

# Perturbation of lignin biosynthesis pathway in *Brassica napus* (canola) plants using RNAi

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Bhinu, V.-S., Li, R., Huang, J., Kaminskyj, S., Sharpe, A. and Hannoufa, A. 2009. **Perturbation of lignin biosynthesis pathway in *Brassica napus* (canola) plants using RNAi.** Can. J. Plant Sci. **89**: 441–453. *Brassica napus* meal contains high levels of lignin, which is one of the most important compositional factors affecting feed utilization by ruminants. We attempted to modify the concentration and composition of lignin in *B. napus* plants using the RNAi approach. Four genes were targeted for silencing by this approach either independently or in combination; caffeic acid *O*-methyltransferase (*COMT*), cinnamate 4-hydroxylase (*C4H*); coumarate 3-hydroxylase (*C3H*); ferulic acid 5-hydroxylase (*F5H*). We successfully developed transgenic *B. napus* lines expressing CaMV35S:*C3H-C4H* RNAi, CaMV35S:*F5H-COMT* RNAi, and Cruciferin:*COMT* RNAi that contained up to 40% less seed lignin in the transgenic seeds compared to the control. Despite successfully achieving suppression of these lignin biosynthesis genes and reduction in lignin content in *B. napus* seeds, we observed minor phenotypic effects on the transgenic plants. In lines carrying the cruciferin: *COMT* RNAi construct we observed a decrease in lignin content (40%) in the seed and anatomical variations when stem sections were examined. While our silencing had no major negative effect on plant growth it resulted in deformation of vessel elements, and minor changes in S-units. Taken together, these results clearly show that by employing RNAi strategy, it is possible to alter seed lignin content and composition in a manner non-detrimental to *B. napus* plants.

**Key words:** *Brassica napus*, cruciferin, lignin, *COMT*, RNAi

Bhinu, V.-S., Li, R., Huang, J., Kaminskyj, S., Sharpe, A. et Hannoufa, A. 2009. **Altération d'un circuit de la biosynthèse de la lignine chez le canola (*Brassica napus*) grâce à l'ARNi.** Can. J. Plant Sci. **89**: 441–453. Le tourteau de *Brassica napus* renferme beaucoup de lignine, un facteur qui, dans la composition des aliments, affecte considérablement son utilisation par les ruminants. Les auteurs ont tenté de modifier la concentration et la composition de la lignine chez les plants de *B. napus* en recourant à l'ARNi. Ils ont ciblé quatre gènes pour court-circuiter, par leur action soit indépendante, soit combinée, la production de lignine, en l'occurrence ceux de la *O*-méthyltransférase de l'acide caféique (*COMT*), de la cinnamate 4-hydroxylase (*C4H*), de la coumarate 3-hydroxylase (*C3H*) et de la 5-hydroxylase de l'acide férulique (*F5H*). Ils ont réussi à créer des lignées transgéniques de *B. napus* exprimant l'ARNi CaMV35S:*C3H-C4H*, l'ARNi CaMV35S:*F5H-COMT* et l'ARNi cruciférine:*COMT* dont les semences contenaient jusqu'à 40 % moins de lignine que celles du cultivar témoin. Bien qu'ils soient parvenus à faire taire l'expression des gènes codant la biosynthèse de la lignine et à réduire la concentration de cette dernière dans les graines du canola, les auteurs ont relevé de légers effets sur le phénotype des plantes transgéniques. Ainsi, chez les lignées portant l'ARNi cruciférine:*COMT*, ils ont noté une diminution de la concentration de lignine (40%) dans les graines, mais aussi des variations anatomiques lors de l'examen des tiges; si elle n'a aucune incidence néfaste majeure sur la croissance de la plante, la suppression des gènes déforme quelques parties des vaisseaux et apporte de petites modifications aux unités S. Ensemble, ces résultats indiquent clairement qu'on peut modifier la concentration et la composition de la lignine des graines en recourant à l'ARNi, sans que les plants de *B. napus* en souffrent.

**Mots clés:** *Brassica napus*, cruciférine, lignine, *COMT*, ARNi

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Seeds of *Brassica napus* (canola) plants are well known for their high value associated with oil and oil-related

**Abbreviations:** *COMT*, caffeic acid *O*-methyltransferase; *C4H*, cinnamate 4-hydroxylase; *C3H*, coumarate 3-hydroxylase; *F5H*, ferulic acid 5-hydroxylase; *CaMV35S*, cauliflower mosaic virus 35S promoter

products. However, the meal remaining after the oil extraction is considered a by-product and used only as a low-grade protein source in the livestock feed industry. The low value of the canola seed meal is attributed mainly to the high levels of anti-nutritional factors such as high fiber/lignin, sinapine and phytate that remain in the seed. For instance, canola meal contains higher amounts of lignin (8% of the meal component) compared with soybean meal (1% of the meal component), and lignins are the most important compositional factor affecting feed utilization by ruminants (Jarrige 1980). Numerous studies suggest that lignins play a role not only through their concentration, but also through their structural characteristics (Jung and Deetz 1993; Besle et al. 1994). The concentration and composition of lignin varies in different plants (Monties 1986) and it may be possible to modify lignins by genetic engineering without interfering with normal plant development (Franke et al. 2002; Baucher et al. 2003; Weng et al. 2008). Previous studies on brown-midrib (*bm*) mutants have revealed that *bm1* mutants show severely reduced cinnamyl alcohol dehydrogenase (CAD) activity. Several other types of brown-midrib (*bm*) mutations have also been described (Anterola and Lewis 2002), one of which is the *bm3* mutant in maize. The *bm3* mutant has, relative to the “normal” phenotype, lower amounts of cell-wall constituents, acid detergent fiber and acid detergent lignin (Muller et al. 1971), as well as an increased forage digestibility (Barnes et al. 1971). One way of modifying lignification is to inhibit enzyme activities of the lignin biosynthetic pathway. CAD has been a common target in tobacco for lignin reduction studies (Sewalt et al. 1997). For example, CAD, which catalyses the final step of the synthesis of lignin monomers, has been successfully inhibited by chemical inhibitors (Grand et al. 1985; Moerschbacher et al. 1990), but the effects on lignin composition and digestibility of the cell walls remain undetermined.

