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Purple Canola: *Arabidopsis PAP1* Increases Antioxidants and Phenolics in *Brassica napus* Leaves

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Anthocyanins, other flavonoids, and phenolic acids belong to a group of plant natural products with antioxidant activity and may play important roles in plant protection against biotic and abiotic stress and in protection against human diseases. In the present study, the *Arabidopsis* regulatory gene *Production of Anthocyanin Pigment 1* (*AtPAP1*) was expressed in *Brassica napus* (canola), and its presence enhanced the antioxidant capacity in transgenic leaves up to 4-fold. Transgenic plants had intense purple coloration, cyanidin and pelargonidin levels were enhanced 50-fold, and quercetin and sinapic acid were 5-fold higher. Consistent with these phytochemical and biological changes, expression for most genes in the flavonoid and phenolic acid biosynthetic pathways was also stimulated.

KEYWORDS: ***Arabidopsis PAP1*; *Brassica napus*; flavonoids; phenylpropanoids; anthocyanins; antioxidant activity.**

INTRODUCTION

The vegetable and mustard *Brassica* species are known for their leaf antioxidant activity (*1*, *2*), which is reduced by cooking due to leaching of polar phenolic compounds (*2*). *Brassica napus* (canola) is a seed crop grown mainly for its oil, but its vegetative tissues also produce a number of glucosinolates, phenylpropanoids, flavonoids, and phytoalexins (*3*, *4*). These substances are now known for their positive effect on health, including antioxidant and antitumor properties (*5*, *6*). Anthocyanins are important pigments in the flavonoid pathway, with colors ranging from orange to red to purple, and potential in the treatment of heart disease (*7*). Recently, anthocyanins and anthocyanidins were shown to reduce apoptosis and inhibit activities of oncogenic transcription factors and protein tyrosine kinase, which, in turn, may have contributed to delayed cancer development in rodents (*8*, *9*).

The potential of *B. napus* leaves to serve as a source of polar antioxidant phenolics is limited by their titer in leaves. *Brassica* species lie within the same family (Brassicaceae) as the fully sequenced and well-annotated model plant *Arabidopsis thaliana*. We have successfully used *Arabidopsis* as a stepping stone to isolate *B. napus* genes that enhance nonpolar antioxidants (carotenoids) in *B. napus* seed oil by suppressing the *DET1* gene (*10*). Others have directly enhanced carotenoids through manipulation of biosynthetic genes (*11*, *12*). We also have expressed *Arabidopsis* genes successfully in *B. napus* to develop flea beetle-resistant "hairy canola" with a dense coverage of seedling trichomes (*13*). These previous studies suggest that *B. napus* could be a candidate for transgenic enhancement of leaf phenolic antioxidants.

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The flavonoid biosynthetic pathway, which is derived from the phenylpropanoid pathway, has been extensively elucidated in *Arabidopsis* (*14*, *15*) (**Figure 1**). Manipulation of flavonoid MYB transcription factors has resulted in significant enhancement of anthocyanin and flavonoids over the past decade. For example, heterologous expression of *DELILA* (*DEL*) and *ROSEAL* (*ROS1*) *MYB* genes from the snapdragon *Antirrhinum majus* stimulated high levels of anthocyanins in the fruit of transgenic tomatoes and enhanced their antioxidant capacity (*16*). Overexpression of the *Arabidopsis PAP1* gene (*AtPAP1*), an *MYB75* anthocyanin transcription factor, led to anthocyanin accumulation in the leaves of *Arabidopsis* (*15*, *17*, *18*). However, *AtPAP1* has been introduced successfully into only one heterologous genetic background, tobacco, where it stimulated massive amounts of anthocyanin and proanthocyanidin (*17*, *18*). It has not been introduced into major food crops, and it does not function in transgenic alfalfa (*18*). Only the *Medicago truncatula* homologue *LAP1* was active at stimulating anthocyanins in alfalfa, *M. truncatula*, and white clover (*19*).

In this study, *AtPAP1* was constitutively expressed in *B. napus* using a CaMV 35S promoter. The levels of cyanidin, pelargonidin, quercetin, and sinapic acid derivatives were evaluated in mature transgenic leaves. As well, the antioxidant capacity of leaves was determined. The results are discussed in relation to the use of *AtPAP1* to improve the potential of *B. napus* to produce pharmaceutical antioxidants and to provide protection against disease.

MATERIALS AND METHODS

General Experiments. All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) (solvents were of HPLC grade). The *B. napus* doubled-haploid line DH12075 was used to develop

