupts the sequence HCGWNS (conserved amino acids are underlined) between the H and C residues. This is significant because the H, G, and S residues are 100% conserved in GT sequences across kingdoms (Kapitonov and Yu, 1999; Miller *et al.*, 1999). Also, the junction between the coding sequence and intron I is in frame whereas the junction between the coding sequence and intron II is out of frame. It is tempting to speculate that the presence of an out of frame intron within a DR sequence, which is within a highly conserved region implicated in substrate specificity, is somehow involved in the unique enzyme properties of F3GalTase.

Primer extension analysis showed that the *F3galtase* gene has two transcription start sites comprised of adjacent cytosine nucleotides. The most 5′-ward of

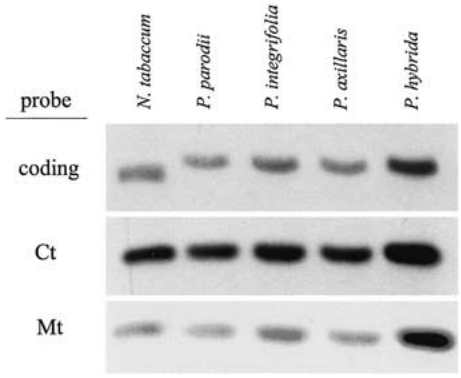


*Figure 5.* Southern blot of genomic DNA from *Petunia parodii*, *P. axillaris, P. integrifolia*, *P. hybrida*, and *Nicotiana tabacum*. The DNA was digested with *Eco*RI/*Bgl*II, size-fractionated, and transferred onto a nylon membrane. The immobilized DNA was hybridized with either the coding sequence, Mt, or Ct probes (Figure 4) as described in Materials and Methods. The size of the restriction fragment that hybrized with the Ct and Mt probes is 1098 bp, whereas the size of the fragment that hybridized with the coding sequence probe is 1163 bp. Re-probing of the membrane with a petunia GAP cDNA and subsequent densitometric quantification showed that 2.9-fold more *Petunia hybrida* genomic DNA was present than genomic DNA in the other lanes.

the two cytosines is shown as the transcription start site in Figure 3. A TATA (TATAAA) box is present at nucleotide −36.

A 28 bp sequence between nucleotides −81 and −53 (Figure 3) is 71% similar to a 30 bp element located in the promoter of the tomato LAT52 gene. This element has been functionally tested and is known to confer pollen specific LAT52 expression (Eyal *et al*., 1995). The *F3galtase* promoter contains potential binding sites that in anthocyanin biosynthetic genes are responsive to the combined action of bHLH and Myb type transcription factors. An 8 bp sequence (−137 to −130) was 87.5% identical to the consensus mammalian bHLH transcription factor binding site (NCANNTGN) and a 6 bp sequence (−152 to −146) was 83.3% identical to the consensus mammalian Myb transcription factor binding site (C/TAACG/TG).

### *The* F3galtase *promoter contains multiple insertions of chloroplast and mitochondrial DNA*

Sequence analysis of the promoter revealed the presence of a 265 bp segment of DNA (−503 to −238) (Figure 3) with 88% and 86% sequence identity to chloroplast (Ct) genomic DNA from *Nicotiana tabacum* and *Spinacea oleracea*, respectively. In addition, there were three separate DNA segments with identity to mitochondrial (Mt) DNA from two different species. A 63 bp (−552 to −489) and a 143 bp (−1775 to −1918) sequence had 92% and 97% identity, respectively, with Mt DNA from *Beta vulgaris*. A 376 bp sequence (−1377 to −999) had 87% identity with *Arabidopsis thaliana* Mt DNA. The occurrence of these sequences in the *F3galtase* gene suggests that homologous regions of both the petunia Mt and Ct genomes migrated into the *F3galtase* promoter.

### *The* F3galtase *gene structure in* Petunia hybrida *progenitors and multiple solanaceous species are identical*

The high level of nucleotide conservation of the Ct and Mt insertions in the *F3galtase* promoter to organellar DNA from other species indicated that either the integration event was relatively recent, or that there had been selective pressure to maintain these sequences unchanged. To distinguish these two hypotheses, we performed gel blot analyses on genomic DNA from *P. axillaris*, *P. integrifolia*, and *P. parodii*. Although the records are unclear as to the exact cross which gave rise to *Petunia hybrida*, there is general agreement that the progenitors of the modern hybrid are included in this group of three (Sink, 1984). If the integration event occurred after speciation of *P. hybrida*, then the organellar DNA sequences would not be present in the progenitors. We also tested *Nicotiana tabacum* reasoning that if the integration event was ancient and did not significantly diverge throughout evolution, then the organellar sequences should be present in more distantly related solanaceous species.