Lignin is an integral cell wall component that supplements cellulose to a greater extent in water-conducting tissues of most vascular plants. It is a complex phenolic heteropolymer resulting from the oxidative polymerization of three types of hydroxycinnamyl alcohols termed monolignols (*p*-coumaryl, coniferyl, and sinapyl alcohols) that vary in their degree of hydroxylation and methoxylation (Baucher et al. 2003). Lignin is the basic unit of xylem and consists of complex polymerized phenylpropane units. The three most important starting compounds are coumaryl alcohol (with an –OH group in position 4 of the phenyl ring), coniferyl alcohol (–OH group in position 4, –OCH<sub>3</sub> in position 3) and sinapyl alcohol (–OH group in position 4, –OCH<sub>3</sub> group in positions 3 and 5). It is predominantly synthesized and deposited in the secondary cell wall of certain specialized cells such as xylem vessels, tracheary elements, phloem fibers, and the periderm. From a functional viewpoint, only vascular plants contain lignin, which provides mechanical strength, aids in resistance to pathogen

attack, and water impermeability to the polysaccharide-protein matrix of the cell wall (Whetten et al. 1998; Rogers and Campbell 2004). Besides its essential structural roles and significance, lignin content and composition also have profound agricultural and environmental consequences. More specifically is the impact of lignin on the optimal utilization of feed because an intimate association of lignin with the cell wall polysaccharides such as cellulose and hemicellulose makes these carbohydrates recalcitrant to ruminant digestion in seed meal (Albrecht et al. 1987). These issues warrant approaches aimed at reducing lignin either by molecular breeding or genetic engineering approaches involving alteration of expression of lignin biosynthesis genes without affecting normal plant development.

Conventional breeding approaches, though useful in introducing desirable traits and achieving favorable results in some plant characteristics, are less straightforward and predictable in outcome than anticipated for altering lignin content. In an era of directed engineering of plant metabolism, suppression of single or multiple genes using antisense or RNAi-mediated approaches offer great potential for the rapid and coordinated suppression of genes in biochemical pathways (Bhinu et al. 2008; Huang et al. 2008). To this end, we report the suppression of four lignin genes involved in lignin biosynthesis namely, caffeic acid *O*-methyltransferase (*COMT*), ferulic acid 5-hydroxylase:caffeic acid *O*-methyltransferase (*F5H-COMT*), coumarate 3-hydroxylase:cinnamate 3-hydroxylase (*C3H-C4H*) either in combination or independently in *B. napus*.

## MATERIALS AND METHODS

### Plant Material

Transgenic *B. napus*, control, wild type (DH12075) and *Arabidopsis thaliana* (Columbia) plants were grown under green house conditions in soil prepared according to the protocol described elsewhere (Yu et al. 2007) at 25°C/17°C, 16 h light/8 h dark cycles. Seeds collected from dry siliques of mature plants were used for lignin analysis. 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<sup>-2</sup> s<sup>-1</sup>.

### 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 (<http://napus.agr.gc.ca>).

### Vector Construction for RNAi

Based on the published cDNA sequences of *Arabidopsis* genes namely, cinnamate 4-hydroxylase (*C4H*, At2g30490), coumarate 3-hydroxylase (*C3H*, At2g40890), caffeic acid *O*-methyltransferase (*COMT*, At5g54160), and ferulic acid 5-hydroxylase (*F5H*, At4g36220), their numerous homologs in *B. napus* were identified in a collection of >150 000 expressed sequence tags (EST) derived from *B. napus* line DH12075 cDNA libraries generated at Agriculture and Agri-Food Canada, Saskatoon Research Center (<http://napus.agr.gc.ca>). The high genetic collinearity between *Arabidopsis* and *B. napus* (Cavell et al. 1998) were valuable in identifying conserved regions between the cDNA clones, and between *Arabidopsis* and *B. napus* (supplementary Fig. 1). These cDNA sequences were used in the design of primers and generation of RNAi constructs for expression in *B. napus*, as described below.

A common strategy was adopted for all the RNAi constructs. Briefly, forward primers having built-in *SpeI*/*AseI* and reverse primers with *Bam*HI and *Swa*I sites were used to generate fragments for RNAi construction (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  $\beta$ -glucuronidase gene in a pGSA1285 vector (CAMBIA, Canberra, ACT, Australia) driven either by a CaMV35S or cruciferin promoter as designated in the construct name. The resulting RNAi vectors were designated 72-146 (*35S:C3H-C4H RNAi*), 72-123 (*Cruciferin:COMTRNAi*) and 72-142 (*35S:*

*F5H-COMT RNAi*) (Fig. 1). All these constructs were confirmed by automated DNA sequencing.

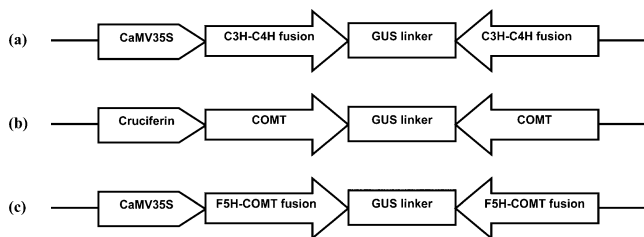
*CaMV35S:C3H-C4H RNAi*: The 900 bp *B. napus* coumarate 3-hydroxylase gene fragment was amplified by PCR from the *Brassica* cDNA clone RL4992 using primers P1 and P2 (Table 1). Likewise, the 616 bp *B. napus* cinnamate 4-hydroxylase fragment was amplified by PCR from the *Brassica* cDNA clone RL7118 using primers P3 and P4. Both the 900 bp and 616 bp fragments were mixed and further used as templates in another round of PCR with P1 and P4 primer pair to create a 640 bp fusion gene product (*C3H-C4H*) that was cloned in pBluescriptII KS vector (digested with *EcoRV*). Common steps as described earlier were followed to generate the RNAi construct pGSAC3H C4 H-RNAi that was designated 72-146.

*Cruciferin:COMT-RNAi*: A 250 bp fragment from the 3' end of *COMT* gene was amplified by PCR from the *B. napus* cDNA clone ESS2134 using primers P5 and P6. The 743 bp cruciferin+G string promoter was isolated from *B. napus* using primer pair P7 and P8 and cloned in pBluescript II KS to generate pBScruciferin. The promoter fragment was excised using *Bg*III-*Sac*I from pBluescript II KS and ligated with *COMT* RNAi fragment to generate pGSACruc-COMT RNAi construct (designated 72-123).

