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MAM gene silencing leads to the induction of C3 and reduction of C4 and C5 side chain aliphatic glucosinolates in *Brassica napus*

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Methylthioalkylmalate (*MAM*) synthases and their associated genes that are extensively investigated in Arabidopsis control the side chain elongation of methionine during the synthesis of aliphatic glucosinolates. A Brassica homolog of the Arabidopsis *MAM* genes was used in this study to analyze the role of *MAM* genes in *B. napus* through RNA interference (RNAi). The silencing of the *MAM* gene family in *B. napus* canola and *B. napus* rapeseed resulted in the reduction of aliphatic glucosinolates and total glucosinolate content. The results indicated that RNAi has potential for reducing glucosinolate content and improving meal quality in *B. napus* canola and rapeseed cultivars. Interestingly, *MAM* gene silencing in *B. napus* significantly induced the production of 2-Propenyl glucosinolate, a 3 carbon side chain glucosinolate commonly found in *B. juncea* mustard. Most transgenic plants displayed induction of 2-Propenyl glucosinolate, however, the absolute content of this glucosinolate in transgenic *B. napus* canola was relatively low (less than 1.00 μmol g^{-1}). In the high glucosinolate content progenies derived from the crosses of *B. napus* rapeseed and transgenic *B. napus* canola, *MAM* gene silencing strongly induced the production of 2-Propenyl glucosinolate to high levels (up to 4.45 μmol g^{-1}).

Isothiocianates, produced by hydrolysis of glucosinolates in Brassica plants have a wide range of biological activities. Some have anti-nutritional and goitrogenic properties in seed meal, sulfroraphane in broccoli is a potent anticarcinogenic compound while other

isothiocianates have the ability to suppress pests and fungi through biofumigation (Fahey et al., 1997; Halkier and Gershenzon, 2006; Zhang et al., 1992). More recently, Clay et al. (2009) found that some glucosinolates involve plant innate immune response and Bednarek et al. (2009) determined that glucosinolates mediate broad-spectrum antifungal defense in plant living cells. In Arabidopsis, the genes controlling aliphatic and indole glucosinolate biosynthesis have been intensively analyzed (Halkier and Gershenzon, 2006). Aliphatic glucosinolates are derived from the elongation of methionine, while indole glucosinolates are derived from tryptophan. Two *MAM* gene clusters encoding methylthioalkylmalate synthases (*IPMS*-like genes) on chromosome 5 in Arabidopsis were suggested to control the elongation of methionine to aliphatic glucosinolates (Benderoth et al., 2006; Kroymann et al., 2001, 2003; Texter et al., 2004, 2007) and other two *IPMS* genes *IPMS1* and *IPMS2* on chromosome 1 mediate leucine biosynthesis through the elongation of methionine in Arabidopsis based on heterologous expression in *E. coli* (de Kraker et al., 2007). In Brassica species, the overexpression of *MAM1* in Chinese cabbage increased the total aliphatic contents in Chinese cabbage (Zang et al., 2008).

In addition to the *MAM* genes that determine the side chain of aliphatic glucosinolates, many other genes involved in glucosinolate biosynthesis have been found. The side chain modification of aliphatic glucosinolates consists of several steps in the biosynthetic pathway and their corresponding genes such as the AOP genes encoding α-ketoglutarate-dependent dioxygenase gene (AOP) have been identified in Arabidopsis and *B. oleracea* (Halkier and Gershenzon, 2006; Li et al, 2003; Li and Quiros, 2003). The gene families encoding cytochrome P450 monooxygenases such as the *CYP79F*, *CYP79B*, *CYP83A* and *CYP83B* gene families are the major gene families controlling aliphatic and indole glucosinolate biosynthesis (Halkier and Gershenzon, 2006). In some cases, the knockout of individual glucosinolate pathway genes leads to the reduction of all aliphatic or indole glucosinolates (Chen et al., 2003; Reintanz et al., 2001). There are also several publications focusing on transcription factors that regulate the biosynthesis of glucosinolates in Arabidopsis including *ATR1*, *AtDof1*, *MYB51*, *MYB28*, *MYB29* and *MYB76* (Celenza et al., 2005; Gigolashvili et al., 2007a, 2007b; Hirai et al., 2007; Sønderby et al., 2007). Apparently, the gene expression level of those regulators is

63 correlated to glucosinolate content in Arabidopsis. However, the homologs of those genes
64 in Brassica species need to be characterized for their possible involvement of
65 glucosinolate biosynthesis.