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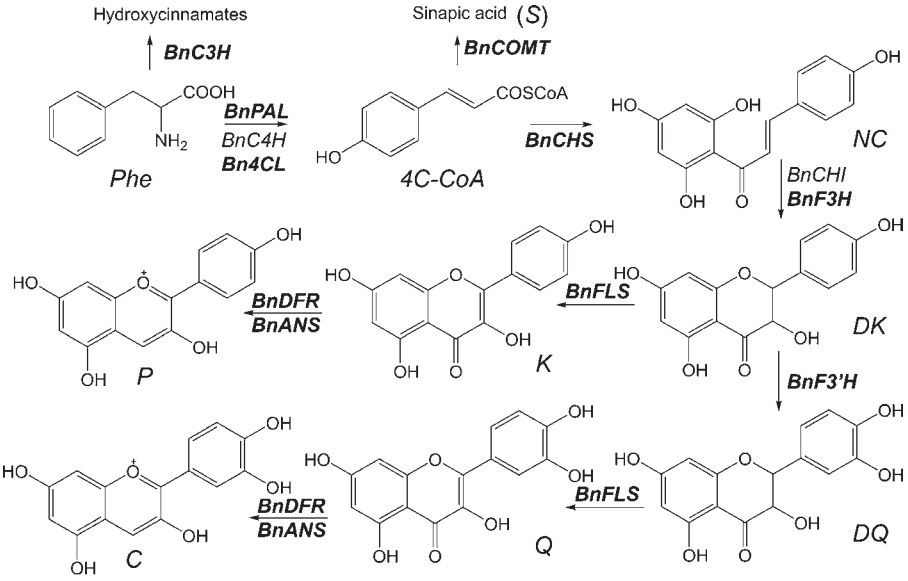

Figure 1. Phenylpropanoid and flavonoid pathways. *BnPAL*, phenylalanine ammonia-lyase; *BnC3H*, cinnamate 3-hydroxylase; *BnC4H*, cinnamate 4-hydroxylase; *Bn4CL*, 4-coumarate:CoA ligase; *BnCOMT*, *O*-methyltransferases; *BnCHS*, chalcone synthase; *BnCHI*, chalcone isomerase; *BnF3H*, flavanone 3-hydroxylase; *BnF3′H*, flavonoid 3′-hydroxylase; *BnFLS*, flavonol synthase; *BnDFR*, dihydroflavonol reducetase; *BnANS*, anthocyanidin synthase; *Phe*, phenylalanine; *4C-CoA*, 4-coumaroyl-CoA; *S*, sinapic acid; *NC*, naringenin chalcone; *DK*, dihydrokampferol; *DQ*, dihydroquercetin; *K*, kaempferol; *Q*, quercetin; *P*, pelargonidin; *C*, cyanidin. Gene names given in bold indicate genes up-regulated by *AtPAP1*.

Table 1. Primers Used in the Experimental Procedures

primer	sequence	primer	sequence
AtPAP1-F	5′-*GGATCC*ATGGAGGGTTCGTCCAAAG-3′	AtPAP1-R	5′-*GAGCTC*CTAATCAAATTTCACAGTCTCTCCATC-3′
BnACT2-Q-F	5′-CATCGGTGCTGAGAGATTCA-3′	BnACT2-Q-R	5′-CACTGAGCACGATGTTACCG-3′
AtPAP1-Q-F	5′-AAATGGCACCAAGTTCCTGT-3′	AtPAP1-Q-R	5′-GAAGCCTATGAAGGCGAAGA-3′
BnPAP1-Q-F	5′-TAAATCGGTGCAGGAAGAGC-3′	BnPAP1-Q-R	5′-ACCGGGTAATCTACCAGCAA-3′
BnANS-Q-F	5′-AAAAAGCGGAATCAGCTCAA-3′	BnANS-Q-R	5′-ACGGATGGTTTCGTCTTCTG-3′
BnDFR-Q-F	5′-CGTCTGCTGGAACGGTTAAT-3′	BnDFR-Q-R	5′-CGTAATCCCAAGCTGCTTTC-3′
BnF3′H-Q-R	5′-AAGCGTCGAACCTCTTGTGT-3′	BnF3′H-Q-F	5′-TCACAAAGCGGAGGAGTTTC-3′
BnBAN-Q-F	5′-ATCAAACCAGCGGTACAAGG-3′	BnBAN-Q-R	5′-TTCGGTCATCACAAGTCCAG-3′
BnF3H-Q-F	5′-TCGACGATGTTGGTGAGAAA-3′	BnF3H-Q-R	5′-CAGGAGGTAACGCGAAGAAG-3′
BnTT19-Q-F	5′-CTCTAGAGCACCGAGCCATC-3′	BnTT19-Q-R	5′-CTTGAGCTCCTCGACCAAAG-3′
BnPAL-Q-F	5′-AATATTCGGCAGCACGAAAG-3′	BnPAL-Q-R	5′-TGTTGGTGTTGAGGAAGCTG-3′
BnC3H-Q-F	5′-GCACGCTTGAACTCTTCACA-3′	BnC3H-Q-R	5′-AACGCAACCGCTCCTAAGTA-3′
Bn4CL-Q-F	5′-CGGTGCTAGCAATGTCGTTA-3′	Bn4CL-Q-R	5′-ACCACGGATGCAAATCTCTC-3′
BnCOMT-Q-F	5′-TCCCGCATCTGAAAGGTATC-3′	BnCOMT-Q-R	5′-TCATCGCTCCAATCATGAAG-3′
35S_F1	5′-CAATCCCACTATCCTTCGCAAGACCC-3′		

independent transgenic lines. All plants were grown in a fertilized soiless mix (Redi-Earth, Grace & Co., Canada) and placed either in a greenhouse supplemented with high-pressure sodium lights or in a controlled-environment growth chamber (Conviron, Winnipeg, Canada) under fluorescent and incandescent lighting (130−150 μeinstein/m^2/s, Licor model LI-185B) with an 18 h photoperiod (22 °C/20 °C light/dark).

