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ORIGINAL PAPER

Targeted modulation of sinapine biosynthesis pathway for seed quality improvement in *Brassica napus*

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Abstract *Arabidopsis thaliana* and other members of the Brassicaceae accumulate the hydroxycinnamic acid esters sinapoylmalate in leaves and sinapoylcholine in seeds. Our recent understanding of the phenylpropanoid pathway although complex has enabled us to perturb the sinapine biosynthesis pathway in plants. Sinapine (sinapoylcholine) is the most abundant antinutritional phenolic compound in seeds of cruciferous species and therefore is a target for elimination in canola (*Brassica napus*) meal. We analysed *A. thaliana* mutants with specific blocks in the phenylpropanoid pathway and identified mutant lines with significantly altered sinapine content. Knowledge gained from *A. thaliana* was extended to *B. napus* and the corresponding phenylpropanoid pathway genes were manipulated to disrupt sinapine biosynthesis in *B. napus*. Based on our understanding of the *A. thaliana* genetics, we have successfully developed transgenic *B. napus* lines with ferulic acid 5-hydroxylase (*FAH*) and sinapoylglucose:choline sinapoyltransferase (*SCT*)-antisense. These lines with concomitant downregulation of FAH and SCT showed up to 90% reduction in sinapine. In addition to reduced sinapine content, we detected higher levels of free choline accumulation in the seeds. These results indicate that it is possible to develop plants with low sinapine and higher choline by manipulating specific steps in the biosynthetic pathway. These improvements are important to add value to canola meal for livestock feed.

Keywords Brassica · Canola · Choline · Phenylpropanoid · RNAi · Sinapine

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Introduction

Brassica napus (canola) seed is well known not only for its valuable oil and oil-related products but also for the nutritional properties of its seed storage proteins (Maenz et al. 2003). These properties include a good balance of essential amino acids as compared to other plant-based proteins and low molecular weight of major storage proteins (a characteristic that is usually associated with low antigenicity; Drew 2004). Also, the protein efficiency ratio is similar to that of known high quality proteins such as beef and milk, and better than those of soybean and other plant proteins (Slominski et al. 1999). Despite its high quality characteristics, canola protein is considered a by-product of oil processing and is used only as a

low-grade protein source and on a limited scale in livestock feed. The high potential value of canola meal remains unrealized and its market share trails those of other oil seeds, such as soybean, mainly due to the high levels of anti-nutritional factors that remain in the seed, including sinapine, fiber and phytate.

Sinapine is found almost exclusively in the seeds of crucifers. Canola seeds contain sinapine at levels ranging from 0.7 to 4% (Blair and Reichert 1984), with about 90% of it present in the embryo (non-hull) fraction (Velasco and Möllers 1998; Wang et al. 1998). Though the biosynthesis pathway of sinapine (Chapple et al. 1992) and the structural genes (Chapple and Ellis 1992) are characterized, the gene encoding the last enzyme in sinapine biosynthesis was only recently cloned in *Arabidopsis thaliana* (Shirley et al. 2001) and *Brassica napus* (Milkowski et al. 2004). The sinapoylglucose:choline sinapoyltransferase (*SCT*) gene, initially cloned in *A. thaliana* encodes a serine carboxypeptidase-like acyltransferase and is expressed only during a brief stage in seed development (Shirley et al. 2001). Disruption of the *SCT* gene in *A. thaliana* was shown to significantly reduce the level of sinapine, and cause the accumulation of sinapoylglucose in the seeds (Shirley et al. 2001; Shirley and Chapple 2003; Huang et al. 2008). Moreover, sinapic acid is a major phenylpropanoid in Brassicaceae that provides intermediates in two distinct metabolic pathways leading to the formation of sinapoyl esters and lignin.

Several beneficial characteristics for sinapine have been reported, including its role in UV protection (Booij-James et al. 2000; Sheahan 1996) and the contributory roles in the synthesis of phosphatidylcholine (Strack 1981). However, sinapine is still largely considered an anti-nutritional factor because of its undesirable attributes. Sinapine causes an unpleasant flavor in the meat and milk of animals fed on canola/*B. napus* (Pearson et al. 1980) and the bitter flavor of sinapine results in poor palatability for livestock (Ismail et al. 1981). Moreover, consumption of sinapine in canola meal by brown-shelled egg layers that are deficient in trimethylamine oxidase imparts a fishy odour to the eggs. Excessive consumption of sinapine could cause serious growth and reproductive problems (Pearson et al. 1980).

In this study, we used *A. thaliana* as a model system to identify rate-limiting steps in sinapine biosynthesis. This was done by determining the levels of sinapine in seeds of mutants with knockouts in genes involved in various steps of the phenylpropanoid pathway. We show that knocking down genes encoding *FAH* and *SCT* caused significant reductions in levels of sinapine. This finding was then successfully applied to the related species *B. napus*, where it caused considerable reduction in sinapine in the seeds.

Materials and methods

Plant material

Wild type and mutant *A. thaliana,* ecotype Columbia, were grown in RediEarth (W.R. Grace & Co., Ajax, Canada) in pots covered with window screens at 25°C, 16 h light/8 h dark cycles under green house conditions. Wild type (DH12075), transgenic *B. napus* and vector control plants were grown in soil prepared under conditions similar to those of *A. thaliana*. Knockout mutants of *A. thaliana* were identified by searching the SALK T-DNA population in the TAIR database (http://arabidopsis.org) using gene-specific sequences, and they included the following lines: for *PAL* (lines SALK70702-20, 21; SALK92252-43, 44) and *C3H* (SALK36132-19, 27; SALK112823-37, 39), *COMT* (lines SALK2373-13, 15; SALK20611-18, 19), *FAH* (SALK 63792-44, 46), and *SCT* (SALK2255-55, 56; SALK18120-11, 13). The *C4H* (SK16293-11) mutant line was isolated by PCR-based methods from the *Arabidopsis* mutant population maintained at Agriculture and Agri-Food Canada, Saskatoon Research Center (http://www.brassica.ca). Seeds were harvested from dry siliques of mature plants of *A. thaliana* and *B. napus*. For *B. napus* transformation, the tissue culture growth room conditions were set to a standard, 22 ± 1°C under 16 h light and 8 h dark cycle with a light intensity of 100 μE/m^2/s. Field trials conducted in the spring and summer of 2006 in Saskatoon complied with the laws and regulations of Canada. Briefly, *B. napus* transgenic lines were seeded in 4 replicates of 2–10 foot rows per line (100 seeds per row) using a randomized plot design that includes *B. napus* cv. Westar control rows, cv. DH12075 greenhouse-grown and cv. DH12075 field-grown rows. Guard rows were maintained in the borders to ensure continuous flowering

during transgenic genotype flowering. Also in between each double row and around the edge of each replicate, barley was planted to decrease outcrossing between cultivars and between replicates. Seeds were harvested at maturity and used for chemical analysis.