*CaMV35S:F5H-COMT RNAi*: A 480 bp *F5H* fragment was amplified by PCR from *B. napus* root cDNA clone RL1627 using primer pair P9 and P10. Similarly, a 230 bp *COMT* fragment was amplified by PCR from *B. napus* root cDNA clone RL1627 using primer pair P5

**Table 1.** List of primers used in this study

Primer	Sequence	Notes
P1	<b>CGACTAGTGGCGCGCCAGAGATGATC</b> AAGAACCCAAGAGTG	Forward primer, to amplify <i>C3H</i> from RL4992, contains <i>SpeI</i> and <i>AseI</i> sites highlighted
P2	AACTCTTCAGCTCCGAACGGAAGCAGCC	Reverse primer for <i>C3H</i> gene from RL4992
P3	CCGTTCCGACCTGAAGAGTTTAGGCCCGAGAG	Forward primer, to amplify <i>C4H</i> from RL7118
P4	<b>GCGGATCCATTAAATGGTGGAGTG</b> GTGAAGGATGTG	Reverse primer for <i>C4H</i> gene from RL7118, contains <i>Bam</i> HI and <i>Swa</i> I sites
P5	<b>GCACTAGTGGCGCGCCATTGCATTATGCTA</b> GCTCACAACCCTG	Forward primer used to amplify <i>COMT</i> from ESS2134 library, contains <i>SpeI</i> and <i>AseI</i> sites
P6	<b>GCGGATCCATTAAATAACAAAGACGGTGA</b> AGTAGACGTACC	Reverse primer for <i>COMT</i> gene from ESS2134, contains <i>Bam</i> HI and <i>Swa</i> I sites
P7	<b>GCAGATCTAAGCTTTTGGCCCTTAATTAT</b> GCTCTCTTTCTAATC	Forward primer used to amplify Cruciferin promoter+G string region and introduce <i>Bg</i> III and <i>Hind</i> III sites
P8	<b>GCGAGCTCTCTAGAATTGTGTGTTTT</b> GGTGATAGATGGATGAAG	Reverse primer used to amplify Cruciferin promoter+G string region and introduce <i>Sac</i> I and <i>Xba</i> I sites
P9	<b>GCACTAGTGGCGCGCCGCTCGTGAAG</b> GCCCGTAATGACC	Forward primer to amplify <i>F5H1</i> from EST RL1627, contains <i>SpeI</i> and <i>AseI</i> sites
P10	GCGGATCCATTAAACTGCGGTTT CGTGATAGGAGGAGTGG	Reverse primer for amplifying <i>F5H1</i> gene from RL1627, contains <i>Bam</i> HI and <i>Swa</i> I sites
P11	<b>GCATAATGCATGCGGTTTCGTGTAGGAGGAG</b>	Reverse primer used for fusion with <i>COMT</i> gene, <i>COMT</i> sequence is shown in bold
P12	<b>CGAAACCGCATGCATTATGCTAGCTCACAACCCTG</b>	Forward primer used for fusion with <i>F5H1</i> gene from RL1627 library, portions of <i>F5H1</i> gene part is in bold
P13	GGAGAGGACACGCTCGAGTATAAGAG	Forward primer to detect DNA insert cloned into the pGSA1285 vector.
P14	CAACGTGCACAACAGAATTGAAAGC	Reverse primer to detect DNA insert cloned into the pGSA1285 vector.
P15	<b>GCTCTAGAAACAAAGACGGTGAAGTAGACGTACC</b>	Reverse primer used for amplifying 3' end of <i>B. napus COMT</i> , contains <i>Xba</i> I site, and employed in PCR screening of transgenic plants.



**Fig. 1.** Schematic diagram of RNAi construct using a GUS linker flanked by partial sequences of the gene targeted for suppression, (a) CaMV35S:*C3H-C4H*, construct 72-146, (b) CaMV35S:*F5H-COMT*, construct 72-142, and (c) Cruciferin:*COMT* construct 72-123 were used in RNAi construct generation.

and P11. These individual PCR products were gel purified and used as template for a second round of PCR to amplify a ~700 bp fusion gene product using the primer pair P12 and P6. The resulting fusion gene product was used in the generation of pGSAF5H-OMT RNAi (designated 72-142) construct using the common strategy described above.

### Transformation of *Brassica napus*

Cotyledon explants of *B. napus* doubled haploid line DH12075 were used for *Agrobacterium tumefaciens* GV3101pMP90-mediated transformation according to (Moloney et al., 1989). Primers that amplify the fusion gene products were used to screen for *C3H-C4H*, and *F5H-COMT* transformants. For the Cruciferin:*COMT* construct, both promoter- and target gene-specific primers were used to eliminate amplification of endogenous gene products. Only those plants shown to be transgenic as determined by PCR were subjected to further analysis. The primers used for PCR were P13 and P14 for 72-146, P9 and P15 for 72-142, and P7 and P15 for 72-123 (Table 1; supplementary Fig. 1).

### Nucleic Acid Analysis

Total RNA was isolated from developing seeds (20–25 d post-anthesis) of *B. napus* plants including lines with constructs 72-146, 72-123, and 72-142, as described in Carpenter and Simon (Carpenter and Simon 1998). Approximately 10 µg of RNA was used for electrophoresis and northern blot analysis was performed according to standard procedures followed in our laboratory (Yu et al. 2007).

### Lignin Analysis

Total lignin content of *B. napus* seeds collected from transgenic and control plants was determined using the thioglycolic acid assay (Lapierre et al. 1995) and by gravimetric determination of lignin using the filter bag technique described below. Briefly, 0.5 g of air-dried seed sample was ground and passed through a 1-m sieve before being bagged. The sealed bags were completely dried before immersing in adequate volume of 72%

H<sub>2</sub>SO<sub>4</sub> (approximately 250 mL) sufficient to submerge the sealed bags. The submerged bags were agitated at 30 min intervals. After an incubation period of 3 h, the sulphuric acid was poured off and the bags were rinsed with tap water to remove all acid. Rinsing was repeated until the pH was neutral and followed by rinsing with ~250 mL of acetone for 3 min to remove water. The bags were dried in an oven at 105°C for 2–4 h, and cooled. Once cooled in a desiccator to ambient temperature, the bags were weighed. The entire bag was ashed in a pre-weighed beaker at 525°C for 3 h or until carbon-free. The weight loss was calculated and the bag ash correction included to determine lignin content using the following formula:

$$\text{Lignin\%} = \frac{[\text{weight after extraction process} - (\text{bag tare weight} \times \text{blank bag correction})]}{\text{sample weight}} \times 100$$

### Fibre Analysis

Fibre content in *B. napus* seeds collected from transgenic and control plants was also determined by using the services of ALS Laboratory that uses the following two methods. The acid detergent fiber (ADF) content was determined using the AOAC method #973.18 described for lignin. The neutral detergent fiber (NDF) was estimated using another validated method [NFTA (1993), pp. 95-103]; which is also a filter bag technique. Briefly, about 0.5 g of dried and ground seed materials were placed in a dry filter bag and samples extracted using repeated acetone. Once the acetone dried off, the bags were immersed in a container filled with acid-detergent solution [20 g cetyl trimethylammonium bromide (CTAB) to 1 L 1 N H<sub>2</sub>SO<sub>4</sub> previously standardized] for 1 h. At the end of the extraction, the bags were thoroughly washed with tap water until the water reached a neutral pH. Subsequently, the bags were soaked again in acetone for 3–5 min, dried in an oven and then weighed. The difference in weights when appropriately corrected provides the %ADF content.