Vector Construction and Plant Transformation. To make a PAP1 expression construct, a 926 bp RT-PCR fragment of the *Arabidopsis AtPAP1* cDNA sequence (AT1G56650) (NCBI reference sequence NM_104541.3) was amplified from leaf cDNA using high -fidelity DNA polymerase *Pfu Phusion* (Invitrogen, CA) and gene-specific primers AtPAP1-F and AtPAP1-R, which included additional restriction enzyme sites *Bam*HI and *Sac*I at their 5′ and 3′ ends, respectively (**Table 1**). The *AtPAP1* PCR product was cloned into pGEM-T Easy (Promega, Madison, WI), then sequenced and inserted using *Bam*HI/*Sac*I sites into a modified pBI-121 binary vector containing the BAR gene (phosphothricin selection) under the control of CaMV35S promoter and designated OXPAP1. Cotyledon explants of *B. napus* DH12075 were used to generate transgenic plants with OXPAP1 using *Agrobacterium tumefaciens* GV3101pMP90-mediated transformation (*20*). Independent transgenic lines that survived herbicide (25 μg/mL Basta) selection were grown in soil in a greenhouse and confirmed for transgene integration and integrity by RT-PCR using the primer AtPAP1-R and a sense primer 35S_F1 (**Table 1**), followed by DNA sequencing. Eleven independent T_0 plants containing the confirmed *AtPAP1* transgene were subjected to further analysis.

DNA/RNA Extraction and RT-PCR. Total genomic DNA was isolated from leaves using the DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Total RNA was extracted using a commercial RNAEasy mini kit (Qiagen, Valencia, CA), reverse transcribed by Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) and amplified using *Taq* DNA polymerase (Invitrogen), and the *Brassica BnACT2* gene was used as an internal control according to the manufacturer's instructions. PCR conditions were as follows: 95 °C for 5 min, 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, followed at the end by 72 °C for 5 min.

Real Time qRT-PCR. RNA preparations described above were used in quantitative real time qRT-PCR reactions. First-strand cDNA was synthesized by reverse transcribing 500 ng of total RNA (as prepared above) in a final reaction volume of 20 μL using random primers and 200 units of SuperScript following the manufacturers' instructions. qRT-PCR primers (**Table 1**) were designed using the online primer design tool OligoPerfect Designer (Invitrogen) based on *Brassica* homologues to corresponding *Arabidopsis* flavonoid and phenolic cDNA sequences at