Chemicals

HPLC grade methanol (J. T. Baker, NJ, USA) was used for all the experiments. Analytical reagent-grade hydrochloric acid, glacial acetic acid and sodium hydroxide were from Pharmacia Biotech (Uppsala, Sweden). The phenylpropanoid standards ferulic acid, *O*-coumaric acid and caffeic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sinapine bisulfate, sinapoyl glucose and sinapic acid were kind gifts from Dr. Alfred Baumert from the Department of Secondary Metabolism, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany and Dr. Ian McGregor, Agriculture and Agri-Food Canada, Saskatoon Research Center.

Estimation of gene copy numbers in *Arabidopsis*

Arabidopsis gene copy numbers were determined by surveying the *Arabidopsis* genome using the BioVis software (http://www.brassica.ca) set to a homology threshold of 80%. BioVis (v 0.5) is an interactive, browser-based SVG application for the visualization and mining of genome data from the model plant *A. thaliana*. It also serves as a *Brassica–Arabidopsis* comparative genomics browser, and was developed at Agriculture and Agri-Food Canada, Saskatoon Research Center.

Vector construction for RNAi and antisense

Based on the published cDNA sequence of target *Arabidopsis* genes namely, phenylalanine ammonia lyase (*PAL*, At3g053260), cinnamate 4-hydroxylase (*C4H*, At2g030490), coumarate 3-hydroxylase (*C3H*, At2g040890), Caffeic *O*-methyltransferase (*COMT*, At5g054160), ferulic acid hydroxylase (*FAH*, At4g 036220) and sinapoylglucose:choline sinapoyltransferase (*SCT*, At5g09640), their homologs in *B. napus* were isolated from a collection of $>150{,}000$ expressed sequence tags (ESTs) derived from *B. napus* line DH12075 cDNA libraries generated at Agriculture and Agri-Food Canada, Saskatoon Research Center, (http://napus.agr.gc.ca). In the case of *SCT,* since no EST could be identified, the gene was isolated by PCR using primers specific to conserved *Arabidopsis* sequences and a *B. napus* cDNA library as template. These cDNAs were used to generate RNAi and antisense constructs for expression in *B. napus* (Table 1).

A common strategy was adopted for all the RNAi constructs. Briefly, forward primers having built-in *Spe*I/*Asc*I and reverse primers with *Bam*HI/*Swa*I sites were used to generate fragments for RNAi construction (Supplementary Table 1). Depending on the gene and the fragment, in all cases, single palindromic repeats of the 5′ and 3′-end PCR products were inserted around a 300 bp spacer of the β-glucuronidase gene in a pGSA1285 vector (CAMBIA, Canberra, ACT, Australia) driven either by a CaMV35S or napin promoter as prefixed in the construct name. The resulting RNAi vectors were designated 72-146 (35S:*C3H–C4H* RNAi), 72-115 (35S:*COMT*-RNAi), 72-142 (35S:*FAH–COMT* RNAi), and 72-145 (35S:*SCT*-RNAi).

A slightly different strategy was employed to generate antisense constructs. Briefly primer pair P7 (having built-in *Spe*I and *Asc*I sites) and P14 (having built-in *Bam*HI and *Swa*I sites) were used to generate the 5′-end fragments of the *FAH* gene (480 bp) from the *Brassica* EST, RL1627. Similarly, a 160 bp fragment from the 3′-end of *SCT* gene was amplified from the *B. napus SCT* clone using primers P12 and P13. These individual PCR products were used as template for amplifying a ~650 bp *FAH–SCT* fusion gene product using the primer pair P7 and P13 and cloned in a binary vector p79-103 (laboratory collection) functional under the influence of napin promoter (Yu et al. 2007). The resulting antisense vector was designated 72-148 (napin:*FAH–SCT* antisense) and used for transformation experiments.

Transformation of *Brassica* and growth conditions

Cotyledon explants of *B. napus* doubled haploid line DH12075 were used for *Agrobacterium tumefaciens* (GV3101pMP90)-mediated transformation (Moloney et al. 1989). *B. napus* plants were grown in a greenhouse (16 h light/8 h dark, 20°C/17°C). Only those plants shown to be transgenic as determined by PCR were subjected to further analysis. The primers used were P15 and P16 for 72-146, P17 and P19 for

Table 1 Details of RNAi and antisense construct generation strategy using *B. napus* genes

Target gene	Amplified fragment	Primers	Source/comments
35S:C3H-C4H RNAi (72-146)	900 bp coumarate 3-hydroxylase (*C3H*)	P1, P2	*B. napus* EST, RL4992
	616 bp cinnamate 4-hydroxylase (*C4H*)	P3, P4	*B. napus* EST, RL7118
	640 bp fusion gene product (*C3H-C4H*)	P1, P4	900 bp *C3H* and 616 bp *C4H* together
35S:COMT-RNAi (72-115)	250 bp 3′-end fragment of *OMT*1	P5, P6	*B. napus* EST, ESS2134
35S:FAH-COMT RNAi (72-142)	480 bp ferulic acid hydroxylase fragment	P7, P8	*B. napus* root cDNA library RL1627
	230 bp *O*-methyl transferase1	P9, P6	*B. napus* root cDNA library RL1627
	~700 bp fusion gene product	P7, P6	480 bp *FAH* and 230 bp *OMT* together
35S:SCT-RNAi (72-145)	~160 bp conserved fragment of *SCT* gene	P12, P13	*B. napus* cDNA (Bn*SCT*) clone containing the 2.1 kb SCT contig
Napin:FAH-SCT antisense (72-148)	480 bp ferulic acid hydroxylase fragment	P7, P14	*B. napus* root cDNA library RL1627
	~160 bp conserved fragment of *SCT* gene	P12, P13	*B. napus* cDNA (Bn*SCT*) clone containing the 2.1 kb SCT contig
	~650 bp *FAH–SCT* fusion gene product	P7, P13	480 bp *FAH* and 160 bp *SCT* together

72-115, P7 and P19 for 72-142, P17 and P13 for 72-145. Primer pair P7 and P13 was used to screen for plants carrying construct 72-148 (see Supplementary Table 1 for list of primers).