The data in Figure 5 show that in all the solanaceous species tested there are Ct and Mt sequences present in the *F3galtase* gene. Further, the pattern of restriction fragments indicates a highly conserved gene structure. These data confirm that the Ct and Mt insertions within the *F3galtase* gene occurred before speciation of the Solanaceae.

### *F3GalTase enzyme activity is present in divergent solanaceous species*

A tobacco *F3galtase* gene virtually identical to the petunia gene suggests that the unique F3GalTase activity expressed in petunia pollen is present in other solanaceous species. To test this possibility we measured F3GalTase activity in extracts from pollen of *Nicotiana tabacum*, *Nicotiana alata*, *Solanum lycopersicum*, and *Solanum tuberosum* (Table 1). The results confirm that the pollen from all four species had F3GalTase activity, albeit at reduced levels compared

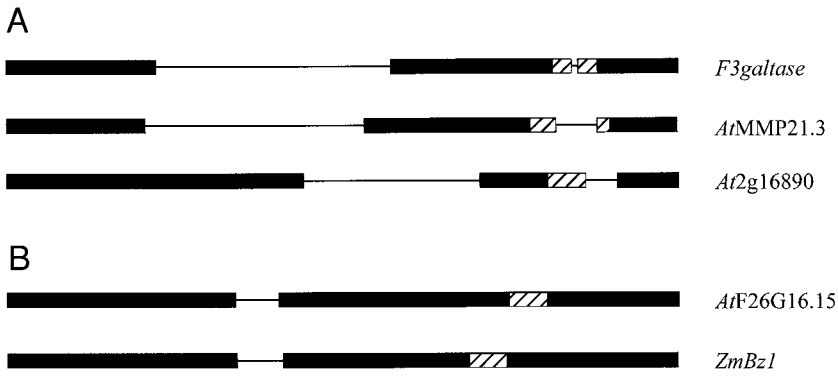


*Figure 6.* Exon/Intron organization of (A.) the *F3galtase* gene and two *Arabidopsis* GT-like genes (*At*MMP21.3 and *At*2g16890) and (B) the *Bz*1 and *Arabidopsis* At26G16.15 genes. Exons are shown as solid black rectangles, introns are thin lines, and the nucleotide sequence encoding the PSPG box is shown as a hatched box.

*Table 1.* Pollen-specific F3GalTase activity and flavonol content in *P. hybrida* and four different solanacaous species.

| Species | F3GalTase activity (nmol · min$^{-1}$ mg protein$^{-1}$) | Flavonol content (pmol per mg anther$^{-1}$) | |
|---|---|---|---|
| | | kaempferol | quercetin |
| *P. hybrida* | 369 | 1445 | 2198 |
| *N. alata* | 11.3 | 393 | 252 |
| *N. tabacum* | 11.1 | 1115 | 303 |
| *S. lycopersicum* | 8.8 | 1007 | 891 |
| *S. tuberosum* | 6.8 | 273 | 151 |

to *Petunia hybrida* pollen. To ensure that the activity was F3GalTase and not the result of an indiscriminant A/FGT activity, enzyme assays were also conducted using UDPglucose as the co-substrate. No flavonol glycosides were detected with UDPglucose confirming that pollen from a number of solanaceous plants contain the unique F3GalTase activity (Table 1). We also determined the flavonol content in pollen from the four solanaceous plants and found that kaempferol and quercetin diglycosides were the major flavonols present. Interestingly, the reduced level of F3GalTase activity in the various Solanaceae does not appear to influence the flavonol accumulation since *N. tabacum* and *S. lycopersicum* had levels comparable to *P. hybrida* (Table 1).

In addition, F3GalTase activity was also assayed in the pollen of a group of monocot and dicot species including *Lilium longiflorium*, *Oryza sativa*, *Zea mays*, *Vitis vinifera*, and *Brassica napus* (data not shown). We were unable to detect F3GalTase activity in any of these plants, further supporting our conclusion that F3GalTase activity is specific to the Solanaceae.