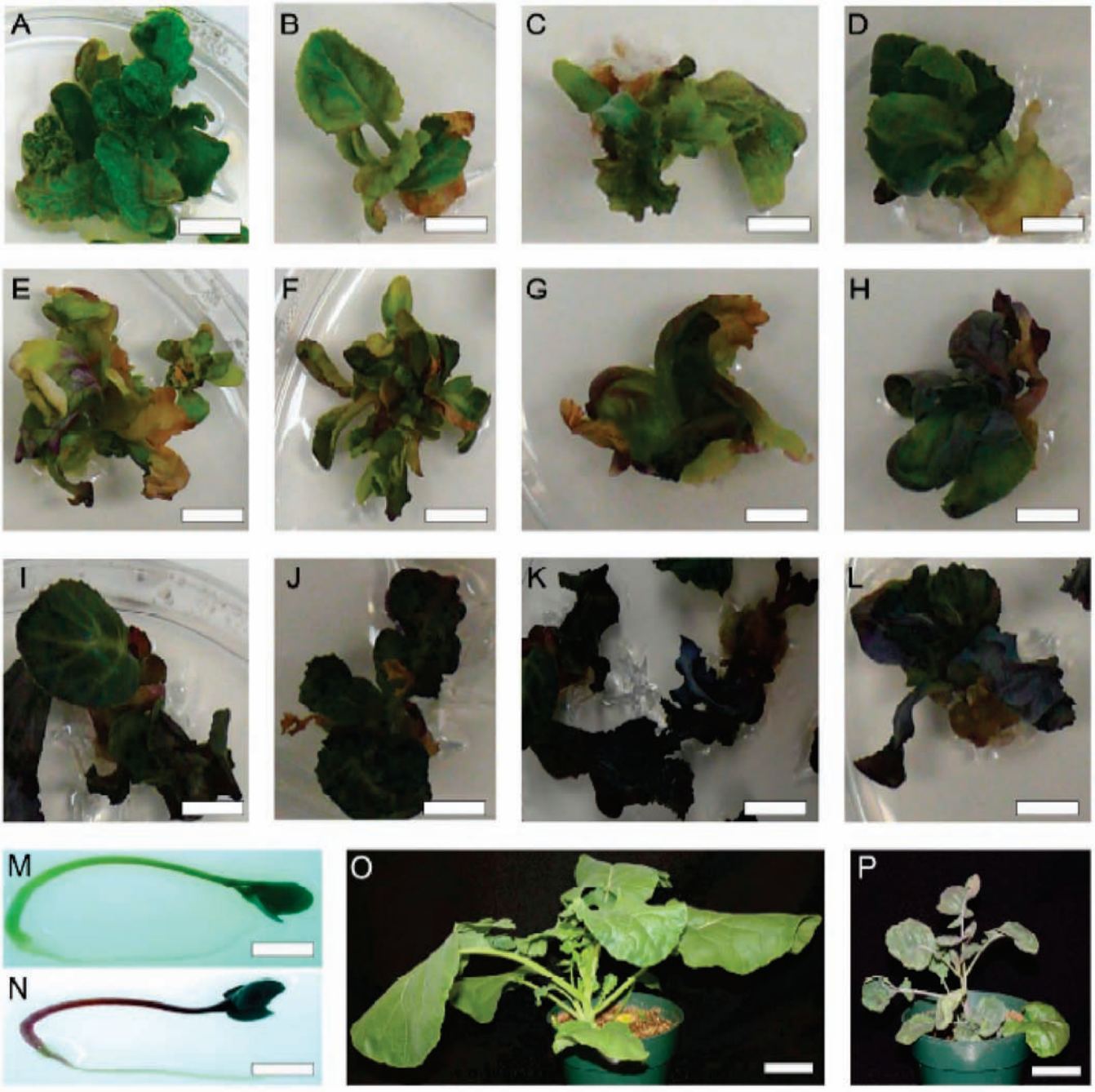

Figure 2. Phenotypes of AtPAP1 transgenic *Brassica napus* (**A**) green DH12075 T_0 control line in tissue culture media (bar =3 mm): (**B**−**L**) independent *AtPAP1* T_0 transgenic plantlets (OXPAP1-01 to OXPAP1-11) showing a range of color phenotypes growing on tissue culture media (bar = 3 mm); (**M**) green DH12075 seedling growing as in **N** (bar = 500 μm); (**N**) purple OXPAP1-08 T_1 seedling at 6 days after planting on MS-agar plates (bar = 500 μm); (**O**) young green DH12075 seedling at 22 days after planting (DAP) on potting mixture (bar = 5 cm); (**P**) small, purple-green OXPAP1 T_1 seedlings grown as in **O** (bar = 5 cm). Other purple T_1 lines have normal growth rates and plant sizes.

www.Brassica.ca. The qRT-PCR mixture contained 8 μL of diluted cDNA, 10 μL of 2xSYBR Green qPCR Master Mix (catalog no. 11735-040, Invitrogen), and 200 nM of each gene-specific primer in a final volume of 20 μL. Control PCR reactions without cDNA templates were also performed for each primer pair. The qRT-PCR reactions were performed using a StepOnePlus Real-Time PCR System (Applied Biosystems) as described (*21*) under the following conditions: 2 min at 50 °C, followed by 2 min at 95 °C, and 40 cycles of 95 °C (15 s) and 60 °C (30 s) in a 96-well optical reaction plate (Bio-Rad Laboratories, Hercules, CA). The specificity of amplicons was verified by melting curve analysis (60−95 °C) after 40 cycles. The levels of various gene transcripts were determined by qRT-PCR using BnACT2 mRNA as an internal reference. Mean expression values were calculated from assays on triplicate RNA preparations from independent plant samples. All PCR reactions displayed efficiencies between 90 and 96%.

Analysis of Anthocyanins, Flavonoids, and Phenylpropanoids. Fresh leaves from each plant (~500 mg) weighed accurately to four decimal places were ground to a powder in liquid nitrogen in a 20 mL Potter (Elvehjem), followed by grinding in 10 mL of methanol/water (50:50; v/v) for 10 min. Following filtration, the pellet was re-extracted overnight at 4 °C in the dark, and then the two extracts were combined and evaporated at 35 °C under vacuum. The dried extract was redissolved in methanol/water/HCl (50:40:10; v/v/v) and boiled for 2 h at 95 °C to release anthocyanins, flavonoids, and phenylpropanoids. After neutralization, 20 μL was analyzed using a Hewlett-Packard Agilent 1100 chromatograph, a G-7120 diode array detector, HP Chemstation ver. 8.01 software,

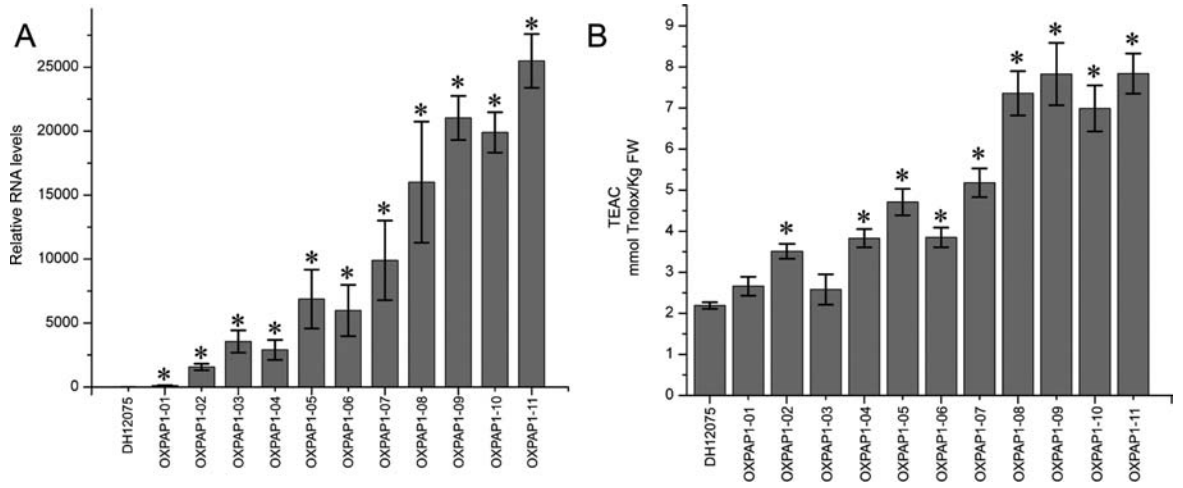

Figure 3. *AtPAP1* gene expression correlates with antioxidant activity in OXPAP1 transgenic *Brassica napus* plantlets: (**A**) quantitative RT-PCR of *AtPAP1* in T_0 *B. napus* plantlets; (**B**) Trolox antioxidant activities (TEAC). Student *t* tests showed significant differences between transgenic plants and DH12075 nontransgenic plants at $p \leq 0.05$ (∗).