Nucleic acid analysis

Total genomic DNA was isolated from leaves of transgenic and control *B. napus* plants using DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Approximately 10 μg of genomic DNA was digested with *Sca*I (for transgenic lines with constructs 72-146; 72-115; 72-145 and 72-142), or *Nco*I (for transgenic lines with construct 72-148). Southern and northern blot analysis of *B. napus* plants was performed as described elsewhere (Gao et al. 2007; Yu et al. 2007). Total RNA was isolated from developing *B. napus* seeds (20–25 days post-anthesis) as described in Carpenter and Simon (1998).

Extraction of methanol-soluble phenolics from *A. thaliana* and *B. napus* seeds and HPLC analysis

Mature and dry seeds of *A. thaliana* and *B. napus* grown under green house conditions were used for chemical analysis. Methanol-soluble phenolics (including major sinapate esters such as sinapoylglucose, sinapoylcholine and sinapoylmalate) were identified using a Waters Alliance 2695 HPLC and UV–Vis spectroscopic comparison with standard compounds from our collection of natural products or from commercial sources. For analysis, either 10 mg (*A. thaliana*) or 50 mg (*B. napus*) of seeds were ground to a fine powder in liquid nitrogen using a pestle and mortar and thoroughly mixed with 15 volumes of extraction solvent (50% methanol, 1.5% acetic acid) for 5 min in a vortexer. The sample was frozen at −80°C for 5–10 min followed by incubation at room temperature for 10 min and centrifugation at 5000 rpm for 5–10 min. The supernatant (containing the methanol-soluble phenolics) was filtered (0.2 μm) and 3 μl was injected on to a Symmetry® 5 μm C18 (3 × 150 mm) reverse phase column (Millipore Canada Limited, Mississauga, Ontario, Canada) and resolved using a 20 min gradient mobile phase of 0.05% trifluoroacetic acid in water and 100% methanol (flow rate: 400 μl/min). Total soluble-phenolic compounds were detected using a Waters 996 photodiode array detector set to 332 nm. Sinapine bisulfate was used as the reference standard. The percent reduction of sinapine was calculated by comparing optical density values of peak areas obtained from seed extracts of transgenic lines to those of non-transgenic parental line grown at the same time under identical conditions. Total soluble-phenolic compounds were also measured using an Ultraspec 3000 (Pharmacia Biotech, Uppsala, Sweden). The supernatant samples were diluted 50 times in extraction buffer and absorbance at 332 nm

Table 2 Genes involved in the phenylpropanoid pathway, their estimated copy numbers and knockout mutants analyzed in this study

Gene	Copy #[a]	Knockout lines analyzed
Phenylalanine ammonia lyase (*PAL*)	4	SALK70702[b], SALK92252
Cinnamate 4-hydroxylase (*C4H*)	1	SK16293[c]
Coumarate 3-hydroxylase (*C3H*)	1	SALK36132, SALK1122823
Caffeic acid *O*-methyltransferase (*OMT*)	1[d]	SALK2373, SALK20611
Ferulic acid hydroxylase (*FAH*)	1[e]	SALK 63792
Sinapoylglucose:choline sinapoyltransferase (*SCT*)	1	SALK2255, SALK18120

[a] Gene copy numbers were estimated using the BioVis software (ver 0.5) (www.brassica.ca) set to a homology threshold of 80%

[b] Lines obtained from the knockout population generated at the SALK institute (http://signal.salk.edu/tabout.html)

[c] Lines obtained by screening AAFC's knockout population (www.brassica.ca)

[d] Copy number estimation done by setting the homology threshold of 80% as done previously. The second hit is a lower level of similarity, i.e., 67%. Hence that is not included in our analysis

[e] Copy number estimation done by setting the homology threshold of 80% as done previously. The second hit is a lower level of similarity, i.e., 77%. Hence that is not included in our analysis

compared with a standard curve of sinapine bisulfate. All extractions were performed in triplicate.

Quantification of free choline in the seeds

Choline was quantified using an established enzymatic release reaction (Takayama et al. 1977). Defatted seed meal was prepared as reported in Chapple et al. (1992). Briefly, about 10 mg defatted seed meal was extracted in 800 µl of water at 100°C for 30 min in sealed tubes. Reactions consisting of 10 µl of aqueous seed extract and 90 µl of choline reaction reagent were incubated at 37°C for 30 min and the absorbance measured at 500 nm. The choline assay reagent consisted of 50 mM Tris–HCl, pH 8.0, 1.2 mg of 4-aminoantipyrine, 2 mg phenol, 0.8 mg $CaCl_2 \cdot 2H_2O$, 25 units of horseradish peroxidase, and 10 U of choline oxidase (Sigma, Oakville, Ontario, Canada). Free choline content in the seed extracts was estimated using choline chloride as the standard in the experiments.

Results

Assessment of sinapine levels in seeds of *A. thaliana* mutants with knockouts in genes affecting the phenylpropanoid pathway

A total of 10 *A. thaliana* mutants with T-DNA inserts in genes affecting phenylpropanoid biosynthesis (Chapple et al. 1992) were collected for this analysis. BioVis software revealed that, with the exception of phenylalanine ammonia lyase (PAL), all *Arabidopsis* phenylpropanoid pathway enzymes that are functionally relevant are encoded by single copy genes (Table 2). However, additional copies of putative genes are likely to be present in the genome given the evolution of plant genomes. Analysis of homozygous seeds of upper pathway mutants indicated only minimal quantitative changes to the level of sinapine content compared with control plants (Fig. 1A). However, disruption of genes coding for the latter steps in the phenylpropanoid pathway, including caffeic acid *O*-methyltransferase1 (*COMT*), ferulic acid 5-hydroxylase (*FAH*) and sinapoylglucose:choline sinapoyltransferase (*SCT*) resulted in a significant decrease in sinapine levels (Fig. 1B). The highest reductions in sinapine levels were observed in seeds of mutant lines with disruption in the *FAH* and *SCT* genes, with the latter having levels barely detectable by HPLC. Given this notable reduction of soluble-phenolics and sinapine alike, we targeted *FAH, COMT, C3H, C4H* and *SCT* genes for silencing in *B. napus* to reduce sinapine content in seeds.