66 In Brassica species, the inheritance of glucosinolate content and glucosinolate profile has
67 been investigated. The glucosinolate biosynthesis pathways and the underlying genes in
68 the pathways have been inferred. In *B. napus*, one locus (*GSL-Pro*) controlling 3-carbon
69 (C3) side chain aliphatic glucosinolates and two loci (*GSL-Elong-A* and *GSL-Elong-C*)
70 controlling C4 and C5 side chain aliphatic glucosinolates were identified through genetic
71 analysis (Magrath et al., 1994). In *B. oleracea*, one locus for C3 (*GSL-Pro*) and one locus
72 (*GSL-Elong*) for C4 side chain aliphatic glucosinolates were identified through the
73 analysis of aliphatic glucosinolate profiles and gene mapping in *B. oleracea* (Li and
74 Quiros, 2002; Li et al., 2003). Furthermore, an expression mutation of *GSL-Elong*
75 Brassica homologs in *B. oleracea* was shown to co-segregate with C4 side chain aliphatic
76 glucosinolates in gene expression analysis (Li and Quiros, 2002). Recently, gene-linked
77 SSR markers were used to find the relation of the functionally characterized Arabidopsis
78 genes to seed glucosinolate content in *B. napus* (Hasan et al., 2008). However, much
79 work still needs to be done to characterize the genes involved in glucosinolate
80 biosynthesis in Brassica species.

81 Conventional plant breeding improved *B. napus* rapeseed oil and meal quality through
82 the development of double-zero *B. napus* rapeseed or *B. napus* canola cultivars in the
83 19070's (Hasan et al., 2008; McVetty et al., 2009; Nesi et al., 2008). Erucic acid was
84 nearly totally eliminated from the oil, which resulted in a healthy edible *B. napus* canola
85 oil with high monounsaturated fatty acid content. Additionally, glucosinolate content in
86 the meal was reduced dramatically to achieve improved *B. napus* canola meal quality.
87 The low glucosinolate trait in *B. napus* canola traces back to Bronowski that had
88 naturally occurring mutations. Bronowski was the only low glucosinolate donor for all
89 current *B. napus* canola cultivars worldwide (McVetty et al., 2009). Compared to the
90 glucosinolate profile observed in traditional *B. napus* rapeseed, only the aliphatic
91 glucosinolates were reduced to a low level in *B. napus* canola, but all glucosinolates are
92 still present and there was minimal change in indole glucosinolate content or profile. For

example, there is still a relatively high content of glucosinolates in *B. napus* canola meal (normally over 10 μm/g seed) and the potential to reduce the total glucosinolate content further is substantial (McVetty et al., 2009). In Brassica species, glucosinolates vary considerably both in content and profile. Glucosinolate biosynthesis in Brassica species is very complex and may be controlled by dozens, or even hundreds of genes, especially in the tetraploid Brassica species, which make it much more challenging to perform gene characterization and gene manipulation. However, the effective manipulation of glucosinolate biosynthesis in Brasscia species would facilitate the development of ultra low glucosinolate lines that would become valuable germplasm for use in *B. napus* canola/rapeseed breeding programs.

RNA interference (RNAi) was used in this study to test the effectiveness and efficacy of gene manipulation of a major *Gsl-Elong* gene family in the biosynthesis of aliphatic glucosinolates in *B. napus*. The results showed that gene silencing of the *Gsl-Elong* gene family resulted in dramatic changes in aliphatic glucosinolate content in *B. napus* canola and a strong induction of C3 aliphatic glucosinolate, not normally detectable in *B. napus* canola/rapeseed.