### *F3GalTase activity is not controlled by* An4

In petunia, *An*1 encodes a bHLH protein (Spelt *et al.*, 2000) and *An4* is a Myb homologue whose activity is confined to the anther wall (Huits *et al.*, 1994; Spelt *et al.*, 2000). Because the *F3galtase* gene contains potential Myb- and bHLH-binding sites, we wished to determine if *An*4 expression in the anther could affect F3GalTase activity in the pollen. If *F3galtase* expression was regulated by *An*4, we would expect that F3GalTase activity would be lower in *an*4/*an*4 plants and highest in *An*4/*An*4 plants.

F3GalTase activity was assayed in pollen extracts from three petunia lines with different *An*4 genotypes. F3GalTase activity was highest in V26, lowest in W78, with intermediate activity in V10 pollen (Table 2). V26 plants are *an*4/*an*4 whereas V10 plants are *An*4/*An*4, suggesting that *An*4 has no effect on the expression of F3GalTase activity in pollen. F3GalTase activity in W78 pollen was lower than in any of the other petunia lines assayed, but we propose that this activity is not limiting due to the high catalytic efficiency of F3GalTase (Miller *et al.*, 1999). This is

*Table 2.* Pollen-specific F3GalTase activity and flavonol content in three petunia lines with different *An*4 genotypes.

| Petunia line | *An*1,4 genotype | Tissue | F3GalTase activity[a] (pmol · min$^{-1}$ mg protein$^{-1}$) | A/F3GT activity[a] (pmol · min$^{-1}$ mg protein$^{-1}$) | Flavonol content[b] (pmol per mg anther$^{-1}$) | |
|---|---|---|---|---|---|---|
| | | | | | kaempferol | quercetin |
| V10 | *An*1, *An*4 | pollen | 210 | nd | 2325 | 868 |
| | | corolla | nd | 89.6 | | |
| V26 | *An*1, *an*4 | pollen | 369 | nd | 1445 | 2198 |
| | | corolla | nd | 60.9 | | |
| W78 | *an*1, *an*4 | pollen | 44.8 | nd | 601 | 538 |
| | | corolla | nd | 17.3 | | |

[a]Flavonols were analyzed following acid hydrolysis of endogenous kaempferol and quercetin 3-*O*-glucosylgalactosides.
[b]Not detected.

supported by the fact that in all of the lines assayed, only flavonol glycosides were detected (Table 2). As expected (Gerats *et al.*, 1985), A/FGT activity was confined to the corolla and showed a direct correlation with the *An*1 genotype (Table 2). We conclude that F3GalTase activity in pollen is not regulated by An4 and propose there may be pollen-specific regulatory factors which control *F3galtase* gene expression.

*The F3galtase gene structure is conserved in a non-solanaceous species*

The *Arabidopsis* genome sequencing project provided us with an opportunity to search an entire genome of a non-solanaceous species for A/FGT genes and determine if any had a structure similar to *F3galtase*. No *Arabidopsis* A/FGTs have been biochemically characterized, but most contain the signature 40 amino acid PSPG box (Li *et al.*, 2001) of all small-molecule GTs from both plant and animal sources (Miller *et al.*, 1999; Vogt and Jones, 2000). Out of about 95 GT-like sequences obtained from the database of *Arabidopsis thaliana* Annotation (http://www-sequence.stanford.edu/ara/ArabidopsisSeqStanford.html), about 48% had genomic structures different than *F3galtase* or *Bz*1 and 45% had similar structures, i.e. a small intron (ca. 100 bp) in the 5′ part of the gene. For example, the F26G16.15 gene contains a single intron of 97 bp in the same location as *Bz*1 (Figure 6B). Only seven *Arabidopsis* sequences had two introns and three exons and of these, two (MMP21.3 and At2g16890) have introns that are similar in size and location to those in the *F3galtase* gene (Figure 6A). Although the level of nucleotide identity between the MMP21.3 and *F3galtase* genes is 43% and the amino acid sequence is 27% identical, the two genes are highly conserved with regard to the exon/intron positions. Because the *F3galtase* and MMP21.3 genes have similar structures, perhaps the MMP21.3 gene encodes a GT with similar substrate usage and catalytic properties to F3GalTase.

## Discussion

The unique structure of the *F3galtase* gene is conserved throughout the Solanaceae. The occurrence of Ct and Mt sequences in the promoter of *F3galtase* is of particular interest by virtue of the potential for these insertions to modulate gene expression by functioning as enhancer or suppressor elements. Blanchard and Schmidt (1995) estimated that 3–7% of plant nuclear DNA contains sequences derived from organellar genomes. They also determined that the average insert size is 117 bp and that all of the genes they studied had either Mt or Ct insertions but never both. Four insertions of relatively large segments of DNA from two different organelles is highly unusual and classifies the promoter region of the *F3galtase* gene as a hot spot for insertions. These elements arise from inverted repeats within the Ct and Mt genomes which are part of an ORF encoding proteins with unknown functions. Perhaps the inverted repeats are more susceptible to enzymes catalyzing the cleavage and recombination events necessary for DNA migration.