Impact of downregulation of *C3H–C4H*, *SCT*, and *COMT* on sinapine levels in *B. napus*

To understand the effect of knocking down phenylpropanoid genes and altering pathway components in *B. napus*, we first employed RNAi-mediated silencing

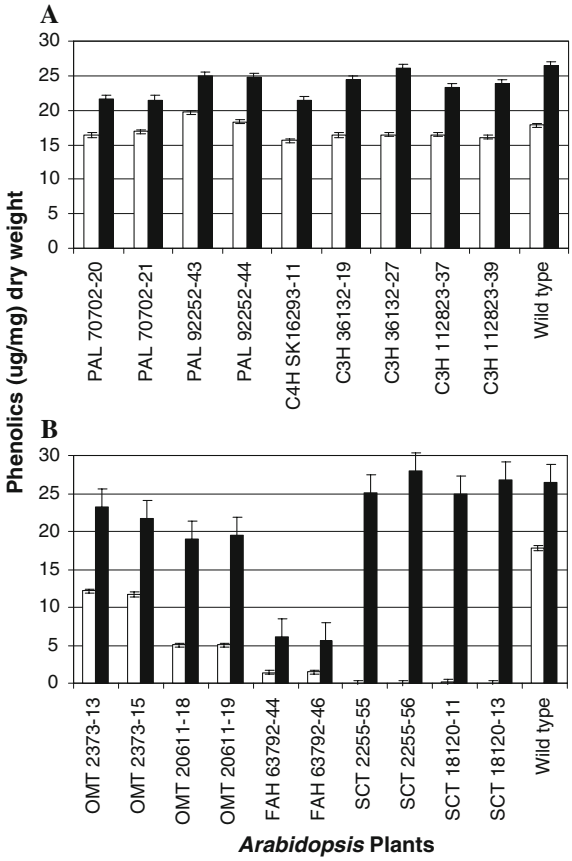

Fig. 1 *A. thaliana* knock-out mutants with altered soluble-phenolic and sinapine content in the seeds. (**A**) Methanol-soluble total phenolic content (■) and sinapine ester (□) in mutants with disruption in upper pathway genes *PAL*, *C4H* and *C3HI*. (**B**) Methanol-soluble total phenolic content (■) and sinapine ester (□) in mutants with disruption in lower pathway genes, *OMT1*, *FAH*, and *SCT*. Error bars indicate standard error of the mean of three individual extractions. Two to four mutant plants were analyzed for each knock-out line

to independently or collectively suppress the expression of 4 genes namely *C3H–C4H*, *COMT*, and *SCT*, involved in sinapine metabolism. We first silenced two genes simultaneously, namely *C4H* and *C3H* to alter the upper phenylpropanoid pathway. When transgenic plants (T_0 generation, DE279, DE290, DE293) carrying these fusion-gene constructs (72-146) were analyzed for seed sinapine content, we observed reduction in the range of only 20–36% compared to control seeds (DH12075). Line DE279 recorded the highest reduction of 36% amongst many plants analyzed (Fig. 2A).

Our analysis of *Arabidopsis* knockout mutants indicated that silencing of the *SCT* gene (At5g09640) resulted in over 95% reduction in sinapine content (Fig. 1B). Based on these results, we down-regulated the expression of the *SCT* gene in *B. napus* by RNAi using a CaMV35S-driven RNAi construct containing a *SCT*-specific 160 bp fragment amplified by PCR. One of these lines, DE324, had 52% reduction in seed sinapine and total soluble-phenolics when compared to untransformed *B. napus*. Other lines such as DE200 and DE209 had only minimal reduction in sinapine in comparison to control plants (Fig. 2B).

We next turned to silencing the *COMT* gene that is not only downstream of both the *C3H* and *C4H* genes in the pathway but also placed above *SCT* in the pathway. *COMT* is known to influence the production of sinapoyl compounds (Bugos et al. 1991). When *B. napus* transformants (containing the 35S:*COMT*-RNAi) were grown and their T_0 seeds analysed (DE127, DE118, DE114), we noticed ~20% reduction in the seed sinapine content especially with line DE127. Other lines such as DE118 and DE114 showed minimal change in sinapine content compared to the control plants (Fig. 2C).

Concomitant down-regulation of *FAH* and *SCT* genes leads to significant reduction of sinapine levels in *B. napus*

A previous study showed that up to 40% reduction in sinapine in *B. napus* seeds could be achieved by expressing an antisense RNA construct for the *FAH* gene (Nair et al. 2000). Given the 52% reduction achieved with suppression of *SCT* (Fig. 2B) and due to the fact that *FAH* is a critical gene in the phenylpropanoid pathway (Meyer et al. 1998; Nair et al. 2000), we hypothesized that concomitant silencing of the *FAH* and *SCT* genes using a napin promoter could lead to an enhanced effect. When both *FAH* and *SCT* genes were silenced, we noticed a significant reduction of sinapine content in seeds in the range of 51–90% (Fig. 2D). Use of a seed-specific napin promoter, coupled with the knocking down of *FAH* and *SCT* expression, seemed by far the most effective strategy given the reduction in sinapine content achieved.

Analysis of sinapine content in *B. napus* T_1 plants

While we were able to achieve reduction in seed sinapine content in transgenic *B. napus* in T_0 plants,