The procedure for determining NDF was similar to the ADF except that the acid-detergent solution was replaced with a neutral-detergent solution [30 g sodium lauryl sulfate, 18.61 g EDTA, 6.81 g sodium tetraborate decahydrate, 4.56 g sodium phosphate dibasic, and 10 mL triethylene glycol in 1 L distilled water], with a pH range of 6.9–7.1.

### Histochemical Analysis of Lignin

Uniform free-hand sections of fresh *B. napus* stems from control and transformants with ~40% reduction in lignin content were subjected to Weisner (Clifford 1974; Pomar et al. 2002) and Maüle (Iiyama and Pant 1998) staining. The same portion of the stem region between the 8th and the 10th node from the surface of the soil (thickness was ~9 mm for plants grown in the greenhouse) was used for sectioning experiments. Transverse sections obtained from the same portion of the stems and softened in 5% vol/vol formaldehyde for 1–2 d were immersed in 2% wt/vol phloroglucinol solution in

ethanol (95%, vol/vol) for 2 min, followed by treatment with 50% HCl for 1 min for the Weisner reaction. For Maüle staining, the sections were submerged for 5 min in 1% wt/vol KMnO<sub>4</sub>, rinsed in water, destained with 30% HCl and treated with a concentrated solution of NH<sub>4</sub>OH. In both cases, the sections were imaged at 12 × or 125 × magnification using an AxioCam HRc camera fitted on SteREO Lumar.V12 stereomicroscope (Carl Zeiss, Gottingen, Germany) equipped with a HBO100 microscope illuminating system and a cold light source KL2500 LCD for intensive illumination, within 20–40 min of staining.

### Statistical Analysis

A completely randomized design was used with three replicates (plants) for each transgenic line (T<sub>0</sub>) studied. Mean differences were analyzed for significance by employing a two-sample *t*-test using the proprietary software SAS version 8.2 (SAS Institute, Inc., Cary, NC) at *P* ≤ 0.05.

## RESULTS AND DISCUSSION

### Plant Transformation with RNAi Constructs

The RNAi constructs driven by either the CaMV35S or the cruciferin promoter (Fig. 1) were mobilized into *Agrobacterium tumefaciens* GV3101pMP90, which was used for plant transformation. Putative transformants exhibiting resistance to herbicide glufosinate (150 g L<sup>-1</sup>, Liberty<sup>TM</sup>) were selected and transferred to a greenhouse. These transformants exhibited a phenotype similar to control plants maintained under similar growth conditions indicating that our RNAi strategy was not lethal. The presence of transgenes in putative transformants was determined by PCR using transgene-specific primers (Table 1) and used in further experiments. Previous studies in our laboratory showed that this strategy had been effective in manipulating levels of secondary metabolites in *Arabidopsis* and *B. napus* (Bhinu et al. 2008; Huang et al. 2008). One transgenic line, RL53, carrying the cruciferin-*COMT* RNAi construct failed to set seeds and hence could not be used for further analysis. While we recognize that plants subjected to tissue culture procedures may undergo some cytological perturbation in the T<sub>0</sub> generation, we did not observe any phenotypic differences between the control and transgenic plants in both generations of *B. napus*. This has been our experience with *B. napus* transformants (Bhinu et al. 2008).

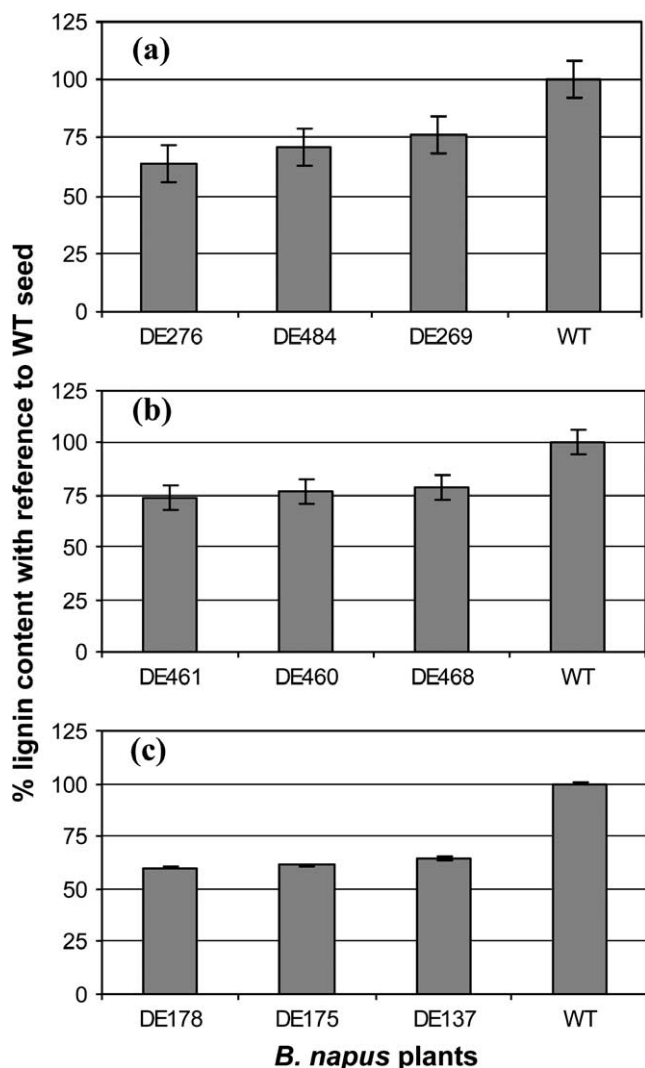
Many phenylpropanoid pathway genes have been used to alter lignin content and composition in numerous plants (Rastogi and Dwivedi 2003), with *COMT* being a commonly targeted gene (Dwivedi et al. 1994; Dwivedi and Campbell 1995) due to its central role in the pathway and its high degree of conservation among dicots (Joshi and Chiang 1998). Our work involving modulation of the phenylpropanoid pathway in *B. napus* provided us with insights into other potential candidate

genes for altering seed lignin content in *B. napus* (Bhinu et al. 2008). Also, in most cases, when some individual genes were used for silencing, we did not observe notable reduction in lignin (data not shown). After preliminary analysis, we selected four *Arabidopsis* gene homologs namely, cinnamate 4-hydroxylase (*C4H*, At2g30490), coumarate 3-hydroxylase (*C3H*, At2g40890), caffeic *O*-methyltransferase (*COMT*, At5g54160), and ferulic acid 5-hydroxylase (*F5H*, At4g36220) to alter the lignin biosynthesis pathway in *B. napus*.

