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ARTICLE

RNAi-Mediated Suppression of *DET1* Alters the Levels of Carotenoids and Sinapate Esters in Seeds of *Brassica napus*

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Carotenoids and sinapate esters in *Brassica napus* affect the nutritional value of the seed. In this study, the *B. napus* regulatory gene DE-ETIOLATED1 (*DET1*), which is a negative regulator of light-mediated responses in plants and affects carotenoid and flavonoid pathways in tomato, was suppressed both constitutively and in a seed-specific manner by RNAi. Constitutive silencing of *DET1* resulted in transgenic seeds with substantially elevated levels of lutein, β-carotene, and zeaxanthin relative to nontransgenic seeds. Levels of these carotenoids were also enhanced but to a lesser extent in seeds of transgenic plants with seed-specific silencing of *DET1*. Moreover, sinapate esters 1,2-disinapoylgentiobiose and 1,2-di-*O*-sinapoylglucose were identified in the seeds using 1D and 2D NMR, as well as ESI-MS spectrum analyses. The levels of 1,2-di-*O*-sinapoylglucose in seeds in both sets of transgenic plants were lower compared to nontransgenic seeds. The results revealed that *DET1* suppression in *B. napus* can increase the levels of carotenoids and reduce the levels of sinapate esters simultaneously in the seeds, thus enhancing their overall nutritional value.

KEYWORDS: *DET1*; RNAi; *Brassica napus*; carotenoid; sinapate ester; seed quality

INTRODUCTION

Brassica napus (canola) is a crop widely grown for its oil and other nutritional components in the seeds. Efforts are underway to produce genetically improved *B. napus* with enhanced levels of nutritional components and reduced amounts of antinutritive factors. *B. napus* seed carotenoid compounds, a group of C40 isoprenoid compounds, are valuable byproducts and have great potential in livestock feed. Carotenoid consumption is increasingly associated with protection from a range of diseases due to their fundamental roles as antioxidants and vitamin A precursors (*1*, *2*). Most of the human intake of carotenoids is obtained from consumption of plant sources.

The carotenoid biosynthesis pathway has been extensively elucidated in higher plants (*3*). Manipulation of some synthetic genes in the pathway (*4*) and chromoplast-related genes (*5*) resulted in significant enhancements of carotenoid accumulation. For example, overexpression of daffodil phytoene synthase (PSY) and a bacterial phytoene desaturase in rice resulted in "golden rice", which accumulated carotenoids (*6*). Overexpression of bacterial or plant endogenous PSY increased carotenoid levels in *B. napus* (*7*), potato (*8*), and *Arabidopsis* (*9*). Manipulating the expression of lycopene β-cyclase (*10*), lycopene ε-cyclase, and β-carotene hydroxylase (*11*, *12*) enhanced β-carotene content in plants. More interestingly, fruit-specific silencing of a tomato photomorphogenesis regulatory gene *HIGH PIGMENT-2* (*HP-2*), an orthologue of the *Arabidopsis* gene *DE-ETIOLATED-1* (*DET1*), increased carotenoid and flavonoid content in tomato fruit without affecting other fruit quality parameters and plant phenotype (*13*). *DET1*, a negative regulator of light-mediated responses in plants, plays pleiotropic roles in plant development through complex interactions to target proteins for proteolysis (*14*, *15*) and to affect chromatin remodeling for photoregulated gene expression (*16*). Approximately 1000 genes are either up- or down-regulated in the *Arabidopsis det1-1* mutant compared to wild type (*17*). *DET1* expression affects several secondary metabolism pathways such as carotenoid and flavonoid pathways in tomato plants even though the molecular mechanisms underlying these effects are not yet fully understood.

Sinapate esters such as sinapoylcholine, sinapoylglucose, and sinapoylmalate are synthesized from a common precursor, sinapic acid, in the phenylpropanoid pathway in cruciferous species. These metabolites contribute bitter taste, poor meal palatability, and unpleasant flavor to the meat and milk of animals fed on a *B. napus* seed meal diet (*18*). Thus, they are considered antinutritive compounds and are targets for elimination or reduction in *B. napus* seed (*19*, *20*). The activities of four enzymes, UDP-glucose (UDP-Glc):*B. napus* sinapate glucosyltransferase (BnSGT1), sinapoylglucose:*B. napus* choline sinapoyltransferase (BnSCT), sinapine esterase (SCE), and sinapoylglucose:malate

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Table 1. Primers Used in the Experimental Procedures

primer	sequence	note
DET1-F3	5′-CG*actagtggcgcgcc*GTCTATAATATGGAAACAAC-3′	*Asc*I/*Spe*I sites italicized
DET1-R4	5′-AT*ggatccatttaaat*TGGATGGAAGAGGAATGAACAT-3′	*Bam*HI/*Swa*I sites italicized
35S_F3	5′-CAATCCCACTATCCTTCGCAAGACCC-3′	PCR screening of transgenic plants
Napin_F5	5′-AGCTCCCAATTTATATTCCCAACGGCAC-3′	PCR screening of transgenic plants
DET1_R2	5′-TCATCGCCTAAAATGGATATTGACGACAG-3′	PCR screening of transgenic plants
qDET1_351F	5′-TTTCCGCAGCAGATAGGCATAGG-3′	transcript quantification
qDET1_480R	5′-TTGCTCCGACCATCTGCACTTC-3′	transcript quantification

sinapoyltransferase (SMT), are closely correlated with the accumulation kinetics of the corresponding metabolites (*21*).