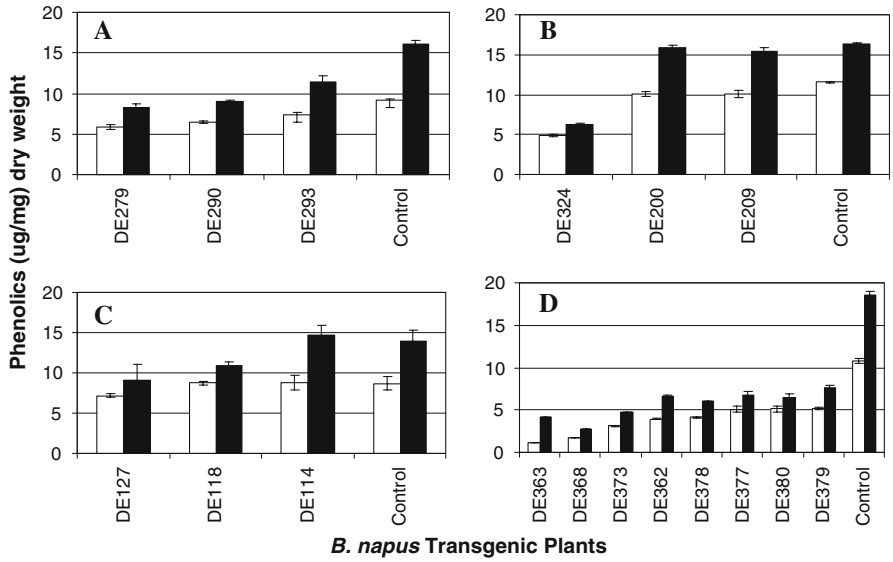

Fig. 2 Seed extract composition of *B. napus* transgenic plants. Methanol-soluble total phenolic content (■) and sinapine ester (□) in T_0 plants carrying RNAi constructs are shown: (**A**) 35S:*C3H-C4H*, construct 72-146, (**B**) 35S:*SCT*, construct 72-145, (**C**) 35S:*OMT1*, construct 72-115, (**D**) napin:*FAH-SCT*, construct 72-148. Error bars indicate standard error of the mean of three individual extractions. A total of three transgenic plants were analyzed for this construct and a total of three extractions per plant were performed

we were interested to know if we could attain trait stability in the next generation (T_1). As a result, we selected seeds of some better performing (low seed sinapine content) T_0 plants and grew at least 2–3 individual T_1 lines under green house conditions. Our results show that these T_1 plants had comparable levels of sinapine reduction (Fig. 3A, Table 3). However, there were slight differences observed such as the accumulation of sinapoyl glucose and sinapoyl malate which are indications of a block at the SCT enzymatic step (refer to Fig. 4 and section "Discussion"). These experiments showed that the reduction achieved through silencing was a stable function in the subsequent generation.

Sinapine reduction is attributed to a reduction in *SCT* transcript levels

Several studies on *SCT* gene expression reported high expression in *Arabidopsis* siliques (Shirley et al. 2001, Shirley and Chapple 2003), and more specifically during the cotyledonary stage and embryo development stages of *B. napus* (Milkowski et al. 2004). In order to verify if the reduction in sinapine content in the present study was due to silencing of the *SCT* gene, RNA extracted from developing seeds of *B. napus* were examined for *SCT* expression using RNA blot hybridization. We observed maximal expression of *SCT* transcripts during 20–25 days after anthesis in *B. napus* seeds, timing which coincides with the cotyledonary stage and embryo development stages (Fig. 3C). We therefore, used this time period to examine the expression of *FAH* and *SCT* expression in several down-regulated transgenic plants from the T_1 generation. Our results show weak expression of *SCT* in all lines containing low levels of sinapine as compared to the wild type control, with marginal reduction in the expression of *FAH* in some plants and a substantial increase in the *FAH* transcript steady state levels in about 50% of the plants despite containing *FAH* antisense (Fig. 3D). This data confirms that sinapine reduction in *B. napus* seeds is attributed to a reduction in *SCT* transcript and to a lesser extent to reduced expression of *FAH*.

Higher levels of free choline in transgenic *B. napus* seeds

The glucose moiety in sinapoylglucose is replaced by choline to form sinapoylcholine; a reaction mediated