and a Zorbax C_{18} column (150 × 4.6 mm, 5 μm i.d., Mississauga, ON, Canada) with a 60 min linear gradient of 5−60% MeOH (for anthocyanidins) or a 40 min linear gradient of 15−100% MeOH (for flavonoids and phenylpropanoids) and a flow rate of 1.0 mL min^{-1}. Flavonoids, anthocyanins, and phenylpropanoids were quantified by calibration of peak areas relative to commercial standards: quercetin (A_{370}), cyanidin (A_{525}), and sinapic acids (A_{325}) (Sigma).

Identical elution conditions were used to purify quercetin, kaempferol, and sinapic acid for structure confirmation by semipreparative HPLC using a preparative Zorbax C_{18} column (250 mm × 25 mm). LC-MS-MS was performed using an Agilent 1100 HPLC coupled with an API Qstar XL pulsar hybrid MALDI-TOF LC-MS-MS system (Applied Biosystems, Fullerton, CA). Purified compounds were dried under nitrogen and then dissolved in 510 μL of freshly opened DMSO-d_6 (99.96 + 0.03 TMS, Sigma). ^{1}H NMR and HMBC spectra were measured with a Bruker Avance 500 MHz NMR spectrometer equipped with a 5 mm inverse triple-resonance TXI probe (Bruker Biospin, Rheinstetten, Germany) at the Structural Sciences Centre, University of Saskatchewan. Chemical shifts were expressed in parts per million (ppm) and coupling constants (*J*) in hertz (Hz).

Trolox Equivalent Antioxidant Capacity of *B. napus* OXPAP1. The antioxidant activities of 50% MeOH extracts of *B. napus* OXPAP1 leaves were measured by the TEAC (Trolox equivalent antioxidant capacity) assay according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The TEAC value was calculated on the basis of the ability of the leaf extracts to scavenge the blue-green $ABTS^{\bullet+}$ radical cation [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate)] relative to the scavenging ability of the water-soluble vitamin E analogue, Trolox.

RESULTS

Generation of Transgenic Plants. *Agrobacterium*-mediated gene transformation and DNA insert sequencing were used to introduce and confirm the CaMV35S-driven *AtPAP1* transgene in 28 independently derived phosphinothricin-resistant *B. napus* DH12075 plants. Eleven independently generated T_0 plantlets exhibited a range of purple coloration over leaves and stems (**Figure 2A**−**L**). Each of these 11 plants showed dramatic induction of the *AtPAP1* gene (the transcript level of DH12075 was assigned as 1, **Figure 3A**). The highest *AtPAP1* expression was detected in leaves of OXPAP1-11, in which the relative RNA level was ∼25000. *AtPAP1* transcript levels from the other lines ranged from 130 to ∼20000. When T_1 seeds from the 11 T_0 plants were grown for 6 days on agar plates or for 22 days on soil, the color changes were maintained. OXPAP1 T_1 plants displayed a purple-green phenotype over the entire surface of all leaves and stems under all growth conditions (**Figure 2M**−**P**). Most OXPAP1 T_1 plants had a growth phenotype similar to that of the nontransgenic DH12075. However, a few appeared smaller and had curled leaves compared with the green DH12705 control plants under greenhouse conditions (**Figure 2P**).

Antioxidant Activities. Differences in antioxidant activity between transgenic and nontransgenic leaves were measured using TEAC. The antioxidant activity of the 50% MeOH leaf extract increased up to 4-fold in transgenic plants compared with nontransgenic control plants (**Figure 3B**). Substantial variation in antioxidant activity also was apparent among the different transgenic plants.

Anthocyanin, Flavonoid, and Phenylpropanoid Derivatives in Transgenic Leaves. To determine whether the increases in antioxidation activity were related to phenolic compounds in the OXPAP1 plants, levels of anthocyanin, flavonoid, and phenylpropanoid derivatives were quantified using HPLC after acid hydrolysis (**Figure 4**). Individual peaks (370 nm) were purified by semipreparative HPLC and confirmed by ^{1}H nuclear magnetic resonance (^{1}H NMR), ^{1}H and ^{1}H correlation spectroscopy (COSY), and heteronuclear multiple bond correlation (HMBC) and MALDI-TOF-MS analysis. Three compounds were firmly determined as quercetin (Q), kaempferol (K), and sinapic acid (S) by these methods. Cyanidin and pelagonidin also were confirmed by comparing their HPLC retention times and UV spectra with authentic standards (Sigma-Aldrich). Compared to nontransgenic leaves, levels of cyanidin and pelagonidin were dramatically induced in the OXPAP1 transgenic plants (**Figure 5A**,**B**), and levels of quercetin and sinapic acid increased up to 300% (from 3.27 to 9.83 mg/g of FW) and 520% (from 1.57 to 6.93 mg/g of FW), respectively (**Figure 5C**,**D**). However, the levels of leaf kaempferols varied among different transgenic lines, most of which showed no significant increase (data not shown). Antioxidation activity correlated with individual compounds types: cyanidin (R^2, 0.9396; $p < 0.05$), pelargonidin (R^2, 0.9129; $p < 0.05$), and sinapic acid (R^2, 0.7274; $p < 0.05$), but correlated strongest with quercetin (R^2, 0.9760; $p < 0.05$). Antioxidation activity did not correlate with kaempferol.