In this study, expression of *DET1* in *B. napus* was suppressed constitutively or in a seed-specific manner using a CaMV 35S promoter and the napin promoter (*22*), respectively. The levels of carotenoid compounds and sinapate ester 1,2-di-*O*-sinapoylglucose were evaluated in mature transgenic seeds, and the results were discussed in relation to the use of *DET1* to improve the nutritional attributes of *B. napus* seed.

MATERIALS AND METHODS

Plant Material and Growth Conditions. In this study, a *B. napus* doubled haploid line DH12075 was used to develop independent transgenic lines. All plants were grown in plastic pots (diameter = 12.5 cm) containing Co−Co Mix plant growth media, which consisted of compacted coconut fiber/peat moss/vermiculite (1:3:3, v/v/v) plus Scotts 15-9-12 "Osmocote PLUS" controlled release fertilizer (Scotts Co. LLC, Marysville, OH). All of the plants were kept in a controlled environment greenhouse (16 h light/8 h dark, 20/17 °C). Upon flowering, plants were bagged to prevent accidental crossing, and mature seeds were harvested from each of the transgenic and nontransgenic plants.

RNAi Vector Construction and Plant Transformation. To make a RNAi construct specific for the *B. napus DET1* gene, *B. oleracea* leaf expressed sequence tag (EST), homologous to the 3′ end (1139−1712bp) of the *Arabidopsis DET1* cDNA sequence (AT4G10180.1) was retrieved from the EST collection at the Saskatoon Research Centre (www.brassica.ca). A pair of primers, DET1_F3 and DET1_R4, was designed on the basis of this EST sequence with the addition of restriction enzyme sites *Asc*I/*Spe*I or *Bam*HI/*Swa*I at their 5′ ends for further cloning (**Table 1**). A 462 bp fragment at the 3′ end of the *B. napus DET1* cDNA was isolated by reverse transcription Polymerase Chain Reaction (RT-PCR) using leaf RNA of DH12075 as a template. The PCR product was then enzymatically end-blunted using the Klenow large fragment of DNA polymerase (Invitrogen, Carlsbad, CA) and cloned into intermediate vector pBluescript KS^+, which was digested with the blunt end restriction enzyme *Sma*I. Single palindromic repeats of the cloned *DET1* fragment were isolated from the pBluescript KS using *Asc*I/*Swa* I and *Bam*HI/*Spe*I and sequentially inserted at the corresponding sites of binary vector pGSA1252 (CAMBIA, Canberra, ACT, Australia) digested with the same two pairs of restriction enzymes, resulting in a 300 bp spacer of β-glucuronidase. This RNAi construct, designated pAZ05, was under the control of cauliflower mosaic virus (CaMV) 35S promoter to obtain constitutive *DET1* suppression in transgenic plants. In pAZ04, the same *DET1* RNAi cassette was under the control of the *B. napus* napin promoter for seed-specific *DET1* suppression (**Figure 1A**).

Cotyledon explants of *B. napus* DH12075 were used for *Agrobacterium tumefaciens* (GV3101pMP90)-mediated transformation (*23*). Putative independent transgenic lines that survived phosphinothricin selection were transplanted into the greenhouse. To confirm the presence of the transgene in the putative transgenic plants, PCR analyses using the antisense primer DET1_R4 and sense primers 35S_F3 or Napin_F5 were conducted (**Table 1**). For each construct, 10−15 T_0 plants containing the transgene as determined by PCR analysis were subjected to further analysis.

DNA and RNA Extraction. Total genomic DNA was isolated from leaves of transgenic and nontransgenic plants using the DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). For RNA extraction of the pAZ05 transgenic plants, fully spread young leaves in shoot-tips were excised and immediately frozen in liquid nitrogen. Leaf RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and DNase I (Qiagen) for on-column DNA digestion according to the manufacturer's instructions. Developing seeds and flowers were also sampled as follows; at the blossom stage, approximately 15 flowers only with fully open petals were labeled and dated for each independent transgenic or nontransgenic plant. Thirty days later, the developing seeds were excised from labeled pods and frozen in liquid nitrogen. Seed RNA was extracted according to the method of Shi and Bressan (*24*).

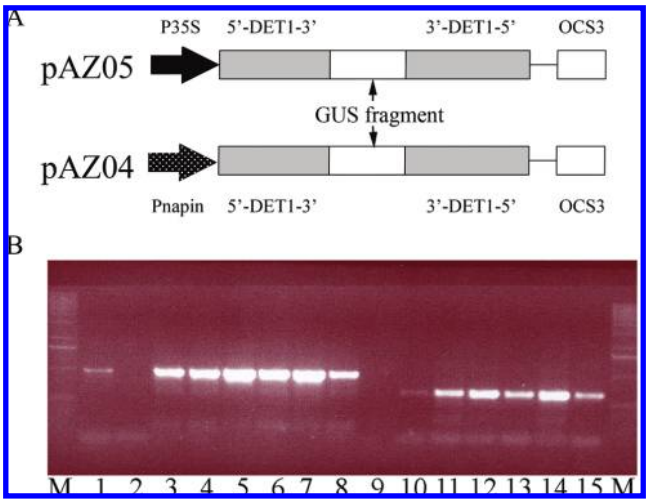

Figure 1. Schematic diagram of *DET1* RNAi cassettes pAZ04 and pAZ05 (**A**) and confirmation of transgene presence in the transgenic plants (**B**). P35S, cauliflower mosaic virus 35S promoter; 5′-DET1-3′ and 3′-DET1-5′, reverse repeats of *DET1* cDNA fragment with 462 bp; OCS3, octopine synthase terminator; Pnapin, *B. napus* napin promoter. Lanes: M, 1kb DNA marker; 1 and 10, positive control using the plasmids containing 35S and napin promoter driven RNAi cassette, respectively; 3−8, independent lines expressing 35S promoter driven RNAi cassette; 11−15, independent lines expressing napin promoter driven RNAi cassette; 2 and 9, empty lanes.

