Identification of a CYP84 Family of Cytochrome P450-Dependent Mono-Oxygenase Genes in *Brassica napus* and Perturbation of Their Expression for Engineering Sinapine Reduction in the Seeds[1]

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CYP84 is a recently identified family of cytochrome P450-dependent mono-oxygenases defined by a putative ferulate-5-hydroxylase (F5H) from Arabidopsis. Until recently F5H has been thought to catalyze the hydroxylation of ferulate to 5-OH ferulate en route to sinapic acid. Sinapine, a sinapate-derived ester in the seeds, is antinutritional and a target for elimination in canola meal. We have isolated three *F5H*-like genes (*BNF5H1-3*) from a cultivated *Brassica napus*, whose amphidiploid progenitor is considered to have arisen from a fusion of the diploids *Brassica rapa* and *Brassica oleracea.* Two cultivated varieties of the diploids were also found to contain *BNF5H3* and additionally either *BNF5H1* or *BNF5H2*, respectively. Whereas all three are >90% identical in their coding sequence, *BNF5H1* and *BNF5H2* are closer to each other than to *BNF5H3*. This and additional data suggest that the two groups of genes have diverged in an ancestor of the diploids. *B. napus* showed maximal *F5H* expression in the stems, least in the seeds, and subtle differences among the expression profiles of the three genes elsewhere. Transgenic *B. napus* with cauliflower mosaic virus 35S-antisense *BNF5H* contained up to 40% less sinapine, from 9.0 ± 0.3 mg in the controls to 5.3 ± 0.3 mg g^{-1} seed. F5H from Arabidopsis and a similar enzyme from sweetgum (*Liquidamber styraciflua*) has recently been shown to have coniferaldehyde hydroxylase activity instead of F5H activity. Thus the supply of 5-OH coniferaldehyde or 5-OH ferulate has a bearing on sinapine accumulation in canola seeds.

Secondary metabolism is indeed essential to the form and function of plants and also to the survival of these sessile members in their diverse and dynamic ecosystems. The products range from relatively simple phenolic esters to the complex, heterogeneous polymers of lignin. These metabolites have a wide range of often disparate functions ranging from providing mechanical strength to pest deterrence to facilitation of symbiosis (Strack, 1997). The general phenylpropanoid pathway is central to secondary metabolism from various perspectives (Dooner et al., 1991; Chapple and Ellis, 1992; Davin and Lewis, 1992; Chapple, 1994; Boudet et al., 1995; Dixon et al., 1996; Douglas, 1996; Mol et al., 1998; Weisshaar and Jenkins, 1998; Whetten et al., 1998; Dixon and Steele, 1999). Secondary metabolism is highly variable not only among plant species but also among different cell types within a plant, and generalizations from specific examples provide a useful framework but not conclusive insights for other systems (Lewis and Yamamoto, 1990; Campbell and Sederoff, 1996). The general phenylpropanoid pathway and the associated pathways are thus essentially a composite.

The phenylpropanoid pathway produces from L-Phe or L-Tyr a number of hydroxycinnamate derivatives by a series of hydroxylations and methylations culminating at one end in sinapic acid in angiosperms (Fig. 1 in Campbell and Sederoff, 1996). Sinapic acid and a number of preceding intermediates are in turn used as the precursors for various compounds (Dixon et al., 1996). Sinapine is one such product derived from sinapic acid (for the pathway, see Chapple et al., 1992). It is found only in crucifer seeds (Regenbrecht and Strack, 1985; Bouchereau et al., 1991), but its role is unknown. Strack (1981) speculated that it is a storage reserve for seedling growth in *Raphanus sativus*, but it does not have this function in Arabidopsis (Chapple et al., 1992). The presence of sinapine in oilseed Brassicas (notably rapeseed/canola) is undesirable because it is an antinutritional component of the seed meal (Bell, 1993). It imparts a bitter flavor, and its metabolite is responsible for the fishy odor in the eggs of certain brown-shelled egg laying hens that are fed on rapeseed/canola meal (Pearson et al., 1980; Ismail et al., 1981). Sinapine is present in all *Brassica* species (Velasco and Möllers, 1998; Wang et al., 1998;) and in nearly all of the crucifers that have been examined (Regenbrecht and Strack, 1985; Bouchereau et al., 1991).

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We have been investigating metabolic engineering as a means to reduce sinapine in canola seeds. Here we present the cloning, characterization, and antisense suppression of the CYP84 family of cytochrome P450-dependent mono-oxygenase genes from *Brassica napus* that resulted in up to 40% reduction in the sinapine content. This work was completed while the available genetic evidence implicated the Arabidopsis ferulate-5-hydroxylase (F5H) in the production of 5-OH ferulate from ferulate, a precursor in the sinapate synthesis pathway (Chapple et al., 1992; Meyer et al., 1996). Very recently, a sweetgum (*Liquidamber styraciflua*) CYP84 member (Osakabe et al., 1999) and the Arabidopsis F5H (Humphreys et al., 1999) have been shown to catalyze the hydroxylation of coniferaldehyde far more efficiently than ferulate when expressed in yeast. The two enzymes, however, show a difference in their substrate spectrum. We discuss our results in light of these new observations.

RESULTS

Isolation of Three Unique F5H cDNA Clones from *B. napus*

Five cDNA clones (BNF5H) hybridizing to an Arabidopsis F5H probe were isolated from a stem cDNA library of *B. napus*. The cDNA inserts in two clones appeared truncated but otherwise identical in sequence to the insert in another clone (*BNF5H1*). Thus only *BNF5H1* and the remaining two clones, *BNF5H2* and *BNF5H3* were characterized further. These three showed nucleotide sequence polymorphism in the open reading frame (ORF) and a significant difference in the 3′-untranslated region (UTR) sequences. The nucleotide sequences of the inserts in these clones have been deposited in GenBank (accession nos. AF214007, AF214008, and AF214009).

The insert in *BNF5H1* (1,880 bp) has a 38-nucleotide 5′-UTR, a 1,560-nucleotide ORF, and a 282-nucleotide 3′-UTR, whereas *BNF5H2* (1,884 bp) has a 51-nucleotide 5′-UTR, a 1,560-nucleotide ORF, and a 273-nucleotide 3′-UTR. The first ATG in the putative ORF of *BNF5H2* is preceded by an in-frame TAG sequence at −36 position relative to the ATG. The flanking sequences in both of the *BNF5H1* and *BNF5H2* (AAT ATG GAG) have the consensus nucleotide present (underlined) in the plant translational initiation context sequence (Joshi et al., 1997). Thus, the ORF of 1,560 nucleotide present in *BNF5H1* and *BNF5H2* is predicted to encode a 520-amino acid polypeptide of 58.5 kD. The third cDNA clone (*BNF5H3*) with an insert of 1,835 nucleotide appeared to be truncated at the 5′ region up to the 18th nucleotide of the ORF. This clone has a 292-nucleotide 3′-UTR.