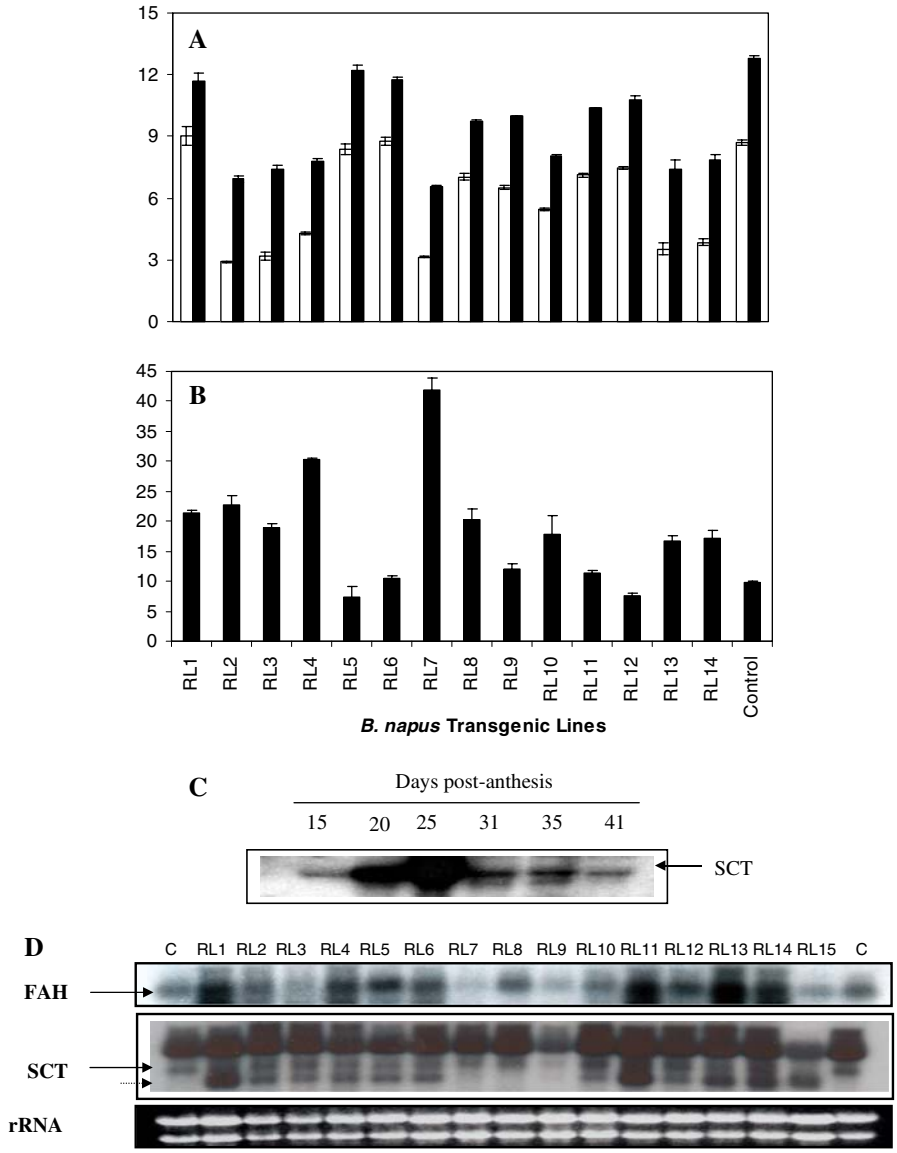

Fig. 3 Evaluation of *B. napus* transgenic T_1 generation for (**A**) stability of the sinapine reduction trait in T_1 lines carrying the napin:antisense-*FAH–SCT* construct 72-148. (**B**) Free choline content of plants carrying 72-148 construct (T_1 generation). Error bars indicate standard error of the mean of three individual experiments. A total of three transgenic plants were analyzed for this construct and a total of three extractions per plants were performed. RL1, RL2, RL3 (progeny of DE362), RL4, RL5, RL6 (progeny of DE363), RL7, RL8, RL9 (progeny of DE373), RL10, RL11, RL12 (progeny of DE379), RL13 and RL14 (progeny of DE384). (**C**) Transcript levels of *SCT* in *B. napus* developing seeds over a period of 41 days in DH12075 (untransformed control plant). (**D**) RNA blot of FAH and SCT in transgenic *B. napus* developing seeds (T_1) carrying napin:antisense *FAH–SCT*. RNA was collected between 22 and 25 days post-anthesis. Lanes RL 1–15 correspond to T_1 transgenic lines, C, refers to control plants used in this study. The short dotted arrow indicates the transcript that is specific to the transgene (*T*-DNA) region harboring *FAH–SCT–NOS* terminator sequence (~950 bp). All transgenic plants were generated in the DH12075 background. RNA extractions were sampled at appropriate stage (between 22 and 25 days) from bulk of three plants for each T_1 line shown. The blot was probed with a 165 bp *SCT*-specific fragment. RL1, RL2, RL3 (progeny of DE362), RL4, RL5, RL6 (progeny of DE363), RL7, RL8, RL9 (progeny of DE373), RL10, RL11, RL12 (progeny of DE379), RL13, RL14 and RL15 (progeny of DE384)

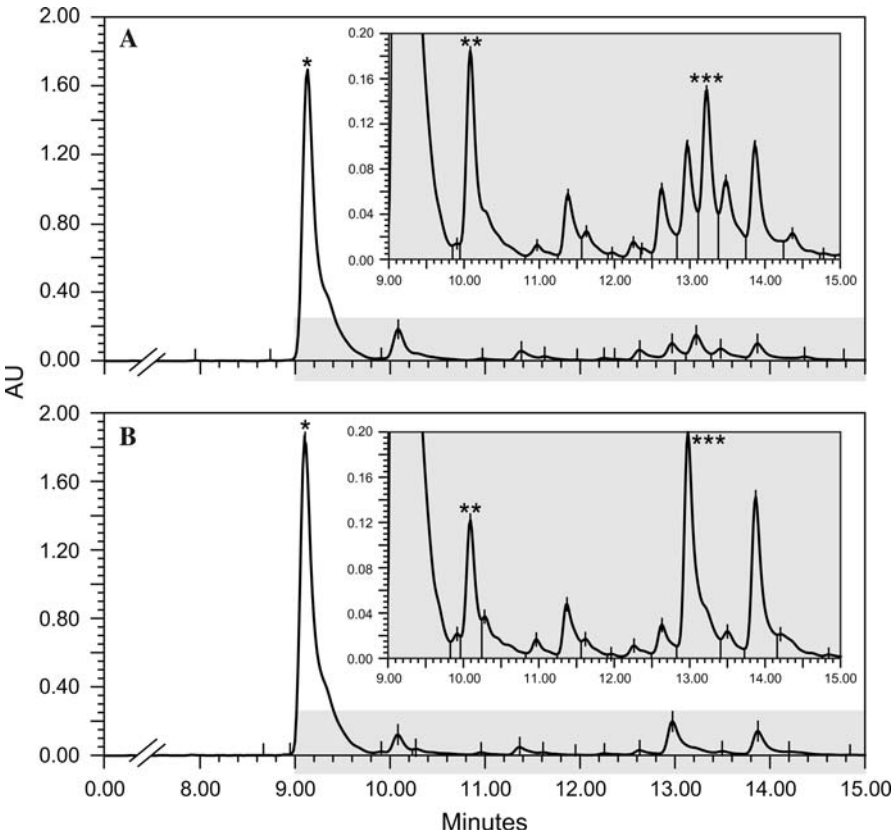

Fig. 4 A typical HPLC profile of methanol-soluble metabolites extracted from *B. napus* seeds. (**A**) Transgenic line RL-7 (T_1) carrying 72-148 (napin: antisense-*FAH–SCT*) construct (**B**) Control plant seeds. The sinapine peak is highlighted (*). Retention times for sinapine, sinapoyl glucose and sinapoyl malate are 9.18, 10.15 and 13.06 min respectively. The inset shows magnification of the time from 9 to 14 min in the chromatogram. Note the change in the levels of sinapoyl glucose (**) and sinapoyl malate (***) in the transgenic plants. Peaks were observed only after 9 min of run and hence the *X*-axis shown is for 8–15 min duration

by SCT. To determine if there was any correlation between sinapine and choline levels, we estimated seed choline content in low-sinapine *B. napus* lines. Free choline content was two to four-fold higher in the seeds of low sinapine lines (T_1 generation) compared to control (Fig. 3B), which is in agreement with a study in *Arabidopsis* (Huang et al. 2008). Free choline was found to be higher in line RL7 (42 μg/g meal; 64% sinapine reduction), mildly reduced in lines RL2 (23 μg/g meal; 63% sinapine reduction), RL3 (19 μg/g meal; 66% sinapine reduction), RL4 (30 μg/g meal; 50% sinapine reduction) and low in RL13 (16 μg/g meal; 56% sinapine reduction), RL14 (17 μg/g meal; 60% sinapine reduction) relative to the control (9.8 μg/g meal), suggesting that greater amounts of free choline have been released in lines with altered sinapine content.

Comparison of green house- and field-grown transgenic plants and their seed sinapine content

Our analysis of seed sinapine levels in transgenic *B. napus* revealed that greenhouse-grown plants carrying the *FAH–SCT* antisense construct had the lowest sinapine content. In order to understand the effect of environmental factors on sinapine reduction and visible agronomic traits, field experiments were carried out in Saskatoon on T_1 lines of the better-performing T_0 plant DE363. When T_1 seeds of plants grown in a greenhouse and a field plot were compared, we observed that the field-grown plants had slightly higher levels of soluble-phenolics (Fig. 5). Apart from this difference, the plants under both conditions had consistent reduction in the sinapine content although field-grown plants exhibited much greater variability. The results suggest that sinapine reduction is not greatly altered by environmental factors. The plants in both conditions were visibly similar in appearance and health.