Real Time Quantitative Polymerase Chain Reaction (qRT-PCR). First-strand cDNA was synthesized by reverse transcribing 300 ng of total RNA in a final reaction volume of 20 μL using random primers and 200 units SuperScript II Reverse Transcriptase (Invitrogen, Burlington, ON, Canada). Real time qRT-PCR analysis of *DET1* transcript levels was performed using the gene-specific primers qDET1_351F and qDET1_480R (**Table 1**) and the Quantum RNA Universal 18S Internal Standard (Applied Biosystems/Ambion, Austin, TX) as the internal reference gene. The qRT-PCR mixture contained 10 μL of diluted cDNA, 12.5 μL of 2× SYBR Green qPCR Master Mix (catalog no. 11735-040, Invitrogen, Carlsbad, CA), and 200 nM of each gene-specific primer in a final volume of 25 μL. Control PCR reactions without cDNA templates were also performed for each primer pair. The qRT-PCR reactions were performed using a DNA Engine Opticon 2 system and software (Bio-Rad Laboratories, Richmond, CA) under the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C in a 96-well optical reaction plate (Bio-Rad

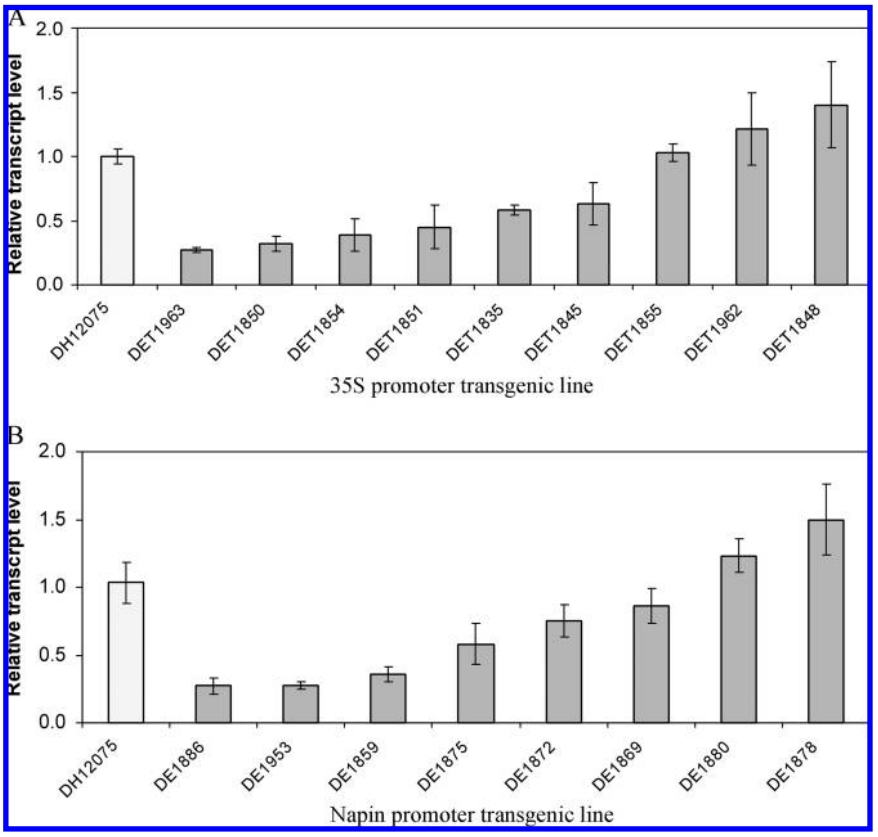

Figure 2. Suppression of *DET1* gene expression in transgenic plants expressing an RNAi cassette under control of the CaMV 35S (**A**) or napin promoter (**B**). The first two young, fully spread leaves of the PAZ05 plants were used for *DET1* transcript analysis. Thirty-day-old developing seeds of the pAZ04 were used for *DET1* transcript analysis.

Laboratories). The specificity of amplicons was verified by melting curve analysis (60−95 °C) after 40 cycles and by agarose gel electrophoresis. All samples were assayed in triplicates from two independent plant samples, and the mean expression values of all the replicates were determined. All PCR reactions displayed efficiencies between 87 and 98%.

Carotenoid Extraction and Quantification. Approximately 200 mg of mature seeds was pooled from each plant and used for carotenoid extraction. Seeds were weighed accurately to four decimal places. Carotenoid extraction and quantitative analysis using high-performance liquid chromatography (HPLC) were carried out as previously described (*12*). Compounds in the eluate were monitored at 450 nm using a photodiode array. HPLC peaks were identified by their retention time and absorption spectra compared to those of known standards. Quantification of carotenoids was conducted using curves constructed from authentic standards. Pure chemical standards β-carotene, lutein, β-cryptoxanthin, zeaxanthin, and violaxanthin were purchased from CaroteNature (Lupsingen, Switzerland). For each independent transgenic line triplicate seed samples were used for carotenoid analysis and analyzed statistically using ANOVA and Duncan's new multiple-range tests.