Flavonoid and Phenolic Gene Expression in Transgenic Plants. To examine the effect of *AtPAP1* heterologous expression on phenylpropanoid and flavonoid pathway genes, transcripts were analyzed using quantitative RT-PCR in one of the lines with

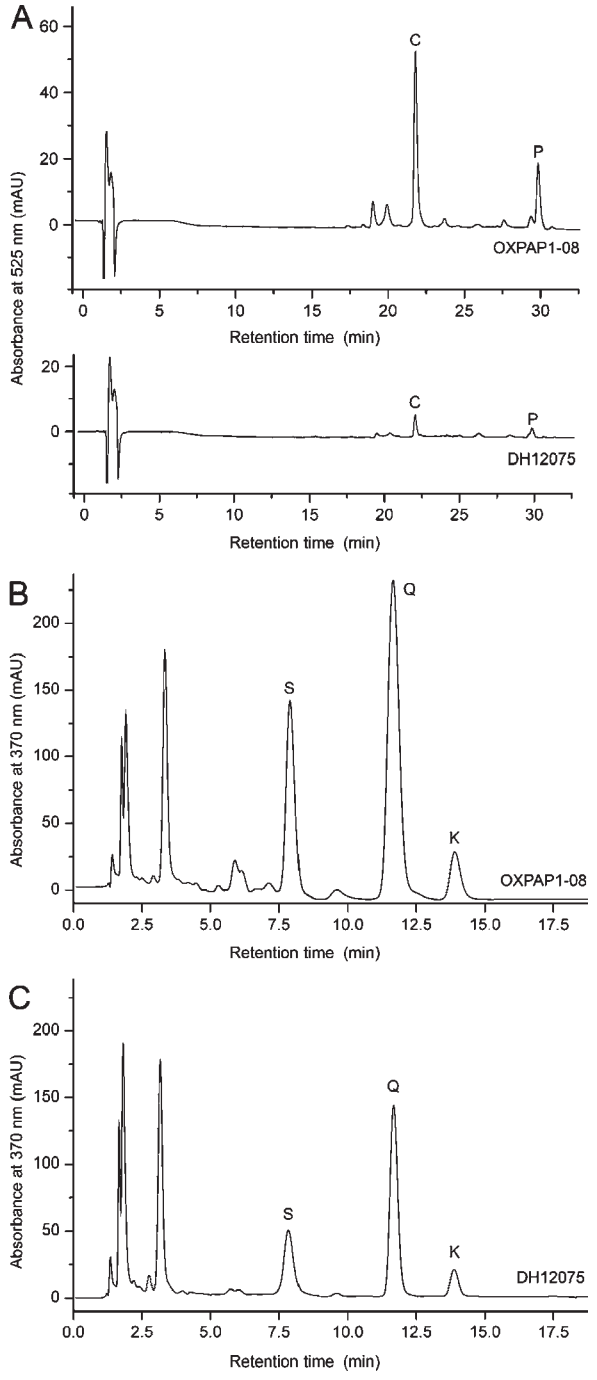

Figure 4. Representative HPLC traces of HCl hydrolysates of *Brassica napus* OXPAP1 plants: (**A**) anthocyanin analysis at A_{525}; (**B**, **C**) flavonoids and phenolics at A_{370}. C, cyanidin; P, pelargonidin; S, sinapic acid; Q, quercetin; K, kaempferol. Traces were very similar in other independent transgenic lines.

intermediate *AtPAP1* expression, OXPAP1-8. The results showed that all selected pathway genes, except *BnPAP1*, were induced (**Figure 6**). The flavonoid biosynthetic genes, *BnTT3* (*DFR*) and *BnTT18* (*LDOX or ANS*), showed strongest up-regulation at ~800-fold higher in transgenic plants compared with DH12075 control plants. Other flavonoid biosynthetic genes, *BnTT6* (*F3H*), *BnTT7* (*F3′H*), and *BnTT19* (*GST*), were induced ~50-fold, whereas *BnBAN* (*ANR*) and *BnPAP1* showed no significant changes. Phenylpropanoid pathway genes, *BnPAL*, *BnC3H*, *Bn4CL*, and *BnCOMT*, also were induced ~30−80-fold compared with their expression in DH12075 (**Figure 6**).

DISCUSSION

Heterologous expression of *AtPAP1* was achieved in *B. napus* OXPAP1 plants and resulted in the enhancement of antioxidant capacity in leaf tissues. OXPAP1 *B. napus* plants also displayed a purple-green phenotype and accumulated substantially increased amounts of anthocyanin, quercetin, and phenylpropanoid derivatives. Our results are consistent with overexpression of *Arabidopsis AtPAP1* in *Arabidopsis* and heterologous expression in tobacco, in which both flavonoid and phenylpropanoid pathways are also affected (*17*, *18*). Our study offers the first direct evidence for the effective stimulation of antioxidant capability in crop plants by *AtPAP1*.