The ORFs in *BNF5H1* and *2* are very similar to each other (98% nucleotide sequence identity; 99% amino acid sequence identity; Fig. 1). They are similar to the Arabidopsis F5H ORF (520 amino acids; Meyer et al., 1996) at the nucleotide (90% identity) and amino acid (93% identity) levels. The near-complete ORF in *BNF5H3* (514 amino acids) also shows the same level of identity to the corresponding portion, but lacked a codon for Pro at position 39, which is present in the other F5Hs. The Arabidopsis F5H is the first member of CYP84, a new family of cytochrome P450-dependent mono-oxygenases (Meyer et al., 1996). Recently, a coniferaldehyde 5-hydroxylase clone from sweetgum has been isolated (Osakabe et al., 1999). This CYP84 member is 72% identical to Arabidopsis and BNF5H protein. The *B. napus* F5Hs also contain a high degree of amino acid sequence conservation in the Pro-rich region immediately following the N-terminal hydrophobic region and in the C-terminal heme-binding region (residues P_{450} FGXGRRX-

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BNF5H1 MESSISQTLS QVIDPTTGIL IVVSLFIFIG LITRΔRRRPPY PPGPRGWPII  50
BNF5H2 ---------- --L------- ---------- ----Δ------ ----------  50
BNF5H3 (      )---- -LL----A-- -I-------- ----Δ---SΔ- ----------  43
ATHF5H ---------- KLS----SLV ---------S F---Δ------ ----------  50
CAld5H -D--LHEA-Δ -PLΔ-M-ΔLF FIIP-LLLL- -VS-L-Q-L-- ----K-L---  48

BNF5H1 GNMSMMDQLT HRGLANLAKK YGGLCHLRMG FLHMYAVSSP DVAKQVLQVQ 100
BNF5H2 ---------- ---------- ---------- ---------- ---------- 100
BNF5H3 ---L------ ---------- ---------- ---------- H--R------  93
ATHF5H ---L------ ---------- ---------- ---------- E--R------ 100
CAld5H ---L------ -----K---Q ----F--K-- ----V---T- -M-R------  98

BNF5H1 DSVFSNRPAT IAISYLTYDR ADMAFAHYGP FWRQMRKVCV MKVFSRKRAE 150
BNF5H2 ---------- ---------- ---------- ---------- ---------- 150
BNF5H3 --I------- ---------- ---------- ---------- ---------- 143
ATHF5H ---------- ---------- ---------- ---------- ---------- 150
CAld5H -NI------- ---------- ---------- -------L-- --L------- 148

BNF5H1 SWASVRDEVD KMIRSVSSNV GKSINVGEQI FALTRNITYR AAFGSACEKG 200
BNF5H2 ---------- ---------- ---------- ---------- ---------- 200
BNF5H3 ---------- ---------- ---------- ---------- ---------- 193
ATHF5H ---------- --V---C-- --P------- ---------- ---------- 200
CAld5H --E------- SAV-V-A--I -STV-I--LV ----K----- ----TISHED 198

BNF5H1 QDEFIRILQE FSKLFGAFNV ADFIPYFGWI DPQGINKRLV KARNDLDGFI 250
BNF5H2 ---------- ---------- ---------- ---------- ---------- 250
BNF5H3 ---------- ---------- ---------- -----S---- ---------- 243
ATHF5H ---------- ---------- ---------- ---------- ---------- 250
CAld5H ----VA---- --Q------I -----WLK-V Δ-----V--N ---GA----- 247

BNF5H1 DDIIDEHMKK KENQNSVDAG DVVDTDMVDD LLAFYSEEAK LVSETADLQN 300
BNF5H2 -------I-- ---------- ---------- ---------- ---------- 300
BNF5H3 ---------- -----T--D- --G------- ---------- -----T---- 293
ATHF5H ---------- -----A--D- ---------- ---------- ---------- 300
CAld5H -K---D-IQ- GSΔKNSΔEEΔ ΔΔ-------- -----G---- Δ---SD---- 291

BNF5H1 SIKLTRDNIK AIIMDVMFGG TETVASAIEW ALTELLRSPE DLNRVQQELA 350
BNF5H2 ---------- ---------- ---------- ---------- --K------- 350
BNF5H3 ---------- ---------- ---------- ---------- --K------- 343
ATHF5H ---------- ---------- ---------- ---------- --K------- 350
CAld5H -----K---- --Δ------- ---------- -M---MK--- --KK------ 340

BNF5H1 EVVGLDRRVE ESDIEKLTFL KCTLKETLRL HPPIPLLLHE TAEDTEIDGY 400
BNF5H2 ---------- ---------- ----N----- ---------- ---------- 400
BNF5H3 ---------- ---------- ---------- ---------- ---------- 393
ATHF5H ---------- --------Y- ---------M ---------- -----S---F 400
CAld5H V--------- -K-F----Y- --V---V--- ---------- ----A-VG-- 390

BNF5H1 FVPKKSRVMI NAFAIGRDKN SWVDPETFRP SRFLEPGVPD FKGSNFEFIP 450
BNF5H2 ---------- ---------- ---------- ---------- ---------- 450
BNF5H3 ---------- --------PK --P-A----- --------A- ---------- 443
ATHF5H -I-------- --------PT --T--D---- ---------- ---------- 450
CAld5H YI-A------ --C------- --A--D---- ----KD---- ---N------ 440

BNF5H1 FGSGRRSCPG MQLGLYALEL AVAHILHCFT WKLPDGMKPS ELDMSDVFGL 500
BNF5H2 ---------- ---------- ---------- ---------- ---------- 500
BNF5H3 -----K---- ---------- ---------- --------A- ----N----- 493
ATHF5H ---------- --------D- ---------- ---------- ----N----- 500
CAld5H ---------- ---------T T---L----- -E-------- --E-N----- 490

BNF5H1 TAPKATRLYA VPSTRLICSV*  520
BNF5H2 ---------- --C-------*  520
BNF5H3 --------F- --------A-*  513
ATHF5H --------F- --T-----AL*  520
CAld5H ---R-I--T-  --P--L-PLY* 511
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Figure 1. Comparison of the deduced amino acid sequence of the *B. napus* F5H (BNF5H) with that of Arabidopsis (Meyer et al., 1996; ATHF5H) and sweetgum (Osakabe et al., 1999; CAld5H). Hyphens indicate identical amino acid, and the variants are as shown. The parenthesis in BNF5H3 denotes incomplete amino-terminal portion, Δ indicates a gap, and the asterisks mark the end.

CXG_{460} of BNF5H1), which is characteristic to P450 mono-oxygenases. Thus, the three BNF5Hs were deemed to belong to CYP84. Further, as alluded to above, the three cDNA clones were considered to represent three CYP84 genes. Additional evidence for this is provided below.

B. napus and Its Progenitors Have at Least Two Groups of F5H Genes

The molecular relationship of *BNF5H1*, *-2*, and *-3* was studied further by examining the 3′-UTR and the ORFs in the three cDNA clones and the introns in the corresponding genomic segments. The nucleotide sequence of the 3′-UTR of *BNF5H1* differs from that of *BNF5H2* by 13% and from that of *BNF5H3* by 37%. Similarly, the 3′-UTR of *BNF5H2* differs from that of *BNF5H3* by 37%. Aside the absence of a Pro codon in *BNF5H3*, the ORFs of the three clones showed nucleotide polymorphism as originally detected by restriction digestion patterns (data not shown) and subsequently by nucleotide sequence analysis.