Analysis of Phenylpropanoids. From each plant, seeds (~200 mg) were homogenized in liquid nitrogen, and then 70% methanol (MeOH) (5 mL per 50 mg of seed powder) was added for phenylpropanoid extraction. The mixture was gently shaken for 30 min at room temperature, and a clear supernatant was obtained after centrifugation at 1864*g* for 10 min. A total of 5 mL of supernatant was dried at room temperature with a stream of nitrogen, and the residue was resuspended in 500 μL of 50% methanol containing 12.5 μg of sinapic acid (catalog no. 49508, Sigma-Aldrich, Oakville, Canada) as an internal standard. The extract was filtered using a 0.2 μm nylon filter, and an aliquot of 20 μL was chromatographed on a Zorbax C_8 column (100 × 2.0 mm, 5 μm, Phenomenex, Torrance, CA) at ambient temperature using a Hewlett-Packard Agilent 1100 HPLC, a G-7120 diode array detector (DAD), and a 120 min linear gradient from 5% aqueous MeOH to 100% MeOH at 0.3 mL/min. UV absorption patterns of the peaks of interest at λ_{280}, λ_{325}, and λ_{370} were similar, so the data at λ_{280} were analyzed for a possible broad detection of phenylpropanoids and flavonoids with Agilent Chemstation ver. 8.01. For each independent transgenic line triplicate seed samples were used for phenylpropanoid analysis and analyzed using Duncan's new multiple-range tests.

For compound identification, 500 g of DH12075 nontransgenic seeds was ground and extracted with 1.5 L of methanol/water (70:30, v/v) three times. The combined extracts were evaporated to near drynesss with a rotory evaporator at 35 °C. The residue was resuspended in methanol/water (50:50, v/v) and separated by HPLC on a preparative Zorbax C_{18} column (150 × 25 mm, Phenomenex) with the same elution gradient and buffer as described above, but the elution rate was set at 3 mL/min. Selected fractions were dried with a stream of nitrogen and then dissolved in freshly opened dimethyl-d_6 sulfoxide (DMSO-d_6) (99.96 + 0.03 TMS, Sigma, St. Louis, MO). Spectra of ^{1}H nuclear magnetic resonance (NMR), correlation spectroscopy (COSY), and heteronuclear multiple bond correlation (HMBC) were measured with a Bruker Avance 500 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) at the Structural Science Center of the University of Saskatchewan. Chemical shifts (δ) were expressed in parts per million (ppm) and coupling constants (J) in hertz (Hz).

RESULTS

Generation of Transgenic Plants. In this study, *Agrobacterium*-mediated gene transformation was performed to introduce CaMV 35S or napin promoter driven *DET1* RNAi cassettes (pAZ05 and pAZ04, respectively) into *B. napus* DH12075. Approximately 30 in vitro plantlets of each construct survived phosphinothricin selection and were transplanted into soil in the greenhouse. PCR was conducted to amplify a transgene fragment from the transgenic plants using the primers specific to the promoters and *DET1* (**Figure 1B**). Sequencing results of the amplified fragments confirmed the transgene presence in the transgenic plants. PCR positive transgenic plants (about 10 from each construct) as well as nontransgenic plants were used for

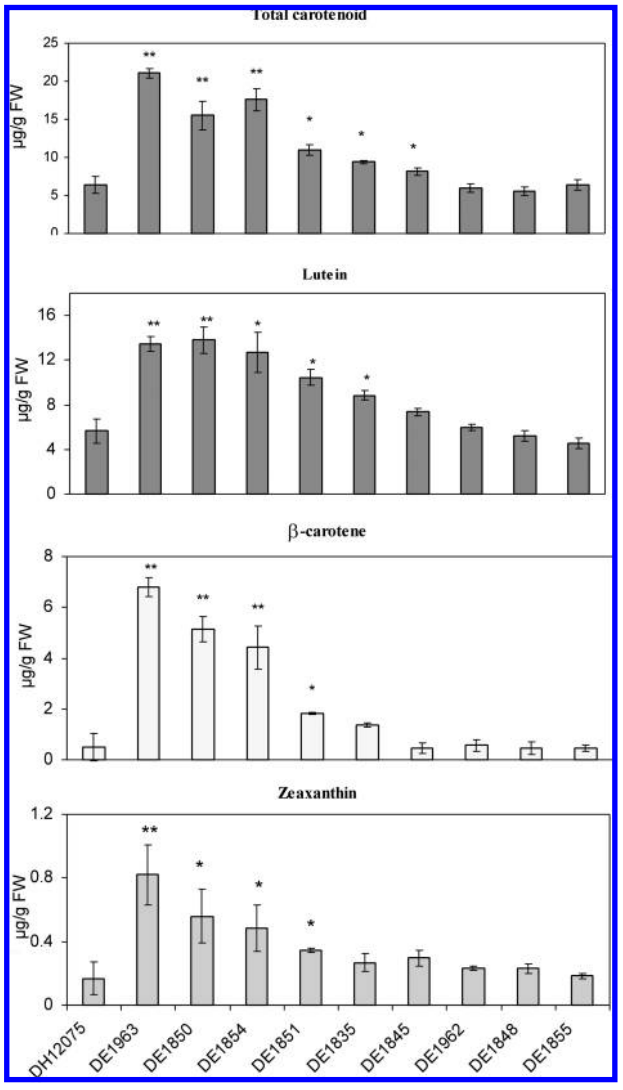

Figure 3. 35S-driven RNAi-mediated suppression of *DET1* expression (PAZ05) enhances carotenoid accumulation in mature seeds of *B. napus* transgenic plants. DH12075, nontransgenic wild type control; FW, fresh weight of dry seeds. Duncan's new multiple-range tests were performed. Significant difference between transgenic plant and wild type is indicated at $p = 0.01$ (**) and $p = 0.05$ (*), respectively.