### Lignin Analysis of Transgenic *B. napus* Seeds

Lignin analysis of *B. napus* T<sub>0</sub> seeds collected from positive transgenic and control plants are summarized in Fig. 2. Lines (DE269, DE276 and DE484) carrying a CaMV35S-driven *C3H-C4H* fusion construct exhibited a 24–36% reduction in lignin content compared to the control (Fig. 2A), while 21–26% reduction was achieved in lines DE460, DE461 and DE468 transformed with *F5H-COMT* fusion construct also driven by a CaMV35S promoter (Fig. 2B). Among the three RNAi constructs that were used to alter lignin biosynthesis, we observed that suppression of *COMT* gene under the control of cruciferin promoter in lines DE137, DE175 and DE178 caused a maximum decline in lignin content in the range of 36–40% compared with control plants (Fig. 2C).

Lignin analysis of *B. napus* seeds collected from control and transgenic T<sub>1</sub> plants are summarized in Table 2. From the transgenic *B. napus* plants analyzed, we observed that progenies of lines DE178 (T<sub>1</sub> plants including RL67, RL68 and RL69), DE484 (T<sub>1</sub> plants include RL70, RL71, and RL72) and DE276 (T<sub>1</sub> plant RL46) showed reduction in lignin content. Lignin analysis in *B. napus* seeds using both the quantitative methods yielded comparable results. Research literature points to variable results with these candidate genes to alter lignin profiles. For instance, over-expression of *C4H* had no effect on lignin content or composition in tobacco, but a 76% reduction in *C4H* activity translated to 63% decrease in Klason lignin content (Sewalt et al. 1997). It is believed that some lignin-specific pathways may bypass the *C4H* step despite the *C4H* being a sequential enzyme in the lignin biosynthesis or catalyze additional reactions in the lignin pathway (Dixon et al. 2001). These data support metabolic channeling at an early stage in the lignin pathway. Unlike *C4H*, *C3H* has been successfully manipulated to achieve lignin reduction in *Arabidopsis* (Franke et al. 2002). As in the case of *C4H*, down-regulation of *COMT* has been achieved in several crop species albeit with varied lignin reduction levels (Baucher et al. 2003). Though the explanation for this variation is unclear, it may be related to the actual level of *COMT* suppression achieved in each transgenic line. However, these data also highlight that caution might need to be exercised when studying a pool of transgenic plants and comparing plants grown in



**Fig. 2.** Seed lignin content of *B. napus* transgenic plants. Seed extracts of transgenic plants carrying RNAi constructs are shown: (a) CaMV35S:*C3H-C4H*, construct 72-146, (b) CaMV35S:*F5H-COMT*, construct 72-142, and (c) Cruciferin:*COMT* construct 72-123. Error bars indicate standard error of the mean of three individual extractions. A total of three individual lines were used in extraction for each construct studied.

different growth conditions and using different analytical techniques for lignin determination.

*COMT* has been identified to play a predominant role in determining the incorporation of S units into the lignin polymer in tobacco and poplar (Li et al. 2000) and is also involved in the methylation of caffeyl aldehyde (Parvathi et al. 2001). *F5H*, on the other hand is a critical gene in the phenylpropanoid pathway (Chen et al. 1999; Parvathi et al. 2001) involved in modifying the relative amount of S units in several crops (Baucher et al. 2003). Our results show that *COMT* plays a fundamental role in lignin synthesis, consistent with

previous observations. Despite the strong activity of the constitutive CaMV35S promoter, our data show that both *C3H-C4H* and *F5H-COMT* combinations driven by this promoter had least effect on lignin perturbation compared to the *COMT* construct.

The NDF content, however, was found to be unaltered in all the plants irrespective of the RNAi constructs used for transformation, but the ADF seemed to vary amongst the lines. We observed variations specifically in lines with low lignin contents, such as RL46 and RL68 (18.5 and 19.5, respectively) amongst others.

*C4H*, *C3H*, *COMT* and *F5H* are reported to be single copy genes in *A. thaliana* based on genomic analyses using BioVis (Ver 0.5) at a homology threshold of 80%. Some in silico analysis reports suggest 17 copies of *COMT* and 2 copies of *F5H* (Costa et al. 2003; Raes et al. 2003) in *Arabidopsis*. However, at higher threshold levels (>80), only one copy could be detected, also favored by their functionality. It is estimated that for every single *Arabidopsis* gene, a minimum of two to four copies are present in *B. napus* genome (Cavell et al. 1998; Parkin et al. 2002). Therefore, in order to better understand the effectiveness of the RNAi strategy and its penetrance, we conducted some preliminary analysis by comparing the two genomes (<http://www.brassica.ca>). 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 1). 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, thus showing that any target to silence *C4H* would have a more 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. *F5H* (At4g36220) gene on the other hand produced moderate hits thus showing that it is not widely expressed as *COMT*. These results provide significant leads because a target design based on *C4H* or *COMT* is likely to work against multiple members and of unrelated gene families in *B. napus*.

### Histochemical Analysis Suggests Alteration in Lignin Content and Composition

Macroscopically there were no phenotypic differences between transgenic lines with reduced lignin content and control plants. Because lignin provides mechanical strength to walls of conducting cells, we used histochemical staining of control and transformant stem sections by Weisner (phloroglucinol) and Maüle (KMnO<sub>4</sub>.HCl) stains. Representative results (of line DE178) are shown in Fig. 3. In each case, staining was observed in subepidermal sclerified parenchyma and metaxylem vessels. When the stem sections from the