A genomic clone was identified by probing a *B. napus* cv Westar library with a *BNF5H1* probe. A preliminary analysis showed that the region corresponding to the central one-third of the ORF contained two introns (data not shown). Two sets of PCR primers (see "Materials and Methods") were used as individual pairs to amplify this region from a *B. napus* genomic DNA sample, and the independently derived amplicons (0.95 or 1.1 kb) were cloned into pBluescript SK^+ (Stratagene, La Jolla, CA). The inserts in nine of these were sequenced completely. Both of the 0.95- and 1.1-kb categories contained two introns and the exon regions. The exon sequence allowed assignment of the amplicons to the three cDNA clones. Three types of amplicons corresponding to BNF5H1, -2, and -3 were found collectively. The congeneric introns thus identified were compared among themselves. The two introns are situated at identical positions in all three genes with respect to the stop codon (Fig. 2). However, the length and nucleotide sequence show significant differences. The introns of *BNF5H1* and *-2* are more similar to each other than to those of *BNF5H3*. Further, the nucleotide sequence dissimilarity scores for the introns shown in Figure 2 support the relationship gleaned from the comparison of the UTRs. Thus these results collectively establish two organizational groups of *BNF5H* genes within the *B. napus* CYP84 family, one comprising *BNF5H1* and -2 and the other *BNF5H3*.

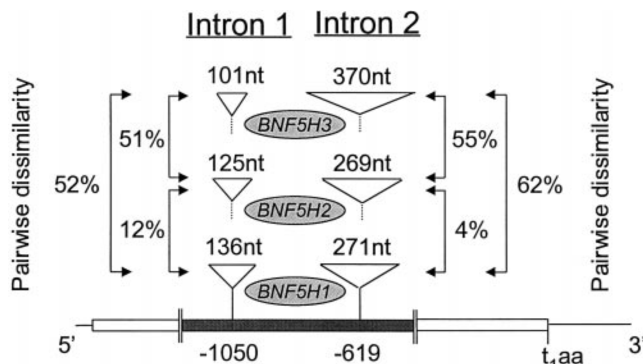

Figure 2. Organizational characteristics of the introns, pertaining to the grouping of the three *BNF5H* genes. The two introns found in the shaded region corresponding to the cDNA are located at identical points with reference to the stop codon (taa; 1,050 and 619 nucleotides upstream) in the predicted ORF. The length of the congeneric introns vary as shown. The nucleotide sequence dissimilarity from pairwise comparisons is shown on the left for intron 1 and on the right for intron 2. The regions corresponding to the unshaded portions of the ORF have not been examined for the presence of introns.

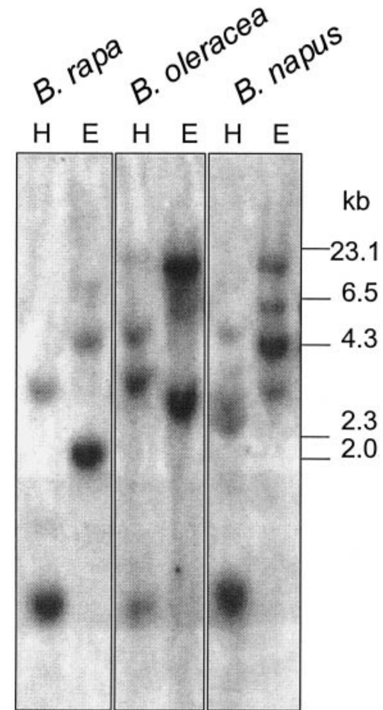

Figure 3. Southern-blot analysis of *BNF5H*-like sequences in the genomic DNA of the amphidiploid *B. napus* and its two diploid progenitor representatives. *Hin*dIII (H) and *Eco*RI (E)-digested genomic DNA were probed with a *BNF5H1* cDNA fragment (nucleotide 256–nucleotide 702). *Hin*dIII-digested bacteriophage λ-DNA size markers in kb are as shown.

The cultivated *B. napus* used here is a descendent of a natural amphidiploid (n = 19) containing the genomes of both *Brassica rapa* (n = 10) and *Brassica oleracea* (n = 9). It was of interest to find the association, if any, of the above gene groups to the diploid species. Southern-blot analysis of genomic DNA detected four bands in *B. napus* and two in each of the two cultivated species of *B. rapa* and in *B. oleracea* (Fig. 3), suggesting the presence of at least four genes in *B. napus* and at least two in the other species. The third band in the *Hin*dIII digest of *B. oleracea* might be due to an additional gene or due to an internal cleavage site in one of the genes. The attribution of the

individual genes to the *B. rapa* or *B. oleracea* lineage was done by amplification of the genomic DNA with gene-specific primers from the coding region. The specificity of these primer pairs was ascertained with the respective cDNA controls. As shown in Figure 4, *BNF5H3* is present in both of the *B. rapa* and *B. oleracea*, *BNF5H1* only in *B. rapa*, and *BNF5H2* only in *B. oleracea*. As expected, *B. napus* yielded the amplicons for all three genes. The above-noted molecular relationship among the three BNF5H genes vis-a-vis the genealogical relationship was confirmed from the conservation of similarity among the amplicons at their nucleotide sequence level. For example, the *BNF5H1* amplicon was more similar to its counterpart from the other two species than to the amplicons of BNF5H2 or BNF5H3 from any of the three species (data not shown). This is noteworthy in view of the closer relationship of *BNF5H1* to *BNF5H2* than to *BNF5H3*.

Expression Profile of the *BNF5H* Genes in *B. napus*

Northern-blot analysis of total RNA from various parts of *B. napus* with an *BNF5H* cDNA probe showed a very abundant transcript of 1.9 kb in the stem and much less in the root, leaf, pod, bud, and the least in the seed tissue (Fig. 5). The expression of other phenylpropanoid pathway genes, Phe ammonia-lyase and cinnamate 4-hydroxylase also followed this general pattern, but the cinnamate 4-hydroxylase gene expression was almost equally abundant in both of the stem and root tissue (data not shown). A 25-cycle reverse transcriptase (RT)-PCR of poly(A^+) RNA with gene-specific primers showed an amplicon of predicted size for each primer pair in all of these tissues, indicating expression of the three genes in these tissues (data not shown). The RT-PCR analysis was repeated with total RNA and with fewer cycles (15-cycle reaction) to provide a better distinction of the relative transcript abundance. A Southern-blot hybridization analysis of the resulting amplicons was done with a common probe as described in "Materials and Methods" (Fig. 6).

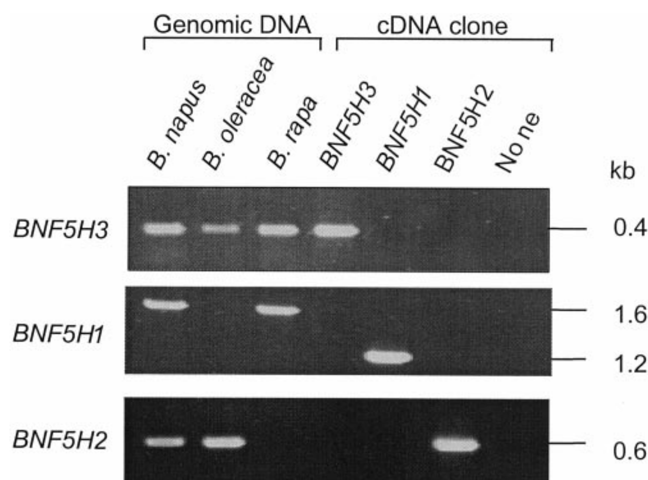

Figure 4. Gene-specific amplification of *BNF5H* sequences from *B. napus*, *B. rapa*, and *B. oleracea* genomic DNA. The positive controls with the *BNF5H* cDNAs and negative control with no template DNA are as shown. The *BNF5H1*-specific primers flank the introns, hence the difference in size between the genomic and cDNA amplicons. The primer sets used here were as follows: set A for *BNF5H1*; set C for *BNF5H2*; and set E for *BNF5H3* (see "Materials and Methods").