MATERIALS AND METHODS

Primer Design

The primers were designed based on the sequence of the broccoli (*B. oleracea*) isopropylmalate synthase (*Gsl-Elong*) gene (NCBI Accession no. AC149635; Gao et al., 2005). Two pairs of primers, FELONG1A, RELONG1A, FELONG1B and RELONG1B located in the first exon of the broccoli *Gsl-Elong* were used to amplify sense and anti-sense fragments (432 bp) with genomic DNA. These amplified fragments, together with an intron fragment, were inserted into the RNAi construct *35S::Elong1*. Another two pairs of primers FELONG2A, RELONG2A, FELONG2B and RELONG2B that are located in exon 6 and exon 10 and span 4 exons at the end of the broccoli *Gsl-Elong* were designed to amplify sense and anti-sense fragments (297 bp) with cDNA to prepare RNAi construct *35S::Elong2*. The intron fragment was amplified with the primers INT5

and INT6 that were designed based on the sequence of the PDK intron in pHellsgate 8 vector. All primers were synthesized by Invitrogen, Toronto, Canada (Table 1).

Preparation of RNAi Constructs

A cassette with sequence 'GCTGAGGAGTTAATTAAGACCTCAGC' was inserted in a binary vector pBI121 to produce a modified binary vector pBI121u. This cassette allowed three PCR fragments to assemble in the vector without ligation. A similar procedure was as described by Nour-Eldin et al. (2006). The 5' universal uracil primers tails were "5'-GGAGTTAAU+" and "5-GGTCTTAAU+" that were added to the forward primers. The 3' universal reverse primer tails "5-ACGAACAGGU+" and "5-ATCCTCTAGU+" were complementary to the tails of two uracil-containing primers INT5 and INT6 that were used to amplify the PDK intron from pHellsgate 8 vector. These two reverse primers were combined with the forward primers to amplify sense and ante-sense gene fragments, respectively.

Vector DNA's were prepared according to the protocol described by Nour-Eldin et al. (2006). pBI121u was first digested with PacI and then, with the subsequent nicking enzyme, Nt.BbvCI (New England Biolabs, Toronto, Canada). A 10 µl mixture of two *Gsl-Elong* gene fragments and the intron fragment were digested with 1 U USER^{TM} enzyme (New England Biolabs, Toronto, Canada). The insert mixture, mixed with vector DNA was incubated 20 min at 37^{o}C followed by 20 min at 25^{o}C and finally transformed into chemically competent *E. coli* DH10B cells. The plasmids of positive clones were isolated and sequenced to confirm the accuracy of the sequence. Finally, the RNAi constructs *35S::Elong1* and *35S::Elong2* were electro-transformed into *Agrobacterium tumefaciens* strain GV3101 and used for *B. napus* transformation.

Plant Transformation and Regeneration

Plant transformation followed the protocol described by Moloney et al. (1989). *B. napus* canola DH line (DH12075) seeds were surface-sterilized for 15 min 4% sodium hypochlorite with 0.1% Tween 20 added as a surfactant. Then, the seeds were washed thoroughly with sterile distilled water and germinated on 1/2 MS (SIGMA, Toronto,

149 Canada) basal medium with 10 g L^{-1} sucrose. Hypocotyls were harvested from 4~5 day
150 old seedlings and cut into 4~6 mm long pieces and placed onto MS medium and
151 incubated 3 days at 25ºC. Agrobacterium was prepared by growing it overnight with
152 shaking at 28ºC in LB medium with appropriate antibiotics, pelleted and re-suspended in
153 the same volume of liquid hormone-free MS with 30 g L^{-1} sucrose medium. The tissue
154 pieces were collected and mixed thoroughly with 10-time dilution of Agrobacterium
155 suspension with hormone-free MS with 30 g L^{-1} sucrose medium. The excess fluid was
156 discarded and the tissue pieces were co-cultured with Agrobacterium on MS medium for
157 5 days. The explants were transferred to MS medium with 20 mg L^{-1} kanamycin to select
158 transformed cells. After a further 2 weeks, the explants were transferred to fresh MS
159 medium. The first shoots developed after 3~4 weeks. The developing green shoots were
160 transferred to MS medium and the elongated shoots were transferred to 1/2MS medium.
161 The rooted shoots were transferred to soli and grown in plant growth chamber.