The single-copy nature of *F3galtase* suggests that regulated expression is dependent solely on elements contained within the gene. Functional testing of the various elements we have identified in the *F3galtase* promoter will be required to determine their role in the temporal and pollen-specific expression of *F3galtase*. One of the first elements to test is the 28 bp sequence because the similarity in size, position, and nucleotide sequence to the 30 bp proximal promoter region of the LAT52 gene argues for a similar function.

Although the *F3galtase* promoter contains bHLH and *Myb*-type binding sites, these sites are not always functional as demonstrated by the finding that the C1 MYB protein preferentially binds to a second, cryptic Myb-binding site in the promoter region of Bz1 (Sainz *et al.*, 1997). Our results from enzyme activity analyses of pollen extracts from petunia plants do not show a positive correlation with the *An*4 genotypes. We would suggest that F3GalTase activity is regulated by novel pollen-specific factors.

The finding that intron II is excised from two virtually perfect, contiguous direct repeats is intriguing. Many transposable elements generate short direct repeats of DNA at the insertion site which can be left behind after excision of the element. Intron II interrupts the PSPG box encoding a highly conserved region of the protein which has been implicated in substrate binding. Perhaps shuffling of DNA in an ancestral gene resulted in the evolution of the highly specific properties of the F3GalTase enzyme.

It is possible that as more gametophyte-specific A/FGT genomic sequences are identified, their structure will be more similar to *F3galtase* than to *Bz*1. The *Bz*1 gene is expressed in both gametophytic and sporophytic tissues and maize pollen, like petunia, requires flavonols to produce a functional germination tube (Coe *et al.*, 1981; Pollak *et al.*, 1995). It appears that in maize, one gene (*Bz*1) has evolved to express a single flavonoid glycosylating activity in all plant tissues. In petunia, and possibly all of the Solanaceae, the single flavonoid glycosylating activity has been replaced by two enzyme activities with different substrate specificities and, we would predict, gene structures. F3GalTase forms only flavonol 3-*O*-galactosides in pollen whereas another gene (the petunia *Bz*1 orthologue described by Vogt and Jones 2000) forms glucosides of both flavonols and anthocyanidins in many tissues.

The occurrence of F3GalTase activity as well as flavonols identical to those found in petunia, in tomato, tobacco, and potato pollen suggests that the *F3galtase* gene co-evolved in these species with the requirement for flavonols in pollen germination (Ylstra *et al.*, 1992; Taylor and Hepler, 1997). The crucial role of flavonols in pollen germination may be the driving force for the conservation throughout evolution of the Solanaceae of the unique gene structure and enzyme activity that characterizes F3GalTase. A critical developmental role for enzymes that glycosylate small molecules is not without precedence. Woo *et al.* (1999) demonstrated that a meristem-localized GT in pea and alfalfa is necessary for proper tissue differentiation and growth. The generation of transgenic plants lacking F3GalTase activity will be an important experiment to assess the role of F3GalTase in the biology of flavonol-induced pollen germination.

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## References

Blanchard, J.L. and Schmidt, G.W. 1995. Pervasive migration of organellar DNA to the nucleus in plants. J. Mol. Evol. 41: 397–406.

Ceska, O. and Styles, E.D. 1984. Flavonoids from *Zea mays* pollen. Phytochemistry 23: 1822–1823.

Coe, E.H. Jr., McCormick, S.M. and Modena, S.A. 1981. White pollen in maize. J. Hered. 72: 318–320.

Eyal, Y., Curie, C. and McCormick, S. 1995. Pollen specificity elements reside in 30 bp of the proximal promoters of two pollen-expressed genes. Plant Cell. 7: 373– 384.

Ford, C.M., Boss, P.K. and Høj, P.B. 1998. Cloning and characterization of *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase, a homologue of the enzyme encoded by the maize *bronze-1* locus that may primarily serve to glycosylate anthocyanidins *in vivo*. J. Biol. Chem. 273: 9224–9233.

Furtek, D., Schiefelbein, J.W., Johnston, F. and Nelson, O.E. Jr. 1988. Sequence comparison of three wild-type *Bronze-1* alleles from *Zea mays*. Plant Mol. Biol. 11: 473–481.