**Table 2. Lignin and fiber content (% ± SEM) of T<sub>1</sub> *B. napus* seeds**

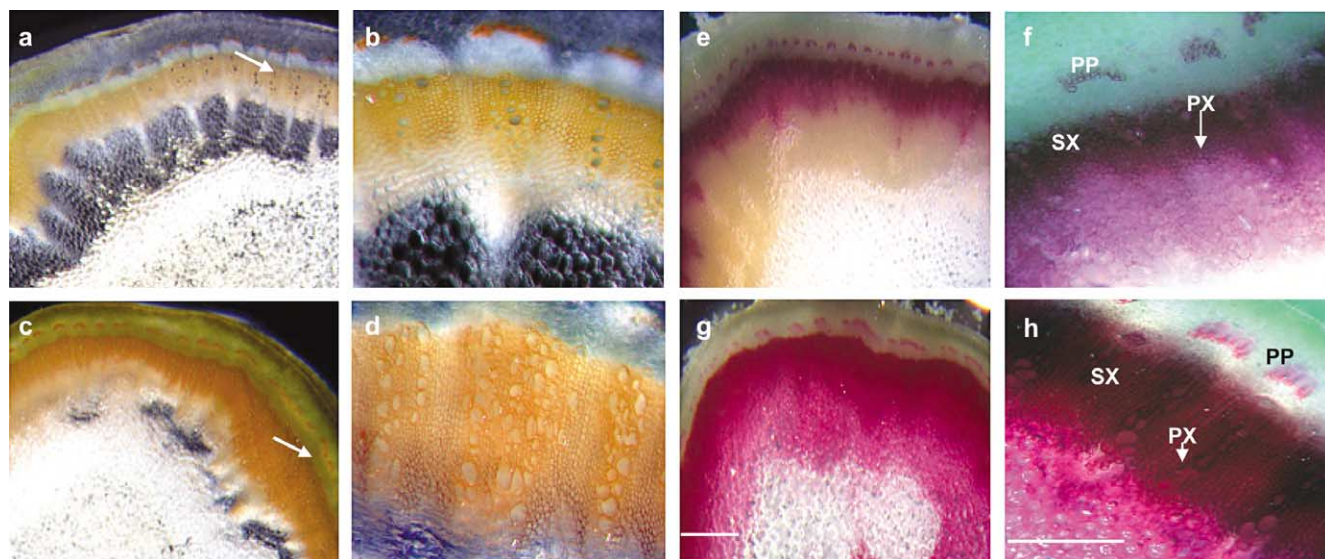
T <sub>1</sub> Plant	ADF (% dry seed) ± 0.1	NDF (% dry seed) ± 0.1	Lignin (% dry seed) ± 0.01
<i>35S:C3H-C4H RNAi</i> (T <sub>0</sub> : DE484) RL70	28.5	32.6	9.28*
RL71	22.2	27.1	8.83*
RL72	22.2	26.3	8.36*
<i>35S:FAH-COMT RNAi</i> (T <sub>0</sub> : DE461) RL58	21.3	25.1	10.1
RL59	24.2	26.0	12.6
RL60	20.3	23.0	10.3
<i>Cruciferin:COMT RNAi</i> (T <sub>0</sub> : DE178) RL67	25.4	29.2	7.11*
RL68	19.5	23.8	6.64*
RL69	23.6	30.0	7.58*
DH12075 Control (Avg)	24.0	28.3	10.2

A total of three samples were analyzed for each transgenic line except line RL53 that failed to set seeds and extraction was not possible. The T<sub>0</sub> parents are shown in parentheses.

\*Statistically significant at  $P \leq 0.05$ .

control and transgenic plants were stained with Mäule reagent, there was light brown stain in the transgenic plants (Fig. 3AB) as compared with an intense brown coloration in the control (Fig. 3CD). The other difference observed was a decreased stain area in transformants compared with control plants (Fig. 3AB). Since Mäule reagent is diagnostic of syringyl (S) units of lignin monomer, our data suggest that only moderate reductions in S units were found in the RNAi lines. Weisner reagent, indicative of aldehyde groups such as C<sub>6</sub>-C<sub>1</sub> benzaldehydes, C<sub>6</sub>-C<sub>3</sub> cinnamaldehydes or more specifi-

cally coniferaldehyde end groups in lignin (Lewis and Yamamoto 1990), revealed variations between the transformants and the control. The stem sections of the transformants were stained brownish-purple (Fig. 3EF) as opposed to pink for the control plants (Fig. 3GH), suggesting the likelihood of an increase in aldehyde end groups in the transformants. A detailed chemical analysis is required to further differentiate the lignin composition. Additionally, the transgenic line also exhibited some slightly collapsed or misshapen primary xylem (PX) vessels (Fig. 3F). The differences in the



**Fig. 3.** Histochemical analyses of lignin in stem sections of transgenic and control plants. (a) Mäule staining of transformant DE178 (12 ×), (b) Mäule staining of transformant DE178 (125 ×), (c) Mäule staining of control (12 ×), (d) Mäule staining of control (125 ×), (e) Weisner staining of transformant DE178 (12 ×), (f) Weisner staining of transformant DE178 (125 ×), (g) Weisner staining of control (12 ×), (h) Weisner staining of control (125 ×). Transgenic primary xylem (PX) appears slightly distorted or misshapen. The secondary xylem (SX) and primary phloem fibers (PP) appear normal.

secondary xylem (SX) and primary phloem fibers (PP) appeared to be minimal. We also noticed that the area of staining, which reflects the total lignin content (Monties 1989), decreased in transformants as compared with the control plants, thereby indicating a decrease in lignin content.

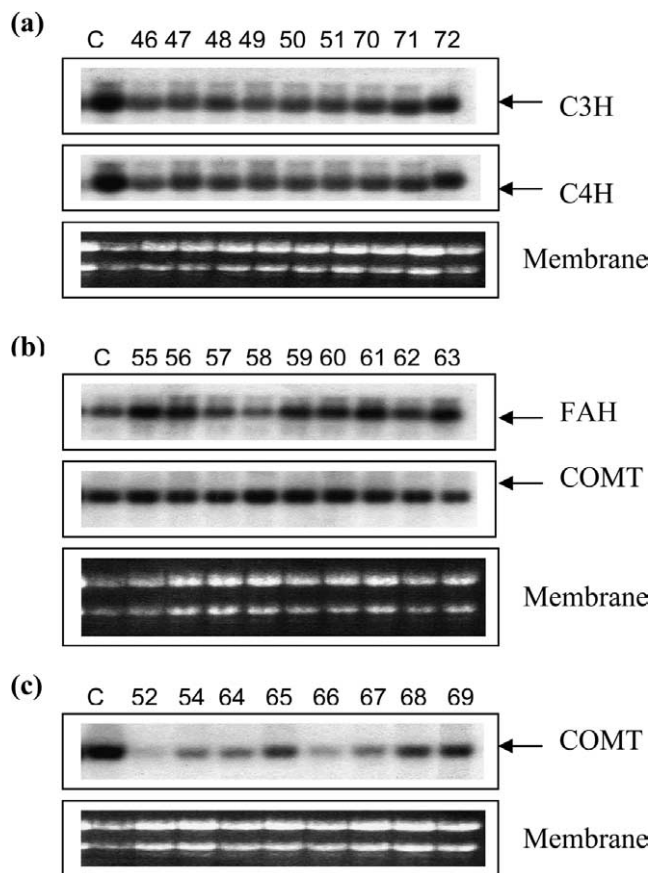
Qualitative assessment of lignification by histochemical staining of stem cross sections of *B. napus* provided additional evidence for the reduction of lignin content as compared with control. This was observed only in lines with reduced lignin content. We also observed some alteration in the lignin composition; however, a change in the composition and content of lignin in the transgenic plants was accompanied by minor changes in the vessel architecture, such as signs of aberration or distorted xylem vessels, which is commonly observed with altered lignin content and composition (Franke et al. 2002). Because the mechanical rigidity of lignin strengthens vessel walls, it is likely that the lack of lignin weakens the vessel walls so that they can no longer withstand the negative pressure generated through transpiration, thus resulting in the collapse of vessel elements. It will be interesting to determine the threshold of lignin reduction beyond which vessel wall strength is affected. However, it appears that even with some deformed vessel elements, plants could still transport water and solutes efficiently to support normal growth.