162 **DNA Extraction and PCR Analysis**

163 The putative T_1 and F_1 transgenic plants were analyzed using PCR to confirm the
164 presence of the transgenes. Plant genomic DNA was isolated from the leaves of each line
165 using modified a CTAB (cety-trimethyl-ammonium bromide) based method (Li and
166 Quiros, 2002). The primer pairs of INDOWN1 and FELONG1B, and INUP1 and
167 FELONG1A were used to identify *35S::Elong1* RNAi transgenic plants; INDOWN1 and
168 FELONG2B, INUP1 and FELONG2A, to identify *35S::Elong2* transgenic plants. PCR
169 reactions were carried out in a 20 µl mixture at 94ºC for 3 min; 35 cycles of amplification
170 at 94ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min. PCR products were visualized by
171 electrophoresis on 1% agarose gel and sequenced to confirm that the inserts were correct.

172 **RNA Extraction and RT-PCR Analysis**

173 RNA was extracted from the leaves of T_1 and F_1 transgenic plants using TRIZOL
174 (Invitrogen, Toronto, Canada). 50~100 ng of total RNA was used to make single strand
175 cDNA using Superscript III reverse transcriptase (Invitrogen, Toronto, Canada) in a 20 µl
176 reaction with oligo (dT18) primers according to the manufacturer's instructions. Primers
177 ELONG934 and ELONG1454 covering exon5 to exon10 of the broccoli *Gsl-Elong* gene

were used for semi-quantitative RT-PCR in a 50 µl reaction at 94ºC for 3 min; 26 cycles for *UBQ* and 36 cycles for *Gsl-Elong* amplification at 94ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min. Primers BRUBQ1 an BRUBQ2 were used to amplify the Brassica ubiquitin gene as a check. PCR products were visualized by electrophoresis on 1% agarose gel and sequenced to confirm that they were the correct ones. Quantification of RT-PCR products was performed with ALPHAEASE FC software (Fischer Scientific, Ottawa, Canada).

Glucosinolate Analysis

Approximately 200 mg seeds were ground in liquid nitrogen. Glucosinolates were extracted and purified by using sephadex/sulfatase protocols previously described (Kliebenstein et al., 2001) with some modifications. The final volume of glucosinolate extract was 400 µl to 500 µl. Fifty microliters of the glucosinolate extract was run on a 5-mm column (Lichrocart 250–4 RP18e, Fisher Scientific, Ottawa, Canada) on a Hewlett-Packard 1100 series HPLC. Compounds were detected at 229 nm and separated with the following programs and aqueous acetonitrile. The program was an 8-min gradient from 1.5% to 7.0% (v/v) acetonitrile, a 4-min gradient from 7% to 15% (v/v) acetonitrile, a 18-min gradient from 15% to 55% (v/v) acetonitrile, a 5-min gradient from 55% to 92% (v/v) acetonitrile, a 5 min at 92% (v/v) acetonitrile, a 5-min gradient from 92% to 1.5% (v/v) acetonitrile, a 3 min at 1.5% (v/v) acetonitrile and a final 4 min at 0%(v/v) acetonitrile. The identity of HPLC peaks was determined on the basis of reference *B. napus* material 'Linnetta" and according to the peaks obtained by Kraling et al. (1990). Each line was run in triplicate. All glucosinolate absorbance data (measured at 229 nm) were converted to micromoles per gram dry weight seeds using response factors determined from the purified standards for each of the glucosinolates (Vinjamoori et al., 2004). Glucosinolate peaks were named and used in the same way in all figures and tables: peak 1, 2-hydroxy-3-butenyl (progoitrin); peak 2, 2-Propenyl (Sinigrin); peak 3, 2-hydroxy-4-pentenyl (napoleiferin); peak 4, 3-Butenyl (gluconapin); peak 5, 4-hydroxy-3-idolylmethyl (4-hydroxy-glucobrassicin); peak 6, 4-Pentenyl (glucobrassicanapin); peak 7, 3-idolylmethyl (glucobrassicin); peak 8, 1-Methoxy-3-indolylmethyl glucosinolate (neoglucobrassicin).