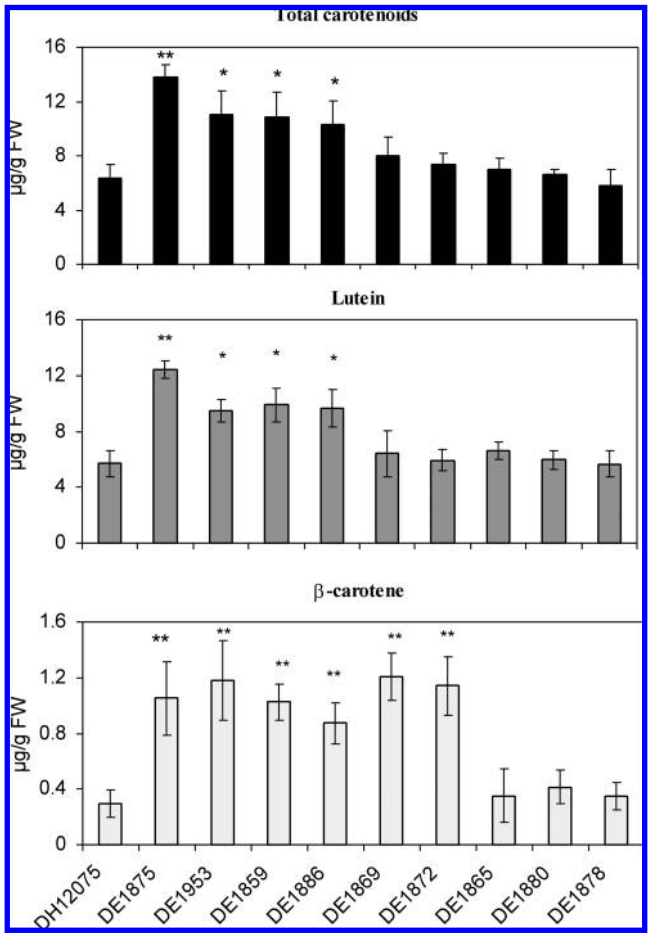

Figure 4. Napin-driven RNAi-mediated suppression of *DET1* expression (PAZ04) results in carotenoid accumulation in mature seeds of *B. napus* transgenic plants. DH12075, nontransgenic wild type control; FW, fresh weight of dry seeds. Duncan's new multiple-range tests were performed. Significant difference between transgenic plant and wild type is indicated at $p = 0.01$ (**) and $p = 0.05$ (*), respectively.

further analysis. Under greenhouse conditions, no visible morphological changes in transgenic plants harboring either cassette compared to nontransgenic plants were observed. However, it was interesting to note that both sets of transgenic seeds had poor germination (40.3 ± 8.6% for pAZ05 and 48.6 ± 9.1% for pAZ04), whereas nontransgenic seeds germinated normally (93.6 ± 6.9%). DE1859, a line overexpressing pAZ04, had 12.3 ± 3.5% seed germination, which was the lowest rate among all of the tested transgenic lines.

Suppression of *DET1* in Transgenic Plants. *DET1* transcript levels in leaves and developing seeds (30 days postanthesis) of transgenic plants were analyzed using real time qRT-PCR. As expected, *DET1* expression was reduced in leaves of most transgenic plants carrying the pAZ05 cassette compared to nontransgenic plants (**Figure 2A**). The lowest *DET1* expression was detected in leaves of DET1963, in which expression was reduced 3.8-fold. Significantly suppressed *DET1* transcript levels from some other lines ranged from 1.6 to 3.1-fold. However, some lines such as DET1962 and DET1848 did not show any significant *DET1* suppression (**Figure 2A**) even though PCR analysis had revealed that they contained the RNAi cassette. Except for seeds of a few transgenic lines carrying pAZ04, most of them had levels of *DET1* transcript 1.7−2.8-fold lower than in nontransgenic seeds (**Figure 2B**).

Carotenoid Levels in Transgenic Seeds. Levels of the three major seed carotenoids, lutein, β-carotene, and zeaxanthin, in mature seeds of transgenic and nontransgenic plants were quantified using HPLC. Compared to nontransgenic seeds, levels of total carotenoids (the sum of the three detected compounds) in the seeds of different transgenic plants carrying pAZ05 increased between 28 and 230%. Lutein and zeaxanthin increased by 30−137 and 61−391%, respectively. The most significant increase was found in β-carotene, ranging from 172 to 1243% (**Figure 3**). Compared to nontransgenic seeds, the levels of total carotenoids, lutein, and β-carotene in mature seeds of the transgenic plants carrying pAZ04 increased by only 34−76, 70−136, and 194−306%, respectively (**Figure 4**). However, the levels of zeaxanthin varied among different pAZ04 lines (data not shown), suggesting that seed-specific napin promoter directed *DET1* silencing might have a limited effect on zeaxanthin accumulation in transgenic seeds. The same phenomenon was also noted in transgenic *Arabidopsis* by Lindgren et al. (*9*). Taken together, our results indicate that pAZ05 is more effective in enhancing the level of carotenoids, especially β-carotene in seeds than pAZ04.