Another interesting aspect is the notable change in the lignin content in the stem of transgenic plants carrying the cruciferin:*COMT*. We believe this is caused partially or in combination due to a leaky expression of the seed-specific promoter in the aerial parts of the plant. The basis of this expression is unknown because lignification of vegetative tissues normally precedes the formation of reproductive tissues. Secondly, genomic imprinting, defined as a genetic phenomenon by which certain genes are expressed in a parent of origin-specific manner, could explain some of our observations given the lineage of the *B. napus* plants used (Rashid et al. 1994) and autoregulation or bypassing of imprinting events (Nowack et al. 2007). Third, we believe there might have been instances of epigenetic silencing, whereby non-mutational gene inactivation or suppression can be faithfully propagated from parents to their progeny. Although we did not notice any abnormalities or differences in the stems of transgenic plants, we observed reduction in lignin content in almost all the lines.

#### Down-regulated Expression of Genes Involved in Lignin Biosynthesis

In order to determine whether the expression of genes involved in lignin biosynthesis was altered by RNAi approach and also to know if gene expression levels correlated with the lignin content (both quantitative and qualitative), we extracted RNA from developing seeds of *B. napus* and examined for *C3H*, *C4H*, *COMT*, and

*F5H* expression using gel blot hybridization. We observed a reduction in the expression levels in all transgenic plants carrying the 35S:*C3H-C4H* construct compared with the control (Fig. 4A); while misregulation with one of the genes did not show any variation (data not shown). In transgenic plants carrying the *F5H-COMT* transgenic lines (Fig. 4B), we noticed greater reduction in the expression levels of *COMT* compared with *F5H* in most lines compared with the control. When we examined the expression of *COMT* in Cruciferin:*COMT* transgenic lines, we observed a marked reduction in the transcript levels in all the lines studied (Fig. 4C). Also, the Cruciferin:*COMT* transgenic plants showed significant reduction in lignin compared with other transgenic lines. These data confirm that our RNAi-mediated transcript suppression was successful, and a concomitant reduction in seed



**Fig. 4.** Northern blot analysis of lignin biosynthesis genes in developing seeds of transgenic *B. napus* ( $T_1$ ) carrying (a) CaMV35S:*C3H-C4H*, (b) CaMV35S:*F5H-COMT* and (c) Cruciferin:*COMT* RNA was extracted from seeds collected between 22 and 25 d post-anthesis. Plant numbers are indicated on the lanes. C, refers to control plant used in this study. All transgenic plants were generated in the DH12075 background. RNA extractions were performed from three  $T_1$  lines for each parent ( $T_0$ ).

lignin content was observed in transgenic *B. napus* plants.

### CONCLUSIONS

In this study, having silenced *COMT*, *F5H-COMT*, and *C3H-C4H* genes in *B. napus* species (AACC,  $n = 19$ ), we achieved lignin reductions to variable levels. Knocking out genes encoding *COMT* had the maximal impact with 40% reduction in lignin as compared with 36 and 26% with suppression of *C3H-C4H* and *F5H-COMT*, respectively. The demonstration of suppression of *COMT* has not only enhanced our knowledge of lignification, but also validates the potential utility of *COMT* over other lignin biosynthesis genes. Because no obvious visible phenotype was associated with reduction in lignin in the *COMT* RNAi plants, it is anticipated that *COMT* may be an ideal target for modification of lignin content and composition in *B. napus*. Additional experiments to study the roles of *COMT* RNAi in influencing S:G lignin monomer ratio in the plants may be interesting. Our findings show alterations in the content and composition of lignin as corroborated by evidence from histochemical staining of stem lignin. Our study reveals that by employing an RNAi strategy, it is possible to alter lignin content and composition in *B. napus* seeds in a manner non-detrimental to the plant.

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**Supplementary Table 1. Abundance of *A. thaliana* homologs identified in *B. napus* EST library collection comprising 150,000 clones (Saskatoon Research Centre)**

Gene ID	ESS	FL	RL	BL	LB	CD	DR	LD	ST	VA	CL	DL	EL	DC	UC
At2g30490 (C4H)	2	–	22	1	–	2	2	1	2	3	–	–	–	–	–
At2g40890 (C3H)	–	–	2	–	–	–	–	–	–	–	–	–	1	1	–
At5g54160 (COMT)	1	5	5	–	2	2	3	2	1	1	3	1	1	–	–
At4g36220 (FAH)	–	1	4	–	–	–	1	2	3	–	2	2	–	–	1

ESS: Tissue: Etiolated seedlings. Development stage – 10-d-old plants. The library was made from seedlings grown at room temperature in the dark for 10 d.

FL: Tissue-developed flowers. The library was made from fresh flowers in early stage of development. Most of the flower receptacle tissue had been removed.

RL: Tissue-root. The library was made from hydroponically grown root tissue.

BL: Tissue-bud. The library was made from buds collected from plants.

CD: Tissue-leaves. Development stage – Four-leaf stage. Cold acclimation in dark. The library was made from plants grown at 20°C days for 16 h and 18°C nights until the four-leaf stage at which point they were put at 4°C in the dark for 4 h.

DR: Tissue-root. Root drought.

LD: Tissue-leaves. Development stage-Young leaves. Leaf drought.

LB: Tissue-floral buds. Development stage – Late stage buds. Complete late stage buds (5 to 8 mm in size) were used for cDNA library construction.

ST: Tissue-stem. The library was made from plant stem tissue cut between growth nodes (auxillary buds were not included).

VA: Tissue-early stage anthers. Anthers were extracted from developing buds when they were 0.5 to 1 mm in size.

CL: Tissue-leaves. Development stage – four-leaf stage. Cold acclimation in light. The library was made from plants grown at 20°C days for 16 h and 18°C nights until the four-leaf stage at which point they were put at 4°C for 4 h.

DL: Tissue-leaf blade. The library was made from the first true leaves that were dead or dying (senescent Leaves).