Supplemental Data

Supplemental Table S1. T1 seed Glucosinolates in 35S::Elong1 and 35S::Elong2 RNAi transgenic B. napus canola plants.

Supplemental Table S2. Glucosinolate contens of T2 seeds in 35S::Elong1 and 35S::Elong2 RNAi transgenic B. napus canola lines.

Supplemental Table S3. Glucosinolate contents of F2 seeds in crosses of 35S::Elong1 and 35S::Elong2 RNAi lines and B. napus rapeseed.

RESULTS

Gene Silencing of the *MAM* Gene Family Changes Glucosinolate Profile and Content in *B. napus*

A USER technology was employed to make RNAi constructs with the modified binary vector pBI121u containing the CaMV 35S promoter. Two RNAi constructs *35S::Elong1* and *35S::Elong2* with the gene inserts located at the beginning and the end of the *MAM* homolog were made based on the sequence of *BoGSL-Elong* in broccoli (Gao et al. 2005) which corresponds to the Arabidopsis *MAM* gene cluster on chromosome 5. Agrobacterium mediated transformation was implemented to obtain transgenic plants and a doubled haploid *B. napus* canola line DH12075 was used for transformation. Over 100 transgenic plants were produced and the inserted DNA in the transgenic plants was confirmed with PCR and sequencing. Except for a few plants that showed chlorotic leaves and which did not survive to maturity, most transgenic plants did not show phenotypic changes during any developmental stage. After harvesting seeds from the transgenic plants, those from 72 T_0 transgenic plants with the *35S::Elong1* construct and 12 transgenic plants with the *35S::Elong2* were used to obtain glucosinolate profile and content through HPLC analysis (Supplemental data S1). Five aliphatic and three indole glucosinolates were detectable in the T_1 seeds of the above 84 transgenic plants (Figure 1).

Compared to the original *B. napus* canola DH line, one of the most obvious changes in the glucosinolate profiles of the T_1 seeds of transgenic plants was the induction of a C3 side chain aliphatic glucosinolate, 2-Propenyl glucosinolate (sinigrin) that was not detectable in the original *B. napus* canola DH line (Figure 1). Fifty-seven of the 84 transgenic plants had detectable 2-Propenyl glucosinolate with content ranging from 0.07 to 1.88 μmol g^{-1}. In contrast, there was an obvious decrease of all other aliphatic glucosinolates including two C4 side chain (2-hydroxy-3-butenyl and 3-Butenyl) aliphatic glucosinolates and two C5 side chain (2-hydroxy-4-pentenyl and 4-Pentenyl) aliphatic glucosinolates. Among all 84 tested T_0 transgenic plants, there were 58 T_0 transgenic plants which showed reduced C4 side chain aliphatic glucosinolate content and 70 T_0 transgenic plants which displayed reduced C5 side chain aliphatic glucosinolate content. In three extreme cases, all C5 side chain aliphatic glucosinolates were eliminated, and in three T_0 transgenic plants the C4 side chain aliphatic glucosinolate content was reduced by approximately five fold (Supplemental data S1). In *B. napus* canola, the induction of a new C3 side chain aliphatic glucosinolate was relatively limited. Among fifty-seven T_0 transgenic plants with induced C3 side chain glucosinolate, there was only one transgenic plant that contained over 1 μmol g^{-1} 2-propenyl glucosinolate while the remaining fifty-six transgenic plants had less than 1 μmol g^{-1} 2-Propenyl glucosinolate. Although there was variation in the content of indole type glucosinolates, most T_0 transgenic plants showed similar levels of indole type glucosinolates as the original DH *B. napus* canola line. The total aliphatic and total glucosinolate content in the T_1 seeds of the majority of transgenic plants were reduced considerably. Fifty-three T_0 transgenic plants contained lower seed glucosinolate content than the *B. napus* canola DH line used for transformation.