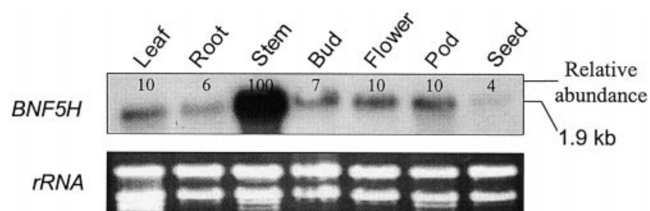

Figure 5. Northern-blot analysis of *BNF5H* gene expression in *B. napus* tissue. Twenty micrograms of total RNA for each sample was electrophoresed, blotted, and probed with a *BNF5H1* (nucleotides 256–702) cDNA probe. rRNA, Ethidium bromide-stained ribosomal RNA. The relative abundance of *BNF5H* transcript was measured in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

In general the stem tissue showed an abundant expression of all three genes, and the pods and seeds showed the least transcript abundance for all three genes. BNF5H3 was expressed nearly uniformly in young and old leaf, root, bud, and flower but much less in pods and seeds. It is interesting that BNF5H2 expression was greater in the seed tissue than in the root, bud, flower, or pod tissue. Also, it showed a high level of expression in mature leaves. BNF5H1 also showed a variation in the expression levels. Thus although all of the tissues that were examined here expressed all three genes, there were differences in the level of expression. This experiment was repeated with different primer pairs that confirmed the expression profile.

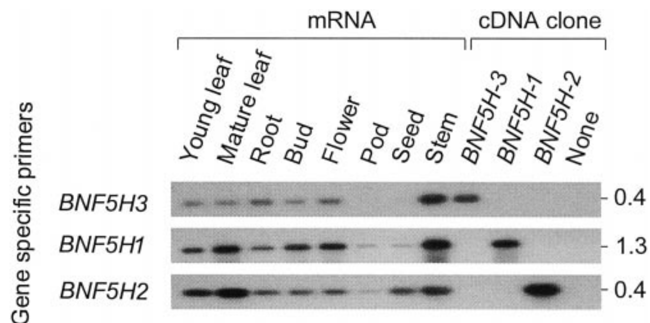

Figure 6. RT-PCR analysis of 10 μg of total RNA with gene-specific primers. The controls with the *BNF5H* cDNAs or no template DNA are as shown. The primer sets used here (see "Materials and Methods") were as follows: set A for *BNF5H1*; set D for *BNF5H2*; and set E for *BNF5H3*. The *BNF5H1*-specific primers used here did not yield an intron-containing amplicon, indicating absence of genomic DNA contamination in the template. Note that the primer set A flanking an intron gave a larger amplicon with genomic DNA as shown in Figure 4. Amplicon size is in kb.

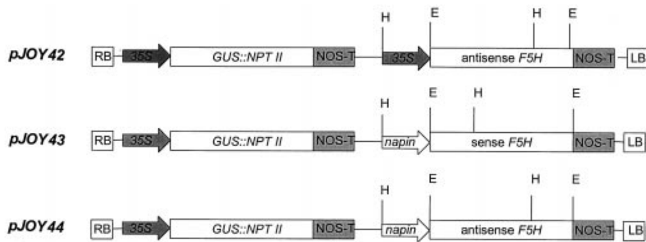

Figure 7. Diagrammatic representation of the T-DNA region of the *A. tumefaciens* plasmids used for the genetic transformation of *B. napus*. E and H, *Eco*RI and *Hin*dIII restriction endonuclease sites, respectively; RB and LB, The right and left borders, respectively, of the T-DNA. 35S, CaMV35S-AMV leader promoter module from Datla et al. (1993); napin, the *B. napus* napin promoter from Kohno-Murase et al. (1994). NOS-T, Nopaline synthase terminator region; GUS::NPTII, the bifunctional fusion gene containing β-glucuronidase and neomycin phosphotransferase II (Datla et al., 1991). These plasmids are derivatives of the pHS723 vector that can replicate in *A. tumefaciens* (Hirji et al., 1996). Southern hybridization of *Hin*dIII-digested genomic DNA with a GUS probe would estimate the number of T-DNA insertions in the transgenics.

F5H Transgenic Lines of *B. napus* and Their Molecular Genetic and Biochemical Attributes

A total of 15 transgenics with pJOY43 (napin-sense *BNF5H1*) and six with pJOY44 (napin-antisense *BNF5H1*) were obtained. *Hin*dIII-restricted genomic DNA of these were probed for T-DNA copy number as noted in Figure 7 to find the lines for generating homozygotes (discussed below). The napin promoter is seed specific, and its activity spans the mid-phase of seed development (Joseffson et al., 1987), during which sinapine is synthesized (Vogt et al., 1993).

The sinapine content of the mature seeds from the primary transgenic lines (T_0 seeds) was assayed on the assumption that perturbation of *BNF5H* gene expression would show an alteration. Not all of the primary lines could be assayed because of the poor seed yield in some. None of the 10 napin-sense *BNF5H1* transgenics showed an enhanced sinapine content, but two of them had much less sinapine than the rest or the control (Fig. 8, see "Primary lines" lot). Four of the five napin-antisense *BNF5H1* lines also showed a reduction (Fig. 8, see "Primary lines" lot). The T_0 seeds from those primary transgenic lines with a single-T DNA insertion were retained for deriving homozygotes: T_0 seeds to give T_1 plants and the seeds from these (T_1 seeds) to give T_2 plants and T_2 seeds therefrom. The homozygotes were identified by their non-segregating β-glucuronidase phenotype from a screening of approximately 40 seedlings from each plant. The average concentration of extractable sinapine in the two homozygotes of the pHS723 transgenic lines (vector control) was 9.0 ± 0.3 mg g^{-1} seed. This value was taken as 100% for all of the comparisons. It was surprising that the T_2 homozygous seeds from napin-sense lines also contained less sinapine (Fig. 8, the "Homozygous lines" lot), but the reduction was not as strong as the apparent reduction in the T_0 samples from the tissue culture-derived plants (Fig. 8, the "Primary lines" lot). The single homozygous napin-antisense line also showed a modest reduction.

We examined the impact of constitutive antisense suppression of *BNF5H* by generating transgenic lines with inverted *BNF5H1* under the control of a CaMV35S promoter (pJOY 42). From 22 transgenic lines, 18 were assayed, and these showed various levels of sinapine in the T_0 seeds (Fig. 9, see "Primary lines" lot). As with the other transgenic lines above, homozygotes were derived, and the seeds from five lines were assayed for their sinapine content. It was