Phenylpropanoid Identification and Quantification. HPLC-UV spectrum analysis of 70% MeOH extract showed at least six distinct peaks in seed extracts between the *DET1* transgenic lines

and wild type *B. napus*. These included sinapoyl glucose as in peak 2. Two compounds corresponding to peaks 3 and 4 were isolated and their structures determined by extensive 1D and 2D NMR, as well as ESI-MS spectrum analysis. Compound **3** (**Figure 5**) was isolated as a pale yellow powder and produced a signal at ESI-MS $[M - H]^-$ m/z 753.5. HMBC analysis showed that H-7,7′ (δ 7.55, 2H, dd, J = 15.6 Hz) was correlated with C-9,9′ (δ_C 167.1) and C-2,2′ and C-6,6′ (δ_C 107.2). The methoxyl group (δ 3.75, 12H, s) was correlated with C-4,4′ (δ_C 147.6) (**Figure 6**).

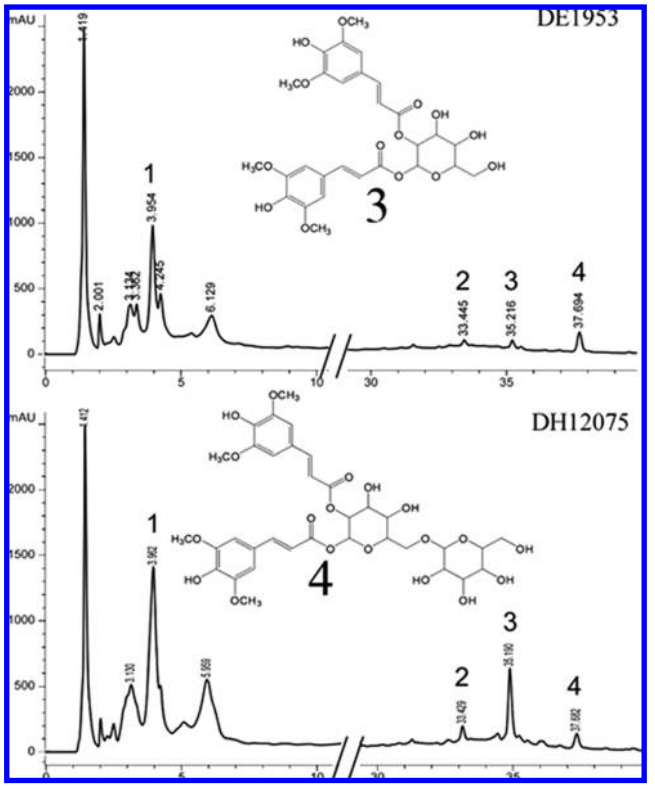

Figure 5. Representative HPLC traces of 70% MeOH extract at 280 nm. Compounds **3**, 1,2-disinapoylgentiobiose, and **4**, 1,2-di-*O*-sinapoylglucose, were isolated from the seeds of *B. napus*.

These data led us to determine that the phenolic part of the compounds had a sinapic acid core. The sugar part of the compound was determined by comparison of chemical shifts and coupling constants with those in the literature (*20*). Thus, compound **3** was determined as 1,2-disinapoylgentiobiose (*25*). Compound **4** (**Figure 5**) showed a similar proton NMR spectrum with compound **3** except for the sugar moiety. The ESI-MS of **4** gave an $[M - H]^-$ ion at 591.4, which was 162 amu less than compound **3**. The reduced molecular mass was consistent with a glucose moiety. Compound **4** was determined as 1,2-di-*O*-sinapoylglucose (CAS Registry No. 91095-79-3) by comparing the NMR data with literature values (*20*). HPLC-UV (λ_{280}) quantitative analysis of the two compounds was performed using sinapic acid as an internal standard. The results showed that seeds of both sets of transgenic lines with reduced levels of *DET1* transcript contained reduced amounts of 1,2-di-*O*-sinapoylglucose (compound **4**) relative to nontransgenic seeds (**Figure 7**).

DISCUSSION

In this study, constitutive and seed-specific silencing of *DET1* was achieved in *B. napus* using CaMV35S and napin promoter driven RNAi cassettes, respectively. In transgenic plants with suppressed *DET1* expression, the levels of carotenoids and sinapate esters that contribute to the overall nutritional quality of the *B. napus* seed were significantly altered, but each was changed in an inverse fashion and in favor of seed nutritional improvement. Our results indicate that *DET1* expression also affects carotenoid and phenylpropanoid pathways in *B. napus* as found in other plants.

In tomato fruits, suppression of *DET1* expression led to a significant increase in lycopene and β-carotene, suggesting that the metabolic flux is strengthened in favor of the β,β cyclization pathway and its derivatives rather than the β,ε cyclization branch leading to α-carotene and lutein (*26*). The present study revealed that levels of both lutein and β-carotene were significantly higher in transgenic seeds than in the control. The level of zeaxanthin in transgenic seeds carrying pAZ05 was also higher. These results clearly demonstrate that in *B. napus* seeds, unlike in tomato fruit, *DET1* suppression results in enhancing both branches of the carotenoid pathway downstream of lycopene.