Gene Silencing is Transferable from Generation to Generation

To determine the stability of gene silencing in the following generations, seven T_1 lines that had relatively strong gene silencing based on the reduction of total aliphatic glucosinolate content were selected for further analysis. Since transgenic and non-transgenic plants segregated in each T_1 line, all T_1 individual plants were checked through specific amplification of the T-DNA insertion using DNA extracted from 4-week

old leaves to identify positive and negative progenies. *Gsl-Elong* gene expression in positive plants of seven T_1 lines was also analyzed through semi-quantitative RT-PCR using the Brassica ubiquitin gene (*UBQ*) as an internal control (Figue 2A). Quantification of the gene expression levels was further analyzed using ALPHAEASE FC software (Figure 2B). The results showed that gene expression was reduced dramatically in T_1 transgenic plants while there was no change in gene expression for the negative check.

The T_2 seeds from selfed T_1 individuals were harvested individually for further glucosinolate analysis. All positive plants from each T_1 line were put into one group and the T_2 seeds from each individual plant were analyzed separately, with each plant considered as a replicate. All plants without T-DNA insertion were put into another group and used as the check. The seeds from each non-transgenic individual plant were analyzed separately, with each plant considered as a replicate. Analysis of variance (ANOVA) was performed for six transgenic groups and one non-transgenic group (Figure 3, Supplemental data S2).

Similar to the results seen in the T_1 seeds, most T_2 seeds from transgenic plants contained detectable 2-Propenyl glucosinolate. Again the absolute amount of this C3 side chain glucosinolate was low in the *B. napus* canola-derived transgenic progeny. Statistical analysis indicated that the induction of the C3 side chain glucosinolate among the tested T_2 transgenic lines was significantly different. Compared with non transgenic plants, the content of C4 and C5 side chain glucosinolates in most T_2 transgenic lines were reduced. In the progenies of *Elong1-1*, the C4 and C5 glucosinolates were reduced dramatically. There was only 1.64 μmol g^{-1} of aliphatic glucosinolates and 3.44 μmol g^{-1} of total glucosinolates, a four-fold reduction from the total glucosinolate content in the original *B. napus* canola line used for transformation.

Glucosinolate Profile and Content in the F_2 Seeds of Transgenic *B. napus* Canola and Non-transgenic *B. napus* Rapeseed

B. napus rapeseed DH line, DH241 with high glucosinolate content (32.72 μmol g^{-1}) was crossed with five T_0 transgenic plants, two of which had the *35S::Elong1* insertion and three of which had the *35S::Elong2* insertion, representing five individual transformation

292 events. The cross of DH241 and DH12075 was also included. In the progenies of the five
293 transgenic parent crosses, there was segregation of transgenic and non-transgenic plants
294 which were confirmed through specific amplification of the T-DNA inserts. The gene
295 silencing effects were analyzed with semi-quantitative RT-PCR using UBQ as an internal
296 control (Figure 2C). Quantification of the gene expression levels was further analyzed
297 using ALPHAEASE FC software (Figure 2D). Compared with the negative check, the
298 expression level of *Gsl-Elong* genes in the F_1 transgenic plants was greatly reduced. After
299 selfing the F_1 plants, the positive individuals from each cross were assigned into one
300 group and five transgenic populations including all positive lines were produced. All
301 negative plants were placed into one group as a negative check and another negative
302 check derived from the cross of non-transgenic *B. napus* canola and rapeseed were also
303 included in the statistical analysis. The glucosinolate contents and profiles of the F_2 seed
304 lots of transgenic and non-transgenic plants were obtained and the seeds of each F_1
305 individual were considered as a replicate to perform ANOVA.