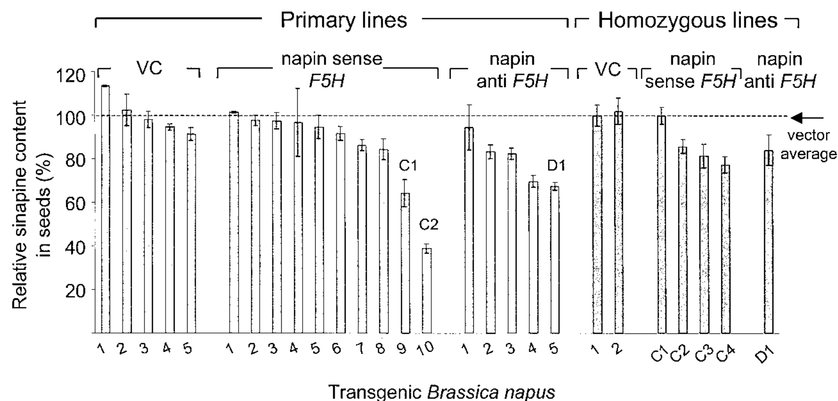

Figure 8. HPLC analysis of the sinapine content of the seeds from transgenic lines with *BNF5H1* (shown in the illustration as *F5H*) under the control of a napin promoter. Three replications were done for each transgenic line. The alphanumeric designation in the bars of the "Primary lines" lot identify the parents of the corresponding homozygotes in the "Homozygous lines" lot. There are additional homozygotes for which no corresponding primary line data were generated here because of the sample size in the parents. The sinapine content in mg g^{-1} mature seeds for the homozygous samples were as follows: vector control (VC) line 1, 9.1 ± 0.1; line 2, 8.8 ± 0.3; C1, 8.9 ± 0.2; C2, 7.6 ± 0.5; C3, 7.3 ± 0.8; C4, 6.9 ± 0.6; and D1, 7.5 ± 1.1. The average of the homozygous vector control, 9.0 ± 0.3 mg g^{-1} mature seeds, was taken as 100% for the relative values shown.

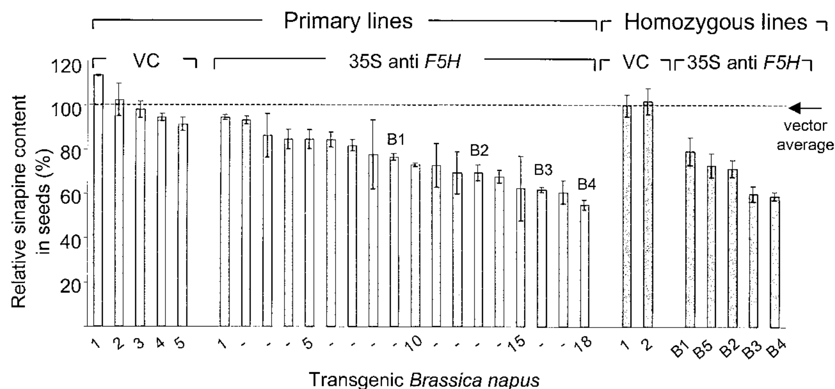

Figure 9. HPLC analysis of the sinapine content of the seeds from CaMV35S-antisense *BNF5H1* (shown in the illustration as 35S anti-F5H) transgenic lines. Three replications were done for each transgenic line. The vector control (VC) is the same as in Figure 8. The sinapine content in mg g^{-1} mature seeds for the homozygous samples was as follows: vector control (VC); line 1, 9.1 ± 0.1; line 2, 8.8 ± 0.3; B1, 7.1 ± 0.8; B2, 6.4 ± 0.6; B3, 5.4 ± 0.5; B4, 5.3 ± 0.3; and B5, 6.5 ± 0.8. The average of the homozygous vector control, 9.0 ± 0.3 mg g^{-1} mature seeds, was taken as 100% for the relative values shown.

reduced in all of them by up to 40% in two lines (Fig. 9, see "Homozygous lines" lot).

The *BNF5H* expression in the homozygotes was analyzed in northern and western blots. The older stem (bottom 10–15 cm) tissue had been found to have the most abundant expression of the native *BNF5H* gene (Fig. 5), and this tissue was examined in the 35S antisense-*BNF5H1* transgenics (Fig. 10). With the exception of line B3, the transgenic lines did not show any significant reduction in the *BNF5H* transcript level.

A similar *BNF5H* gene expression pattern was also observed when the top 15-cm stem was probed (data not shown). Napin-sense *BNF5H1* transgenic seeds showed a very abundant *BNF5H* transcript level as compared with the vector-alone transformed plants (Fig. 10), as expected for the expression characteristic of this promoter (Joseffson et al., 1987). Thus, one of the 35S antisense lines had a slightly diminished *BNF5H* transcript level, whereas all of the napin-sense lines had a very high level in the seeds. We then examined the level of BNF5H polypeptide in the 35S antisense *BNF5H1* transgenics by immunoblot analysis of crude extracts. The stem tissue of the 35S antisense *BNF5H1* transgenic plants (except line B1) showed lower levels of F5H polypeptide than the vector-transformed plants (Fig. 11).

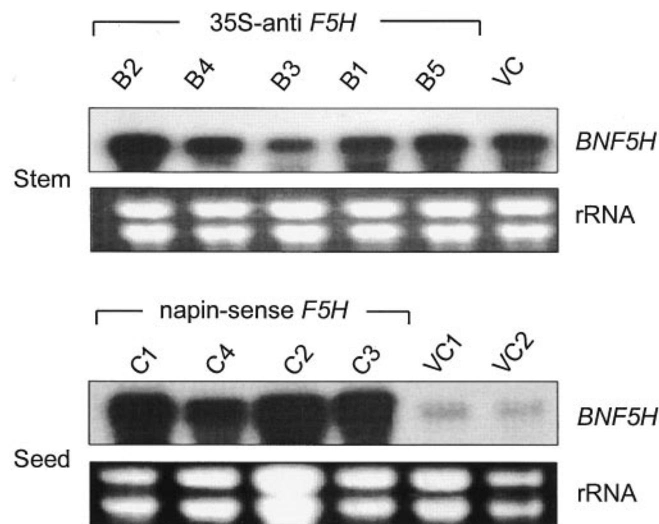

Figure 10. Northern-blot analysis of homozygous (T_2) transgenics with a *BNF5H* probe. Fifteen micrograms of total RNA from the bottom 10-cm portion of stem tissue from each of the 35S antisense-*BNF5H1* transgenics or from the seeds of each of the napin sense-*BNF5H1* transgenic lines of *B. napus* was electrophoresed, blotted, and probed; the seeds from the latter were collected over a 20- to 30-d period after flowering in each line. VC, Empty vector transgenic line control; rRNA, ethidium bromide-stained ribosomal RNA. Here and in subsequent figures, the alphanumeric designations B1 through B5 and C1 through C4 denote homozygous transgenic lines (T_2), whose lineage has been referred to in Figures 8 and 9.

Immunoblot analyses of the seed extracts from the wild-type or vector-alone transgenic lines did not identify a clearly visible polypeptide corresponding to the one found in the stem tissue, indicating that its presence in the seeds is at the detection limit of this method (data not shown). Although the seeds from the napin-sense lines contained a very high level of BNF5H transcript, there was no detectable increase in the BNF5H polypeptide.