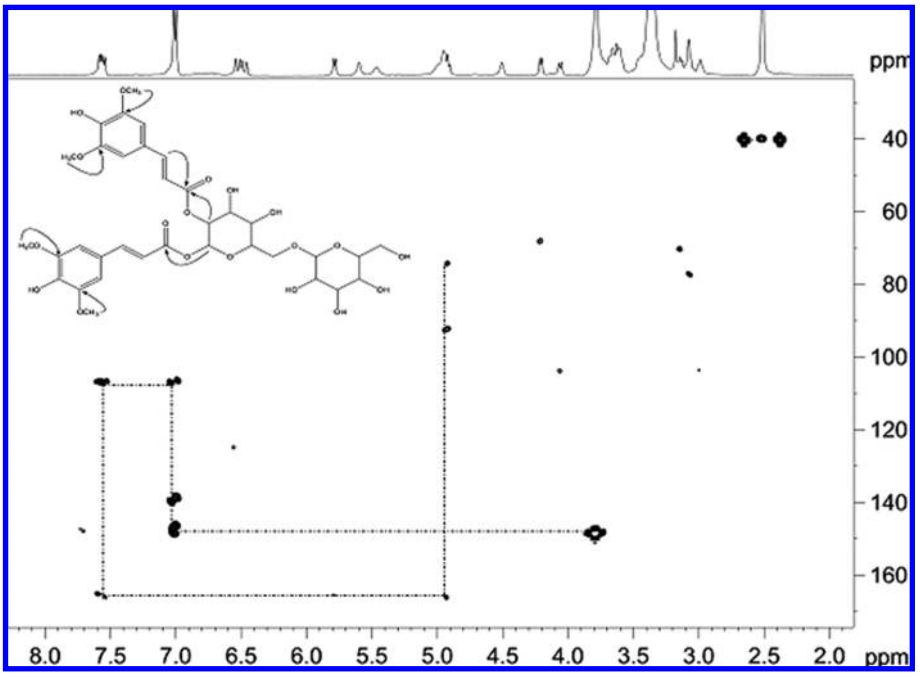

Figure 6. HMBC spectrum of compound **3**.

In the present study, among five phenylpropanoids with altered levels in transgenic *B. napus* seeds, two sinapate esters, 1,2-di-*O*-sinapoylglucose and 1,2-disinapoylgentiobiose, were identified. More interestingly, the levels of 1,2-di-*O*-sinapoylglucose were reduced in the transgenic seeds compared to nontransgenic control. As a sinapate ester, 1,2-di-*O*-sinapoylglucose may contribute to the bitterness of *B. napus* meal and to other nutritional deficiencies. The presence of 1,2-di-*O*-sinapoylglucose in *Arabidopsis* along with expression of genes catalyzing its biosynthesis has been reported in the literature (*27*, *28*). Within the phenylpropanoid pathway, 1,2-di-*O*-sinapoylglucose is located downstream of sinapic acid and is indirectly linked to flavonoid biosynthesis (**Figure 8**). Although *DET1* suppression in tomato led to increased levels of flavonoids and other phenolic compounds in the fruit (*13*), sinapate esters are not among them due to their exclusivity to crucifers. In our study, a preliminary quantitative analysis of flavonoid levels in transgenic seeds suggested that the levels of at least one flavonoid compound, a quercetin derivative, were significantly increased (13.0 ± 3.18 mg/g) in the transgenic seeds carrying 35S driven RNAi cassette and slightly increased (8.03 ± 1.21 mg/g) in those carrying napin promoter driven cassette compared to nontransgenic plants (5.03 ± 1.91 mg/g) ($p = 0.05$). We hypothesize that increased levels of this flavonoid compound might be correlated with the decrease in sinapate ester 1,2-di-*O*-sinapoylglucose. All of these related compounds are synthesized from the common precursor *p*-coumaric acid through the phenylpropanoid pathway and the metabolic flux toward the synthesis of some phenylpropanoids might affect the production of other phenolic compounds, including flavonoids (**Figure 8**).