306 All negative plants did not have detectable levels of C3 side chain glucosinolate, similar
307 to that observed in the T_2 population. The gene silencing effects of all T_0 transgenic
308 plants were judged on the basis of glucosinolate content and profile and those T_0
309 transgenic plants with the strongest gene silencing were selected for making crosses and
310 producing the F_2 seeds. As expected, the induction of C3 side chain glucosinolate was
311 strong in all five F_2 transgenic populations with the C3 side chain glucosinolate content
312 ranging from 2.66 to 5.47 μmol g^{-1} (Supplemental data S3). Compared with the negative
313 controls, there was a significant reduction of C4 glucosinolates in three and significant
314 reduction of C5 in four out of five F_2 populations, respectively. However, there was one
315 F_2 population DH241 X (*Elong1-1*) that had a significant reduction of aliphatic and total
316 glucosinolate content at the 1% significance level and another cross DH241 X (*Elong2-*
317 *49*) which showed a significant reduction of aliphatic and total glucosinolate content at
318 5% significance level (Figure 4, Supplemental data S3).

319 **Discussion**

In Arabidopsis, the *MAM* genes encoding methylthioalkylmalate synthases are the genes that control the side chain elongation from methionine to different side chain aliphatic glucosinolates. Specifically, *MAM1* and *MAM2,* located on chromosome 5 control C3 and C4 side chain aliphatic glucosinolates (Benderoth et al., 2006; Kroymann et al., 2001, 2003; Texter et al., 2004, 2007). In this study, two RNAi constructs that contain two different regions of a *B. napus* homolog of the Arabidopsis *MAM1* and *MAM2* genes were used to silence the *MAM* gene family in *B. napus*. Surprisingly, glucosinolate profile changes included both the induction of a C3 side chain glucosinolate and the reduction of C4 and C5 glucosinolates in both *B. napus* canola (low aliphatic glucosinolate content) and *B. napus* rapeseed (high aliphatic glucosinolate content) progeny, respectively. It was further confirmed that the *MAM* genes are involved in the side chain elongation of methionine to C4 and C5 aliphatic glucosinolates in *B. napus*. However, our results indicated that the *MAM* genes might not be the only gene family that controls the side chain elongation of aliphatic glucosinolates.

Total aliphatic glucosinolates may not be eliminated by the manipulation of the *MAM* gene family in *B. napus*. In this study, the major goal of *MAM* gene silencing was to reduce total glucosinolate content in *B. napus* canola for meal quality improvement. Similar results were obtained in both *B. napus* canola and rapeseed where a C3 side chain aliphatic glucosinolate was induced, at the same time the C4 and C5 side chain aliphatic glucosinolates were reduced. The change in total glucosinolate content in *B. napus* canola transgenic progeny was more dramatic than in *B. napus* rapeseed transgenic progeny. Five of six T_2 populations in *B. napus* canola displayed statistically significant reductions in aliphatic glucosinolate content, but only one of five F_2 populations showed statistically significant decreases of aliphatic glucosinolate contents in the crosses of *B. napus* canola with low glucosinolate content and *B. napus* rapeseed with high glucosinolate content. Therefore, total glucosinolate content in *MAM* RNAi transgenic plants can be dramatically reduced in *B. napus* canola, but less so in *B. napus* rapeseed.