All of the T_2 plants were normal for their vegetative and reproductive phases of growth in a controlled cabinet (data not shown). *B. napus* leaves contain a sinapoylmalate synthase activity, which is implicated in the synthesis of sinapoyl malate from sinapate-

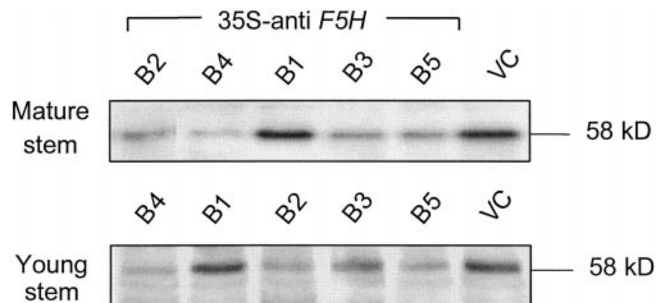

Figure 11. Immunoblot analysis of 35S antisense-*BNF5H1* stem extracts. Fifteen micrograms of total protein extracts was boiled, separated on SDS-PAGE, blotted, and probed with rabbit antisera raised against a truncated recombinant BNF5H produced in *Escherichia coli*. VC, Vector-alone transgenic line. Mature and young stem refer to bottom 10 to 15 cm and top 15 cm of stem, respectively. Equal loading of protein was verified by amido black staining of the membranes.

derived precursors (Strack et al., 1990). Thus the 35S antisense F5H lines should show a reduction in the sinapoyl malate content if the *BNF5H* gene product(s) were involved in the biosynthesis of sinapate. We found a reduction in the sinapoyl malate content of the T_2 plants by up to 25% relative to the vector-alone transgenics (data not shown).

DISCUSSION

Multiplicity of the CYP84 Family Members in *B. napus* and Its Progenitors

We have identified two groups comprising BNF5H1 and -2 in one and -3 in the other. Another very closely related gene in the *BNF5H3* group might have escaped detection here. The *BNF5H* genes identified here add three more members to the CYP84 family, which previously contained the Arabidopsis F5H (Meyer et al., 1996) and subsequently a sweetgum coniferaldehyde hydroxylase (Osakabe et al., 1999). We have further shown that the two groups of the *B. napus* CYP84 genes are unlikely to have converged from the hybridization of the two diploid parents. Instead, the two groups must have formed prior to the divergence of *B. oleracea* and *B. rapa*. The greater divergence between *BNF5H3* and either of the other two genes suggests that this duplication might have occurred very early in the lineage of the *Brassica* spp., whose ancestor remains unknown (Truco et al., 1996).

A large number of genes exist as families in *Brassica* spp. In some cases the gene multiplicity has been attributed in part to the amphidiploid nature of *B. napus* (Slocombe et al., 1994). However, the diploids or their ancestor may have undergone a polyploidy event (Kianian and Quiros, 1992; Truco et al., 1996; Anderson and Warwick, 1999). The presence of two groups of *BNF5H* genes in the diploid *Brassica* spp. might also be due to such polyploidy. The presumptive genetic basis aside, the closer similarity of *BNF5H1* to *BNF5H2*, despite their genealogical separation, suggests that the genes got "fixed" for whatever functional requirements. That the gene-specific primers designed for *B. napus* sequences indeed amplified DNA from the cultivars of *B. rapa* and *B. oleracea* chosen at random further illustrates this conservation. It will be interesting to study the biochemical attributes of the *BNF5H* gene products, especially in view of the observation that P450 monooxygenases differing very little in their primary structure can exhibit different activities (Wachenfeldt and Johnson, 1995).

Expression Pattern of the CYP84 Members

The observation that the stem tissue had the most abundant steady-state level of *BNF5H* mRNA resembles the expression profile of *F5H* in Arabidopsis (Ruegger et al., 1999) and other phenylpropanoid metabolism-related genes (Lee and Douglas, 1996; Bell-Lelong et al., 1997; Mizutani et al., 1997; Hu et al., 1998; Inoue et al., 1998). It is consistent with a role for F5H in lignification. Despite the production of sinapoyl esters in the seeds, the expression of *BNF5H* here was no greater than that in the roots, pods, buds, or flowers. Thus, there was no evidence for the ester accumulation being supported by an enhanced *BNF5H* transcription level in the seeds. In contrast we observed a higher level of transcripts in the roots than in the seeds. Canola-associated microbes have been found in the rhizosphere (Siciliano et al., 1998), and hitherto unidentified phenylpropanoid compound(s) in the roots may have a role in these interactions. Whereas similar gene expression pattern with Phe ammonia-lyase (Wanner et al., 1995), cinnamate 4-hydroxylase (Bell-Lelong et al., 1997), 4-coumarate:coenzyme A ligase (Lee et al., 1995), and chalcone synthase (Schmid et al., 1990) might be linked to defense-related synthesis of flavonoids and phytoalexins, perhaps *BNF5H* gene expression is also pertinent to this.

Against the modest difference in the spatial expression profile of the three *BNF5H*, it is interesting to note the ubiquitous expression of *BNF5H3*, a gene that occurs in all three *Brassica* spp. The presence of two *BNF5H3* genes in *B. napus*, one from each of the two fusion parents, may be a contributing factor. Given the minor differences in the gene sequences, it will be necessary to determine the expression profile of the three promoters transgenically to discern the differences, if any, in the temporal pattern, and to gain further insights into the spatial expression pattern.

Reduction in the Sinapate Ester Content of the Transgenics

In the 35S-antisense *BNF5H* plants there was a noticeable reduction in the BNF5H polypeptide level and a reduction of the sinapine content by up to 40%. The *BNF5H* transcript level was affected in only one of the transgenics. This suggests that the antisense *BNF5H* transcripts did not affect the transcription of the endogenous *BNF5H* gene or the stability of the transcript as much as the translation of BNF5H protein was affected. Antisense suppression results generally in a concurrent reduction of both of the transcript and the polypeptide (Bourque, 1995). However, there is at least one report where the transgenic plants suffered a substantial reduction in the amount of polypeptide without encountering a similar effect on transcription (Temple et al., 1993). The mechanism of antisense suppression is not completely elucidated; antisense transcripts may hybridize with the endogenous sense transcript, destabilize the formation of the ribosomal complex, and prevent translation (Mol et al., 1994).

The homozygous napin-antisense *BNF5H* line showed a reduction of only 17%. Obviously, more

lines are required to address the impact of seed-specific antisense suppression, but the additional T_0 lines from this study (Fig. 8) are not suitable owing to their multiple transgene copies and genetic segregation (data not shown). The modest reduction of the sinapine content in the napin transgenic lines might be due to variability or suppression of the native BNF5H genes. The transgene transcript was abundant in the sense lines but there was no increase in the polypeptide level. We have not identified the basis for this result.

The generalized pathway for phenylpropanoid metabolism depicts F5H catalyzing the formation of 5-hydoxyferulate, a precursor of sinapate, and sinapate in turn as the precursor for sinapine and for sinapoyl CoA in two bifurcated pathways (Chapple et al., 1992; Meyer et al., 1996, 1998; Ruegger et al., 1999; Fig. 1 in Campbell and Sederoff, 1996). Sinapoyl CoA has been considered as the precursor for sinapyl alcohol, which is then polymerized into syringyl (S) lignin. This inference has, however, been challenged very recently (Chen et al., 1999; Humphreys et al., 1999; Osakabe et al., 1999). Osakabe et al. (1999) demonstrated that the sweetgum CYP84 product carries out the hydroxylation of coniferaldehyde (*ConAld*) to 5-OH *ConAld* approximately 140 times more efficiently than that of ferulate to 5-OH ferulate, thus prompting the term ConAld hydroxylase to refer to the enzyme. Further, *ConAld* non-competitively inhibits ferulate hydroxylation. Humphreys et al. (1999) have simultaneously shown that *ConAld* (K_m = 1 μM), but not ferulate (K_m = 1 mM), is the preferred substrate for the Arabidopsis F5H, which is 72% identical in its primary structure to the sweetgum *ConAld* hydroxylase.