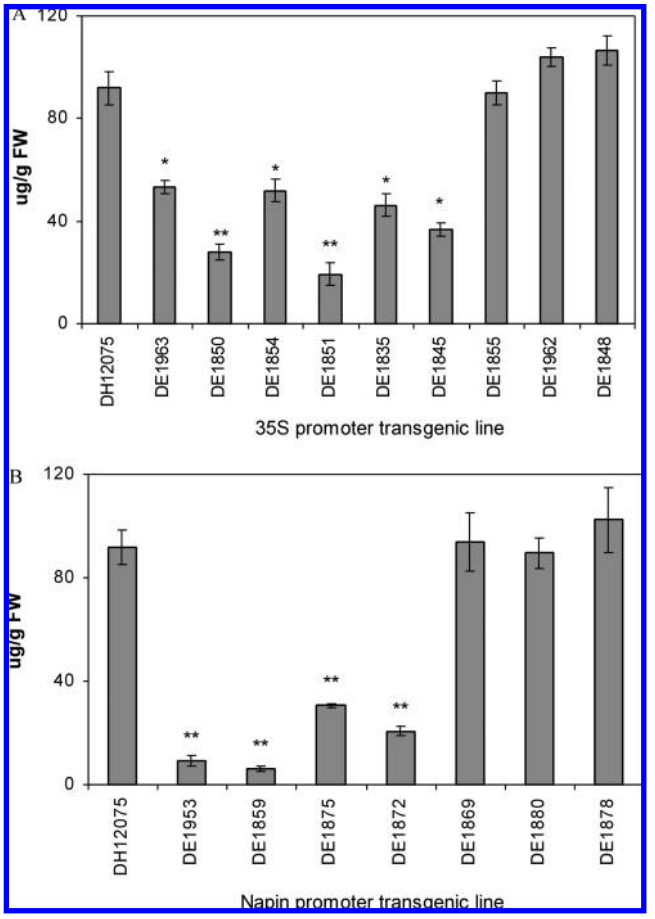

Figure 7. Decreased 1,2-disinapoylgentiobiose levels in mature seeds of *B. napus* transgenic plants carrying CaMV35S (**A**) or napin (**B**) promoter cassette. DH12075, nontransgenic wild type control. Duncan's new multiple-range tests were performed. Significant difference between transgenic plant and wild type is indicated at $p = 0.01$ (**) and $p = 0.05$ (*), respectively.

The *Arabidopsis* partial loss-of-function mutant *det1-1* exhibits light responses in darkness such as short hypocotyls, open cotyledons, and high anthocyanin levels (*29*). In addition, *det1-1* plants show reduced apical dominance, day-length-insensitive flowering (*30*), and defects in germination, expression of light-regulated genes, and chloroplast development (*31*). Compared to *det1-1* darkness phenotypes, the tomato *hp-2* mutant (loss of function of orthologue gene *HIGH PIGMENT*) displayed only minor phenotypes in the dark, and these were only slightly noticeable (*32*). In contrast, transgenic tomato plants with different extents of *DET1* post-transcriptional silencing exhibited a wide range of phenotypes compared to wild type. Modified immature tomato fruit phenotypes range from normal wild type, to intermediate blotchy, to uniform dark green fruit. The severity of these phenotypes is inversely correlated with the expression levels of *DET1*. Similarly, whole plant phenotypes varied from wild type, to light green plants, to intermediate dark green plants, to very dark green, bushy plants (*33*).

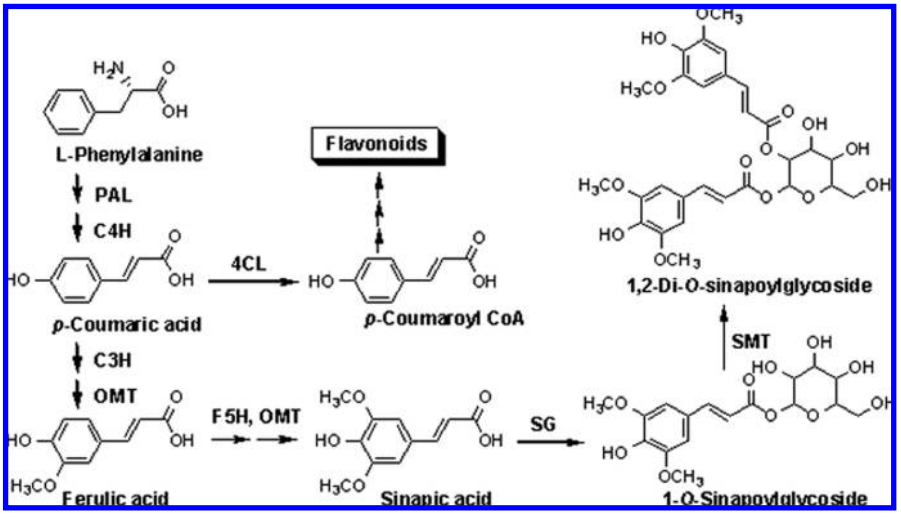

Figure 8. Phenylpropanoid pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, cinnamate 3-hydroxylase; OMT, *O*-methyltransferases; F5H, ferulate-5-hydroxylase; SG, sinapoyl glycosyltransferases.

In this study, seeds of some lines of both sets of modified *B. napus* plants exhibited poor germination rates. This might be related not only to the germination defect of *Arabidopsis det1-1* seeds on sugar-free media, which can be rescued by *DET1* suppressor *DET3* knockout (*34*), but also to the delayed germination of *Arabidopsis* seeds with increased levels of carotenoids and abscisic acid due to seed-specific overexpression of phytoene synthase gene (*9*). The effect of *DET1* silencing on *B. napus* seed germination should be further characterized. Possible approaches such as *DET3* expression manipulation, a chemical inducible promoter system application, or addition of sugar in the transgenic seed germination media can be tested to ensure normal germination of the *DET1* silenced *B. napus* seeds. Unlike tomatoes, the *B. napus* plants with suppressed *DET1* transcript levels did not display visible morphological changes when compared to the nontransgenic control, even though the levels of carotenoid compounds in both sets of transgenic plants were higher than in control plants. Moreover, the CaMV 35S promoter resulted in higher seed carotenoid increases than the seed-specific napin promoter. The wide range of phenotypes found in tomato fruit, leaves, and whole plants is well correlated with the extent and tissue specificity of *DET1* suppression (*13*, *33*). Differences in the extent of changes in carotenoid levels in the two types of transgenic *B. napus* seeds likely are related to differences in the strength and modes of action of the two promoters, because CaMV35S leads to constitutive expression in whole plants whereas napin promoter is largely limited to seeds (*22*).

In conclusion, our results showed that *DET1* suppression in *B. napus* can increase levels of carotenoids and reduce the content of sinapate esters simultaneously in seeds, thus enhancing their overall nutritional value.

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