Discussion

Over the past decade attempts have been made to reduce sinapine levels using traditional approaches albeit with limited success. Recently, a combination

Table 3 Sinapine content of *B. napus* transgenic lines (T_1 generation) carrying other RNAi constructs

Construct	T_1 Line# (Parent)	Mean Sinapine (fw µg/mg) ± SE	% Reduction in Sinapine compared to control
72-145	RL16 (DE200)	6.421 ± 0.15	26.3
	RL17 (DE200)	6.805 ± .020	21.9
	RL18 (DE200)	4.586 ± 0.09	47.3
	RL19 (DE324)	6.852 ± 0.20	21.3
	RL20 (DE324)	7.226 ± 0.14	17.0
	RL21 (DE324)	6.505 ± 0.08	25.3
72-146	RL22 (DE279)	5.222 ± 0.10	40.0
	RL23 (DE279)	4.012 ± 0.19	53.9
	RL24 (DE290)	7.064 ± 0.10	18.9
	RL26 (DE290)	7.635 ± 0.17	12.3
72-115	RL30 (DE114)	7.225 ± 0.18	17.0
	RL31 (DE114)	7.435 ± 0.08	14.7
	RL32 (DE114)	8.130 ± 0.17	6.7
	RL33 (DE118)	8.342 ± 0.54	4.27
	RL34 (DE118)	8.415 ± 0.11	3.43
	RL35 (DE127)	7.771 ± 0.04	11.46
72-142	RL37 (DE264)	7.389 ± 0.14	15.2
	RL38 (DE264)	7.008 ± 0.20	19.58
	RL39 (DE264)	6.395 ± 0.38	26.6
	RL40 (DE455)	7.190 ± 0.14	17.48
	RL41 (DE455)	7.818 ± 0.15	10.28
	RL42 (DE455)	7.374 ± 0.3	15.38
	RL43 (DE466)	7.402 ± 0.05	15.06
	RL44 (DE466)	7.846 ± 0.06	9.96
	RL45 (DE466)	7.260 ± 0.07	16.68
Control		8.714 ± 0.32	NA

of metabolic engineering and plant breeding techniques have been used to achieve up to 40% reduction in sinapine content in seeds of *B. napus* by antisense RNA down regulation of ferulic acid 5-hydroxylase (Nair et al. 2000), and up to 76% reduction by RNAi silencing of sinapate glucosyltransferase (*SGT*) (Husken et al. 2005). Efforts were also made towards the identification of low sinapine *Brassica* germplasm to generate low sinapine varieties by classical plant breeding (Velasco and Möllers 1998). Development of *B. napus* germplasm with reduced sinapine content is more economically sustainable than reducing sinapine by chemical treatments of the meal because of the cost of processing (Husken et al. 2005). About 80–85% reduction in sinapine content in seed is considered sufficient for use in animal feed. Given the need for drastic reduction of sinapine content in seeds, we employed a genetic engineering approach to achieve reduction in seed sinapine content.

Using the high genetic and molecular collinearity that exists between the genomes of *Arabidopsis* and *Brassica* species (Cavell et al. 1997; Parkin et al. 2002; Paterson et al. 2001; Ryder et al. 2001; Vanoosthuyse et al. 2001); we identified rate-limiting steps in the phenylpropanoid pathway in *A. thaliana* and successfully altered this pathway in *B. napus*. Our results show that it is effective to extrapolate *Arabidopsis* genome information to engineer *B. napus* plants. We also show that it is possible to modulate sinapine biosynthesis in a controlled manner to achieve a range of reduction in seed sinapine content in *B. napus*. Gene copy number is an important factor to be considered when analyzing knockout mutants. For instance, *PAL* is encoded by multiple genes in *A. thaliana* (three genes, Liang

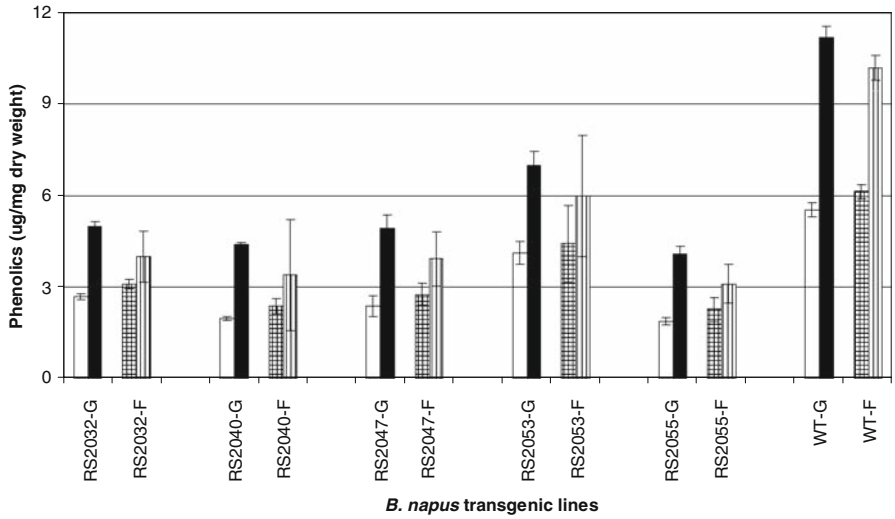

Fig. 5 Assessment of genotype and environmental (G × E) effects on phenolic and sinapine content in the progeny (T_1) of transgenic parent DE363 carrying the construct 72-148 (napin: *FAH–SCT* antisense). Greenhouse-grown plants (G), phenolic content (■) and sinapine ester content (□); Field-grown plants (F), phenolic content (▦) and sinapine ester content (▥). Error bars indicate standard error of the mean of three individual extractions. A total of three transgenic plants were analyzed for each line

et al. 1989; four genes, Costa et al. 2003); therefore disrupting or mutating one of them may be inadequate to block the biosynthesis of sinapine. However, this may not be the case with *Arabidopsis C3H* and *C4H*, which are encoded by single copy genes (Raes et al. 2003). Mutations in *PAL*, or *C4H* genes had no noticeable impact on sinapine biosynthesis, however, *C3H* has been reported to have some effect on the sinapine pathway (Abdulrazzak et al. 2005). This could be due either to the presence of alternative biosynthesis routes for sinapine precursors or non-specific enzymatic biosynthesis (Bell-Lelong et al. 1997; Mizutani et al. 1997; Nair et al. 2002). The *C3H* or *C4H* genes are also involved in a complex signal cascade that receives different signals (Bell-Lelong et al. 1997; Nair et al. 2002; Raes et al. 2003). Recent evidence indicates that differential expression of *C3H* and *C4H* genes is associated with *cis*-acting regulatory elements, and the divergent isoforms play distinct roles in phenylpropanoid metabolism (Lu et al. 2006; Reddy et al. 2005).