What, then, is the basis for our observation that antisense suppression of the CYP84 members in *B. napus* diminished sinapine synthesis in the seeds? Sinapine is accumulated mostly in the cotyledons in *B. napus* (Fenwick, 1979). If the enzymatic properties of *B. napus* F5H are similar to that of the sweetgum *ConAld* hydroxylase, a supposed absence of *ConAld* in the non-lignified cotyledons would explain the participation of the *B. napus* enzyme in ferulate hydroxylation in vivo. Alternatively, as Humphreys et al. (1999) have speculated for Arabidopsis, an aldehyde oxidase might yield sinapate from 5-OH *ConAld*-derived sinapoyl aldehyde, and thus any constraint on the flux to 5-OH *ConAld* would also diminish sinapine synthesis. Clearly, a biochemical characterization of the *BNF5H* gene products is necessary to address the potential functional differences among them.

As Lindberg and Negishi (1989) have shown, a single amino acid substitution in the coumarin 7 hydroxylase, a P450 mono-oxygenase, can expand its substrate specificity to include a steroid. It is interesting that the Arabidopsis F5H but not the sweetgum gene product (both from a yeast expression system) can hydroxylate coniferyl alcohol (Humphreys et al., 1999; Osakabe et al., 1999), suggesting potential differences in the catalytic repertoire of CYP84 enzymes as well. It will also be informative to compare the activity of the CYP84 gene products from various species, and thus the identification of three independent *BNF5H* genes will be useful in this regard. The apparent minor differences among the *BNF5H* gene products may have some enzymological implications.

Implications of BNF5H Down-Regulation for Canola Meal Improvement

Canola meal is a significant source of protein supplement in animal feed. Our results show that it is possible to achieve a measure of reduction in the sinapine content by down-regulating BNF5H, thus offering an avenue for improving the meal quality. Besides sinapine reduction, this approach may offer other collateral benefits. Cherney et al. (1991) had found that the brown midrib mutants of maize and sorghum, which are lower in the relative content of syringyl lignins, are more digestible as forage. The digestibility of forage is inversely related to its lignin-derived methoxyl content (Dixon et al., 1996). The syringyl lignin contains two methoxyl groups on each aromatic ring, in contrast to the guaiacyl lignin that contains one. Thus, the feed would be more digestible if it contained less syringyl lignin even if the total lignin content of a feed material, typically guaiacyl + syringyl lignins, remained unaltered. The F5H null mutant of Arabidopsis has very little syringyl lignin. However, it is interesting that cauliflower mosaic virus promoter-directed production of F5H in this mutant restores the syringyl lignin content, and the use of a cinnamate 4-hydroxylase promoter instead renders the lignin almost completely syringyl type (Meyer et al., 1998; Marita et al., 1999). Thus, the down-regulation of the *F5H* genes in *B. napus* may have a favorable impact on the lignin composition and meal digestibility, which are yet to be determined.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Brassica napus cv Westar, *Brassica oleracea* cv Horizon, and *Brassica rapa* cv Green Valiant plants were used in this study. The plants were planted in Rediearth (Grace Horticultural Products, Ajax, Canada) potting medium, watered with a fertilizer solution (0.2 g/L N:P:K, 20:20:20; Plant Products Company, Brampton, Canada), and grown in a chamber under 16-h light/25°C and 8-h dark/20°C cycle. The light intensity was 380 μmol m^{-2} s^{-1} photosynthetic photon flux density. The samples were collected, frozen in liquid nitrogen, and stored at −80°C until needed.

Chemicals

Purified sinapate esters used as the reference standards were initially the kind gifts of Drs. D.I. McGregor (Agriculture and Agri-Food, Saskatoon, Canada) and B.E. Ellis and S.X. Wang (University of British Columbia, Vancouver), and subsequently were isolated and purified from canola by Ms. K. Gossen (National Research Council-Plant Biotechnology Institute, Saskatoon, Canada). HPLC grade solvents were purchased from J.T. Baker (Phillipsburg, NJ), and all of the other reagents were of reagent grade from Sigma (St. Louis).

DNA and RNA Methods

The nucleic acid-related enzymes used were from Life Technologies (Burlington, Canada). Oligonucleotide synthesis and DNA nucleotide sequencing were conducted at the National Research Council, DNA Technologies Unit. A *B. napus* stem cDNA library in Uni-ZAP XR vector (Stratagene) constructed in our laboratory according to the supplier's instruction manual was screened with an Arabidopsis *F5H* cDNA probe (Meyer et al., 1996; kind gift of Dr. C.C. Chapple, Purdue University, West Lafayette, IN) to isolate the *B. napus F5H* cDNA clones. The plasmids from the phages were isolated according to the supplier's instruction manual. The clones with insertions >1.5 kb were selected for determining the nucleotide sequence in both directions. LASERGENE Biocomputing Software (DNASTAR, Inc., Madison, WI) was used for sequence analysis.

Genomic DNA was isolated from leaves using a Nucleon PHYTOPURE plant DNA extraction Kit (Amersham, Buckinghamshire, UK). Fifteen micrograms of genomic DNA digested with restriction endonucleases was separated by 1% Tris [tris(hydroxymethyl)aminomethane]-acetate EDTA agarose gel electrophoresis (Sambrook et al., 1989) and transferred onto a GeneScreen Plus membrane (NEN Life Science Products, Boston). The membrane was prehybridized at 42°C for 3 h in 10 mL of prehybridization buffer (Sambrook et al., 1989; 50% [v/v] formamide, 5× Denhardt's reagent, 5× SSC, 0.1% [w/v] SDS, and 100 μg mL^{-1} denatured salmon sperm DNA). [^{32}P]dCTP-labeled *F5H* probe was synthesized from a fragment of *BNF5H1* cDNA (nucleotides 256–702) using a rediprime II random primer labeling kit (Amersham). Hybridization with the probe was done overnight in 10 mL of hybridization buffer (prehybridization buffer plus 2% [w/v] dextran sulfate). After hybridization, the membrane was rinsed twice with a solution of 0.2× SSC and 0.1% (w/v) SDS (Sambrook et al., 1989), washed twice with 0.2× SSC and 0.1% (w/v) SDS for 15 min at 50°C, and once with 0.1× SSC and 0.1% (w/v) SDS for 30 min at 65°C. It was then exposed to an x-ray film (Fuji Photo Film, Tokyo) for 1 to 5 d with Quanta III intensifying screens (DuPont, Wilmington, DE).

Total RNA from various tissues was isolated using TRIzol reagent (Life Technologies) according to the supplier's protocol. Northern-blot analysis was done with 15 μg of total RNA that was electrophoretically separated and transferred to a GeneScreen Plus membrane (Sambrook et al., 1989). The *BNF5H* probe was prepared as above.

PCR Analysis

The introns in the genomic segments were amplified with the following primer pairs: pair 1, for BNF5H1 and BNF5H2, 5′-CGAGTCATGGGCTTCTGTT-3′ and 5′-TATCGCTGACGCTACCGTTCC-3′; pair 2, for BNF5H3, 5′-AGATGAGGAAAGTGTGT-3′ and 5′-CCGTAATAACTCCGTTAAG-3′. The first primer in each of these pairs and in the primer sets outlined below is based on the sense strand, and the second primer is derived from the non-coding strand of the cDNA clone sequences deposited in GenBank (see "Results"). PCR was set up in a 50-μL volume that contained 250 ng of genomic DNA as template, 1× PCR buffer (Life Technologies), 1.5 mM $MgCl_2$, 200 μM each dNTP, and 50 pmol of each gene-specific primer. The samples were heated at 94°C for 3 min to denature the template, then cooled to 72°C, and immediately 2.5 units of *Taq* DNA polymerase was added. The amplification was conducted for 30 cycles in a DNA thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA) at a setting of 94°C for 45 s, 56°C for 1 min, and 72°C for 2 min for each cycle and a final extension step at 72°C for 10 min. The positive controls contained as the template 20 ng of *BNF5H1*, *-2*, or *-3* cDNA clones, and the negative control contained only the primers in question.