Numerous studies have reported the induction and regulation of anthocyanin and flavonoid biosynthesis and shown that endogenous and exogenous factors, such as sugar, hormones, or abiotic stresss, can promote their accumulation (*22*−*24*). In higher plants, the anthocyanin pathway is regulated by a suite of transcription factors that include Myb, bHLH, and WD-repeat proteins (*14*, *15*). Heterologous expression of *Lc* and *C1*, paired *bHLH* and *MYB* genes regulating anthocyanins in maize, resulted in tomato fruit containing increased levels of flavonols, but not anthocyanins or phenolic acids (*25*). This suggests that specific regulation of flavonoid and phenolic end-products may differ between plant species, such that heterologous genes may not always induce any or all parts of these pathways.

Microarray analysis of *Arabidopsis* overexpressing *AtPAP1* identified several genes coordinately up-regulated in leaves in response to elevated expression of this *MYB* transcription factor, including *DFR*, *ANS*, and *TT19* (*26*). Bioinformatic analysis on promoter fragments of genes up-regulated in the *AtPAP1 Arabidopsis* revealed that a common cis-regulatory motif was required for AtPAP1-dependent transactivation (*27*). In the present study, we showed that heterologous expression of *AtPAP1* in *B. napus* resulted in elevated anthocyanins, phenolic acids, and flavonols and a coordinated up-regulation of a wide range of biosynthetic genes in these pathways, although the *BnPAP1* and *BnBAN* genes were not affected. This suggests that *AtPAP1* may interact with *B. napus* regulatory genes, such as *TT8* or *TTG1*, to perform similar functions in both *Arabidopsis* and *B. napus*. Future analysis using both the *AtPAP1* and the *BnPAP1* in one-hybrid systems with *B. napus* flavonoid and phenylpropanoid gene promoters, analysis of coordinately regulated genes (*27*), and evaluation of *B. napus GUS fusion* lines mutated in these promoters should determine the position and sequence of cis-regulatory motifs that interact with *PAP1* genes.

Flavonoids and anthocyanins are small molecules that possess nutraceutical properties (e.g., antioxidant activity) and may have practical application in biotherapies for heart disease and cancer (*8*, *9*, *28*). As secondary metabolites, the flavonoid level in most common crops is probably inadequate to develop these plants into sources of these agrichemicals. In the present study, heterologous expression of *AtPAP1* in *B. napus* increased antioxidant capacity by 4-fold and the concentration of anthocyanins, flavonoids, and phenylpropanoids up to 5-fold than normally present in leaves. The new canola OXPAP1 lines should be tested for the effect of AtPAP1 on seed antioxidant levels found in *B. napus* (*29*) and for changes to long-term seed storage. The transgenic leaves could provide characterized material linked to specific antioxidant levels, which could be tested for a range of biological properties that depend on the quenching of oxygen free radicals. Such properties could include plant resistance to pathogens, drought, flooding, and saline conditions and protection against UV, air pollution, and pesticide damage, which are conditions that affect crop survival and yield (*4*, *30*). In practical