The *COMT* gene encodes the caffeic acid *O*-methyltransferase, a bifunctional enzyme capable of performing two *O*-methylations at the C5 position (Bugos et al. 1991) or at the C3 position (Ye et al. 2001) of the aromatic compound. Such methylation events are likely to influence the type and amount of phenolics in the tissues, and therefore capable of affecting sinapine content. We observed that silencing the *COMT* gene caused slight reduction even though it is placed early in the sinapine biosynthesis pathway, likely because caffeic acid *O*-methyltransferase1 (*COMT*) is involved in redundant functions for sinapoyl malate biosynthesis in *Arabidopsis* (Do et al. 2007). It is unclear how a deficient redundant *COMT* could lead to a reduction in the sinapoyl malate content in *Arabidopsis* plantlets. Another study reported the presence of 17 copies of *COMT* gene in *Arabidopsis* (Costa et al. 2003), of which only one is involved in sinapyl alcohol biosynthesis (Jaeck et al. 1996). These studies suggest that *COMT* is involved in the formation of methylated soluble phenolics, particularly sinapoyl compounds in crucifers.

Given our interest in ascertaining that reduction of sinapine was due to silencing of sinapine pathway genes, we analyzed the expression of *SCT* in developing *B. napus* seeds. Our results show that maximal expression of *SCT* transcripts were detected in seeds between 20 and 25 days after anthesis in *B. napus,* coinciding with the cotyledonary stage and embryo development stages. Previous studies have reported that the *SCT* transcript levels were high in siliques and absent in other plant tissues (Shirley et al. 2001, Shirley and Chapple 2003). Furthermore, the transcript levels

of *SGT1* and *SCT* in *B. napus* seem to determine changes in the corresponding enzyme activities in the developmental regulation of the metabolism of this sinapate ester (Milkowski et al. 2004). At early embryogenesis stages, i.e., globular, torpedo or late torpedo stage, there is no detectable expression of *SCT* whereas the *SGT1* transcript could be found in small quantity during these stages (Milkowski et al. 2004). Other studies reported that the gene encoding *SCT* was subjected to pronounced transcriptional regulation during seed development (Shirley et al. 2001, Shirley and Chapple 2003). Its expression was restricted to seed development and was nearly undetectable in the earliest stages of development, while transcripts increased to reach their highest levels in the developed embryo stage. As seed development progressed, a dramatic and rapid decrease of the *SCT* transcript level was observed. Later stages (26–35 days) were shown to contain about 10% of the amount of *SCT* transcript found prior to 25 days (not shown).

All five genes (*C4H*, *C3H*, *COMT*, *FAH* and *SCT*) are reportedly single copy genes (functional) in *A. thaliana* based on genomic analyses using BioVis (Ver 0.5) with a homology threshold set to 80%. Because of the polyploid nature of *B. napus*, for every single *Arabidop*sis gene, a minimum of two–four copies are estimated to be present in *B. napus* genome. Since our RNAi strategy in *B. napus* was based on *Arabidopsis* genome information, and with multiple copies of *Arabidopsis* homologs expected in *B. napus*, we conducted some preliminary analysis by comparing the two genomes (http://www.brassica.ca) to understand the effectiveness of our RNAi strategy and its penetrance in *B. napus*. As a result of genome-wide comparison, we identified several homologs of *A. thaliana* genes in the *B. napus* EST collection comprising $>150{,}000$ clones (Supplementary Table 2). For the *C4H* (At2g30490) gene, we identified homologs in diverse tissue types including anther, while *C3H* (At2g40890) gene homologs were limited to the roots, embryo and cotyledons, indicating that any target to silence *C4H* would have a pronounced effect on the *B. napus* genome than a *C3H* (homolog) target. *COMT* (At5g54160) gene showed the maximal number of *B. napus* homologs, however, they were diverse, i.e., identified in multiple tissues ranging from embryo to anther to roots. *FAH* (At4g36220) gene on the other hand produced moderate hits thus showing that its expression is not as wide-spread as *COMT*. *FAH* homologs were observed in diverse tissues such as flowers, leaves, stem, root and cotyledons. *SCT* homologs were restricted to seeds (young, developing and mature) and anther tissues. These results provide useful leads because a target design based on *C4H* or *COMT* is likely to work against multiple members of unrelated gene families in *B. napus*, thus resulting in some unexpected observations. Similar results have been obtained in other studies involving *B. napus* transgenic plants (Bhinu and Hannoufa, unpublished).

Sinapine is synthesized via a transfer reaction that replaces glucose moiety with choline. Choline in plants is synthesized from its precursor phosphoethanolamine, and the first committed step is methylation of phosphoethanolamine catalyzed by phosphoethanolamine *N*-methyltransferase, PEAMT (EC 2.1.1.103) (Cruz-Ramirez et al. 2004). By knocking out the *SCT* gene in *Arabidopsis* seed, it was possible to eliminate the anti-nutritional compound sinapine and simultaneously increase free choline more than twofold (Huang et al. 2008); similar results were reported with *Arabidopsis SNG2* mutant allele (Shirley et al. 2001). Given the interest in choline, we examined our transgenic lines for: (1) the presence of bound forms of phenolic acids (sinapoyl choline) and (2) free choline accumulation. We hypothesized that with choline being used in the synthesis of sinapoyl choline, a reduction in sinapine content would elevate free choline levels. In line with our hypothesis, lines carrying construct 72–148 (T_0 and T_1 generations) had significantly lower levels of sinapine and higher amounts of choline compared to the non-transformed plants (Fig. 3A and B). Plants with both low sinapine and higher choline levels may provide better quality canola seeds that add value to the meal and animal feed industry. Overall, our analysis suggests that genes affecting later rather than earlier steps in the biosynthesis pathway have more impact on qualitative and quantitative profiles of phenylpropanoids in *B. napus* seeds and that they can double the advantage to producers by simultaneously altering the sinapine and choline contents.

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