Poly(A$^+$) mRNA was isolated from 5 mg of total RNA from various tissues using an Oligotex mRNA midi kit (Qiagen, Mississauga, Canada). Two micrograms of the eluted mRNA sample was used for the first strand cDNA synthesis by Superscript RNase H$^-$ Reverse Transcriptase (Life Technologies) following the manufacturer's protocol. The single-stranded cDNA was dissolved in 100 μL of Tris-EDTA buffer, the PCR was set up with 2.5 μL of cDNA template as described above, and amplification was conducted for 25 cycles.

RT-PCR with total RNA isolated from various tissues was done as follows. First strand cDNA was synthesized from 10 μg of total RNA by Superscript RNase H$^-$ Reverse Transcriptase (Life Technologies). The reaction was stopped by incubating at 68°C for 10 min, and the total volume made up to 50 μL with deionized water. PCR was set up in a 50-μL reaction volume containing 1 μL of the above cDNA preparation as the template, 1× PCR buffer (Life Technologies), 1.4 mM $MgCl_2$, 200 μM each dNTP, 50 pmol of each of the two gene-specific primers for BNF5H1, -2, or -3, and 2.5 units of platinum *Taq* DNA polymerase (Life Technologies). After an initial denaturation of DNA for 2 min, amplification was conducted for 15 cycles as described above except that the annealing temperature was at 61°C for 1 min. The positive controls contained 20 pg of relevant BNF5H cDNA as the template, and the negative control contained the primers but no template. After agarose gel electrophoresis, Southern-blot hybridization was done with a probe derived from *BNF5H2* (nucleotides 1,310–1,695) that hybridizes to all three *BNF5H* amplification products.

Specific primer sets for each of the three *BNF5H* genes were used: set A and B for *BNF5H1*; set C and D for *BNF5H2*, and set E and F for *BNF5H3*. The nucleotide sequence of these sets are as following: set A [5′-TTCTCGAACCGAC-

CAGCT-3′ and 5′-ACAAATAAGGCGCGTGCT-3′]; set B [5′-GAGTGGGCATTGACTGAATTAC-3′ and 5′-CCATACCAACCACCTTTCC-3′]; set C [5′-GGGCATTGACTGAGTTAT-3′ and 5′-CCACAGCTCAAGAACCATC-3′]; set D [5′-CGCGACAAGAACTCTTGGGTTGAT-3′ and 5′-CCACGTTCAAGAACCATCAACC-3′]; set E [5′-ACCCTAAATCTTGGCCTGACG-3′ and 5′-CCATACCTACTACCAACCCTTCG-3′]; set F [5′-TTAGAACCGGGAGTAGC-3′ and 5′-CCTATTACCAACCCTTCG-3′].

Genetic Transformation of Plants

The plasmids for *Agrobacterium tumefaciens*-mediated transformation were constructed by inserting a 1.8-kb *Eco*RI fragment of the *BNF5H1* cDNA (this fragment lacks the poly[A] region) in the sense or antisense orientation (Fig. 7). All of the plasmids are based on the binary vector pHS723 constructed in this laboratory (Hirji et al., 1996). *A. tumefaciens* GV3101 [pMP90] (Koncz and Schell, 1986) derivatives with the pJOY plasmids were obtained by the freeze-thaw method described by An et al. (1988). Genetic transformation of *B. napus* hypocotyl explants and regeneration of plants were according to Moloney et al. (1989). Putative transgenics were selected by kanamycin resistance and confirmed as transgenics by 5-bromo-4-chloro-3-indolyl-β-glucuronic acid-based β-glucuronidase (GUS) assay (Jefferson et al., 1986) afforded by the bifunctional selection marker (GUS::neomycin phosphotransferase II; Datla et al., 1991) within the T-DNA (Fig. 7). The primary lines with a single-copy of the transgene were identified following Southern blot of *Hin*dIII-restricted DNA and probing with a *GUS* gene fragment. These were selfed to obtain T_0 seeds. Ten T_1 plants were selfed to obtain T_2 seeds. Seedlings from T_2 seeds were screened by the GUS assay to identify homozygous T_2 seed lots.

Immunoblot Analysis

Rabbit antisera were raised against a recombinant BNF5H1 protein, lacking the first 170 amino acids of the deduced nucleotide sequence from the *BNF5H1* ORF, produced in *Escherichia coli* BL21(DE3) pLysS cells carrying a derivative of the plasmid pRSET (Invitrogen, Carlsbad, CA). The polypeptide was purified according to the supplier's instructions prior to immunizing the rabbits. One gram of pulverized *B. napus* stem tissue was ground with 1.5 mL of buffer (50 mM HEPES [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], pH 7.5, 1 mM EDTA, pH 7.5, and 10% [v/v] glycerol). The extract was centrifuged at 6,000*g* for 30 min at 4°C, and the supernatant was assayed for protein concentration by the modified Bradford method (Bio-Rad, Hercules, CA). Fifteen micrograms of protein-equivalent of the extract in the gel loading solution (50 mM Tris, pH 6.8, 2.3% [w/v] SDS, 5% [v/v] β-mercaptoethanol, and 10% [v/v] glycerol) was boiled for 10 min and separated in SDS-PAGE (10% acrylamide, w/v) in duplicate gels. The polypeptides were electroblotted onto a polyvinylidene difluoride membrane (Hybond-P, Amersham), and the membrane was probed with the BNF5H antisera, followed by horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham), and chemiluminescent detection with a ECL+Plus western-blotting system (Amersham) according to the supplier's instructions. The duplicate membrane and also the immunoprobed membrane after chemiluminescent detection were stained with 0.1% (w/v) amido black in a 45% (v/v) methanol-5% (v/v) acetic acid solution to verify equal loading and transfer of protein.

HPLC Analysis of Phenolics

Ten seeds, including the seed coat, or 10 mg of freeze-dried leaves from 21-d-old seedlings were ground in 500 μL of 80% (v/v) methanol, incubated at 4°C for 1 h, and frozen at −80°C for 1 h. The extract was thawed and centrifuged at 20,000*g* for 15 min to obtain the supernatant. The pellet was re-extracted with 500 μL of 80% (v/v) methanol at 4°C for 1 h and centrifuged as above. The two supernatants were pooled, and 20 μL of the pooled extract was run on an HPLC column (Nucleosil C18 AB, Alltech, Deerfield, IL) using an acetonitrile/phosphoric acid (1.5% [v/v]) gradient of 10% to 30% over a 35-min period in an HPLC instrument (Vista 5500, Varian, Palo Alto, CA). UV A_{330} was detected with a Polychrom (model 9065, Varian). The average of sinapine and sinapoyl malate contents per milligram of vector alone transformed transgenic seeds or leaves was calculated as 100%. All of the other data were calculated as percentage sinapine or sinapoyl malate content of vector control plants. The SE was calculated from three replications for each transgenic plant.

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