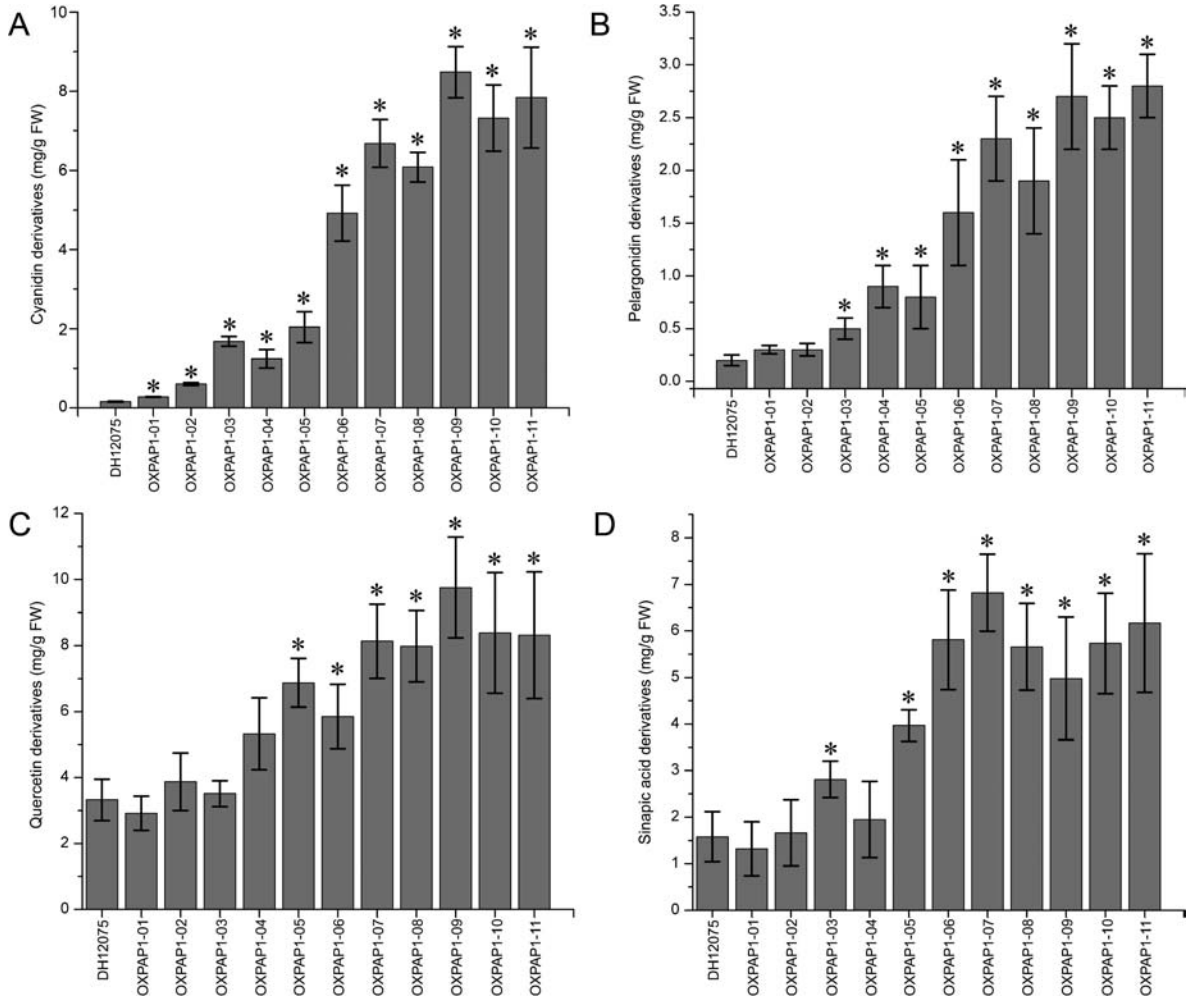

Figure 5. Quantitative analysis of anthocyanidins, flavonoids, and phenolics in HCl acid hydrolysates of *Brassica napus* OXPAP1 plants: (**A**) cyanidin derivatives; (**B**) pelargonidin derivatives; (**C**) quercetin derivatives; (**D**) sinapic acid derivatives. Student *t* tests showed significant differences between transgenic plants and DH12075 nontransgenic plants at $p \leq 0.05$ (∗).

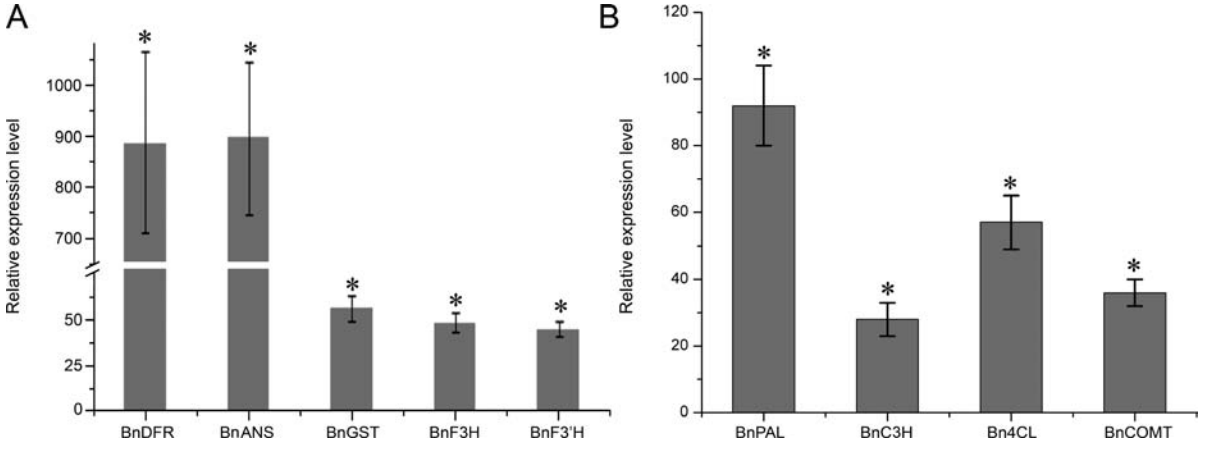

Figure 6. Quantitative RT-PCR of flavonoid and phenylpropanoid biosynthetic gene expression in *Brassica napus* OXPAP1-08 T_0 plants: (**A**) *TT3* (dihydroflavonol reductase, *BnDFR*), *TT18* (anthocyanidin synthase, *BnANS* or *BnLDOX*), *TT19* (glutathione *S*-transferase, *BnGST* or *BnTT19*), *TT6* (flavanone 3β-hydroxylase, *BnF3H* or *BnTT6*) and *TT7* (flavonoid 3′-hydroxylase (*BnF3′H* or *BnTT7*); (**B**) phenylalanine ammonia-lyase (*BnPAL*), cinnamate 3-hydroxylase (*BnC3H*), 4-coumarate-CoA-ligase (*Bn4CL*), and coumaroyl-*O*-methyl transferase (*BnCOMT*). Results of expression represent the average of data, and SD values were calculated from the results of three independent experiments. The levels of various gene transcripts were determined by qRT-PCR using BnACT2 mRNA as an internal reference. Expression patterns were very similar between OXPAP1-08 and other *AtPAP1*-transgenic *B. napus* plants. Student *t* tests showed significant differences between transgenic plants and DH12075 at $p \leq 0.05$ (∗).

terms related to human nutrition and health, the new "purple" *B. napus* also could be crossed to lines enhanced with carotenoids and tested as crushable or extractable material for treatment of chronic diseases such as cardiovascular disease and cancer.

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