Gerats, A.G.M., Vrijlandt, E., Wallroth, M. and Schram, A.W. 1985. The influence of the genes An1, An2, and An4 on the activity of the enzyme UDP-glucose:flavonoid 3-*O*-glucosyltransferase in flowers of *Petunia hybrida*. Biochem. Genet. 23: 591–598.

Huits, H.S., Gerats, A.G., Kreike, M.M., Mol, J.N., Koes, R.E. 1994. Genetic control of dihydroflavonol reductase gene expression in *Petunia hybrida*. Plant J. 6: 295–310.

Kapitonov, D. and Yu, R.K. 1999. Conserved domains of glycosyltransferases. Glycobiology 9: 961–978.

Lee, H.-S., Singh, A. and Kao, T.-h. 1992. Rnase X2, a pistil-specific ribonuclease from *Petunia inflata*, shares sequence similarity with solanaceous S proteins. Plant Mol. Biol. 20: 1131–1141.

Li, Y., Baldauf, S., Lim, E.-K. and Bowles, D.J. 2001. Phylogenetic analysis of the UDP-glycosyltransferase multigene family of *Arabidopsis thaliana*. J. Biol. Chem. 276: 4338–4343.

McKnight, S.L., Gavis, E.R., Kingsbury, R. and Axel, R. 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25: 385–398.

Miller, K.D., Guyon, V., Evans, J.N.S., Shuttleworth, W.A. and Taylor, L.P. 1999. Purification, cloning, and heterologous expression of a catalytically efficient flavonol 3-*O*-galactosyltransferase expressed in the male gametophyte of *Petunia hybrida*. J. Biol. Chem. 274: 34011–34019

Mo, Y., Nagel, C. and Taylor, L.P. 1992. Biochemical complementation of chalcone synthase mutants defines a role for flavonols in functional pollen. Proc. Natl. Acad. Sci. USA. 89: 7213–7217.

Pollak, P.E., Hansen, K, Astwood, J.D. and Taylor, L.P. 1995. Conditional male fertility in maize. Sex. Plant Reprod. 8: 231–241.

Ralston, E.J., English, J.J. and Dooner, H.K. 1988. Sequence of three bronze alleles of maize and correlation with the genetic fine structure. Genetics 119: 185–197.

Sainz, M., Grotewold, E. and Chandler, V. 1997. Evidence for direct activation on an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb-domain proteins. Plant Cell 9: 611–625.

Sink, K.C. 1984. Taxonomy. In: K.C. Sink (Ed.) Petunia. Springer-Verlag, Berlin, pp. 3–9.

Spelt, C., Quattrocchio, F., Mol, J.N.M. and Koes, R. 2000. *anthocyanin1* of *Petunia* encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell 12: 1619–1631.

Strommer, J., Gerats, A.G.M., Sanago, M. and Molnar S.J. 2000. A gene-based RFLP map of petunia. Theor. Appl. Genet. 100: 899–906.

Taylor, L.P. and Hepler, P.K. 1997. Pollen germination and tube growth. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 461–491.

Taylor, L.P. and Jorgensen, R. 1992. Conditional male fertility in chalcone synthase-deficient petunia. J. Hered. 83: 11–17.

Vogt, T, and Taylor, L.P. 1995. Flavonol 3-*O*-glycosyltransferases associated with petunia pollen produce gametophyte-specific flavonol diglycosides. Plant Physiol. 108: 903–911.

Vogt, T., Wollenweber, E. and Taylor L.P. 1995. The structural requirements of flavonols that induce pollen germination of conditionally male fertile *Petunia*. Phytochemistry 38: 589–592.

Vogt, T. and Jones, P.2000. Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends Plant Sci. 5(9): 380–386.

Wise, R.P., Rohde, W. and Salamini, F. 1990. Nucleotide sequence of the *Bronze*-1 homologous gene from *Hordeum vulgare*. Plant Mol. Biol. 14: 277– 279.

Woo, H.-H., Orbach, M.J., Hirsch, A.M. and Hawes, M.C. 1999. Meristem-localized inducible expression of a UDP-glycosyltransferase gene is essential for growth and development in pea and alfalfa. Plant Cell. 11: 2303–2315.

Ylstra, B., Turaev, A., Benito Moreno, R.M., Stöger, E., van Tunen, A.J., Vicente, O., Mol, J.N.M. and Heberle-Bors, E. 1992. Flavonols stimulate development, germination and tube growth of tobacco pollen. Plant Physiol. 100: 902–907.