EL: Tissue-embryos without seed coat. Development stage – mid to late embryos (4–6 mg). Seeds were collected when they were still very green (mid to large stage, cotyledons were formed). The seed coats were removed and the remaining tissue was used for cDNA library construction. After initial screening, the most abundant redundant clones were screened out using 22 oligos designed to match napins (including albumins), cruciferins, oleosins, trypsin inhibitor 2, cytosolic GAPDH, cyclophilins, HSP70, desaturase, and CAB (LHCP).

DC: Tissue – cotyledon. Damaged cotyledons. The library was made from cotyledons damaged by insect feeding (open field plot), before any leaves had grown.

UC: Tissue – cotyledon. Undamaged cotyledons. The library was made from cotyledons showing no damage from insect feeding (open field plot),

<b>C3H gene</b>			
		<b>Seq-P1</b>	
At2G40890	.....CACGACAGCGATAACAGCGGAATGGGGCATGGCGGAAATGATCAAGAATCCAAGAGTGCA	1680	
RL4992	.....CACAAACAGCTATAACAGCAGAATGGGCCATGGCAGAGATGATCAAGAACCCAAGAGTGCA	266	
	*****		
At2G40890	ACAAAAAGTGAAGAAGAGTTCGACAGAGTGGTTGGACTTGACCGGATCTTAACCGAGGC	1740	
RL4992	ACAAAAAGTGAAGAAGAGTTCGACAGAGTGGTTGGACTTGACCGGATCTTAACCGAGGC	326	
	*****		
At2G40890	AGATTTCTCCCGCTTACCTTACTTGCAATGCGTGGTGAAGAGTCATTACGGCTGCATCC	1800	
RL4992	AGACTTCTCAGCTTACCTTACCTCAATGCGTGGTAAAGAATCATTACAGACTCCACCC	386	
	*****		
At2G40890	TCCAACGCCTTAATGCTACCTCACCGAAGCAACGCAGATGTCAAGATCGGAGGCTATGA	1860	
RL4992	TCCAACGCCTTAATGCTACCTCACAGATCCAACGCACACGTCAGATCGGAGGCTACGA	446	
	*****		
At2G40890	TATTCCTCAAAGGATCAAACGTTTCATGTGAATGTGTGGGCTGGCTAGAGACCCGGCTGT	1920	
RL4992	CATCCCCAAAGGCTCAAACGTTCCAGTGAACGTTCTGGGCGTGGCTAGAGACCCGGCTGT	506	
	**		
At2G40890	ATGGAAAAATCCATTTGAGTTTAGACCAGAGAGATTCCTGGAAGAAGATGTTGACATGAA	1980	
RL4992	GTGGAAAAACCCATTAGAGTTTAGACCAGAGAGATTCCTGGAAGAAGATGTTGACATGAA	566	
	*****		
		<b>Seq-P2</b>	
At2G40890	GGGTATGATTTTAGGCTGCTTCCGTTTGGAGCTGGAAGAAGCGGTTTGTCCCGTGCACA	2040....	
RL4992	AGGTATGATTTTAGGCTGCTTCCGTTTGGAGCAGGAAGAAGGTTTGTCCCGTGCACA	626.....	
	*****		
<b>C4H gene</b>			
		<b>Seq-P3</b>	
At2G30490	TGGTGGCTAGCAAAACACCCCAACAGCTGGAAGAAGCCTGAAGAGTTTAGACCAGAGAGG	1620	
RL7118	TGGTGGCTAGCAAAACACCCCTGAGAGCTGGAAGAAGCCTGAAGAGTTTAGGCCGAGAGG	260	
	*****		
At2G30490	TTCTTTGAAGAAGAATCGCACGTGGAAGCTAACGGTAATGACTTCAGGTATGTGCCATTT	1680	
RL7118	TTCTTTGAAGAAGAGGCACACGTGGAAGCGAACGGTAATGACTTTAGGTATGTGCCGTTT	320	
	*****		
At2G30490	GGTGTGGACGTCGAAGCTGTCCCGGATTTATATTGGCATTGCCATTTTGGGGATCACC	1740	
RL7118	GGTGTGGACGTCGAAGCTGTCCCGGATTTATATTGGCATTGCCATTTTGGGGATCACC	380	
	*****		
At2G30490	ATTGGTAGGATGGTCCAGAACTTCGAGCTTCTTCCCTCCAGGACAGTCTAAAGTGGAT	1800	
RL7118	ATTGGTAGGATGGTACAGAACTTCGAGCTTCTTCCCTCCAGGACAGTCTAAATGGAT	440	
	*****		
		<b>Seq-P4</b>	
At2G30490	ACTAGTGAGAAAGGTGGACAATTCAGCTTGACATCCTTAACCACTCCATAATCGTTATG	1860	
RL7118	ACTTCTGAGAAAGGTGGACAGTTCAGCTTGACATCCTTACCACCTCCACCATCGTAATG	500	
	**		
<b>COMT gene</b>			
		<b>Seq-P5</b>	
At5G54160	ACACCAGACTCAAGCCTCTCAACCAAAACAAGTAGTCCATGTCGATTGCATATATGTTGGCT	960	
Ess2134	-----TATGCTAGCT-----	10	
	**** *		
At5G54160	CACAATCCCGGAGGCAAAGAACGAACCGAGAAGAGTTTGAGGCATTAGCCAAAGCATCA	1020	
Ess2134	CACAACCCCTGGAGGCAAAGAGAGGACCGAGAAGAGTTCGAAGCATTGGCTAAAGGATCA	70	
	*****		
At5G54160	GGCTTCAAGGCATCAAAGTTGCTGCGACGCTTTTGGTGTTAACCTTATGAGTTACTC	1080	
Ess2134	GGCTTCAAAGGATCAAATGTTGCCTGCAATGCTTTTGGTGTTACGTTATGAGCTGCTC	130	
	*****		
At5G54160	AAGAAGCTCTAAAA-ACAAACAATGTTCCATGAAGATGAT-TTATATGTAAACATTA-T	1137	
Ess2134	AAAAAGATGTAAACACACACAATCCATGTAATAATGATATATATGTAAACATTCGT	190	
	** * * * *		
		<b>Seq-P6</b>	
At5G54160	CTCATA-----TCTCCTTCCACGGT-----	1157	
Ess2134	CTCATGGTACGCTCTACTTCCACGGTCTTTGTTTAAAACTACTATGTTAATAATGGTTAT	250	
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Supplementary Fig. 1. Alignment highlighting nucleic acid-level conservation between *Arabidopsis* genes and *Brassica napus* EST. Conserved sequences were used to design primers for RNAi vector construction.