Glucosinolate biosynthesis is a multiple step process with different precursors resulting in various glucosinolates. Several enzymes modify the side chains of glucosinolates to produce novel glucosinolates after desulfoglucosinolates are formed. Although the

expressional and functional changes of any genes in the pathways of glucosinolate biosynthesis may affect total glucosinolate contents as demonstrated from the overexpression transformation in some reports and the down-regulated expression through the RNAi transformation in this study, two major classes of genes apparently contribute to total glucosinolate contents (Halkier and Gershenzon, 2006). One class identified to involve the biosynthesis of glucosinolates belongs to the cytochrome P450 gene family encoding P450 monooxygenases with the members including *CYP79F1*, *CYP79F2*, *CYP79B2*, *CYP79B3*, and *CYP83B1*. Another class of important regulators of glucosinolate content and profile belongs to transcription factors such as *MYB28*, *MYB29*, *MYB76*, *ATR1/MYB34*, *MYB51*, and *MYB122* that regulate the expression of the genes involved in glucosinolate biosynthesis. For example, a disrupted *CYP79F1* mutation results in the elimination of short side chain aliphatic glucosinolates (Reintanz et al., 2001) and a double mutant of *MYB28* and *MYB29* eliminates all aliphatic glucosinolates in Arabidopsis (Beekwilder et al., 2008). Unfortunately, most of these genes have pleiotropic effects, which may cause unwanted traits in *B. napus* canola if they are used to manipulate glucosinolate content. In the RNAi transgenic plants of the *MAM* gene in *B. napu*s in this study, morphological changes were rarely observed. The only apparent changes involved glucosinolate contents and profiles.

It should be noted that the *Gsl-Elong* gene family may not be the one responsible for the glucosinolate content reduction in *B. napus* canola observed in this study since the knock-down regulation of its expression via RNAi did not significantly change the total glucosinolate content in *B. napus* rapeseed. Most likely, the genes such as the aforementioned two classes of genes may contribute to the observed glucosinolate content change in *B. napus* canola. However, it is still challenging to determine what genes are responsible for the glucosinolate differences between *B. napus* rapeseed and *B. napus* canola since many genes could be the candidates for the change of glucosinolate content in *B. napus* canola. For example, four Brassica paralogs of the Arabidopsis *MAM* genes and 56 homologs in total corresponding to the Arabidopsis genes involved in the biosynthesis of glucosinolates were identified through genome-wide sequence analysis of *B. rapa* (Zang et al., 2009).

RNAi of the *Gsl-Elong* gene family is an effective method to change glucosinolate profiles in *B. napus.* Since all Brassica species contain extensive gene duplications, gene redundancy hinders the development of new genotypes with beneficial phenotypes. In particular, this is even more difficult in polyploidy species such as *B. napus* where every gene has at least two members. The diverse bioactivities of glucosinolates are related to specific glucosinlolates so specific end uses require that some glucosinolates must be reduced or eliminated while others must be increased. For mustard production and use of mustard in biofumigation, C3 side chain aliphatic glucosinolates such as 2-propenyl glucosinolate must be increased since 2-propenyl gluosinolate is beneficial to the quality improvement in this crop. 2-propenyl glucosinolate provides the flavor in mustard and is more effective in biofumigation since it is a short side chain aliphatic glucosinolate with its corresponding isothiocianate more active in soil (Matthiessen and Shackleton, 2005). Through RNAi, the 2-propenyl glucosinolate was induced dramatically in this study with its content reaches approximately 40-50% of total aliphatic glucosinolates. In wild-type *B. napus*, C3 side chain glucosinolates are not detectable. In contrast, in newly resynthesized *B. napus* using a *B. oleracea* parent containing C3 side chain aliphatic glucosinolates, the C3 side chain glucosinolates are induced to a level of 20 to 30% of total aliphatic glucosinolates (Magrath et al., 1993). Since plant transformation frequently produces a wide range of phenotypes, large scale of transformation is often necessary to develop the best transgenic plants for practical uses. Among the transgenic plants obtained in this study, the induction of C3 side chain glucosinolate was quite different. However, the gene silencing effect was closely related to the phenotypic change and strong *Gsl-Elong* gene silencing contributed to an effective induction of 2-propenyl gluosinolate. This induction is stably transferable between *B. napus* canola and rapeseed and between generations and so could be used in Brassica breeding.

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509 **Legends of figures**

510 Figure 1. A to D show the HPLC chromatograms of glocosinolate profiles in *B. napus*
511 seeds: A, canola line DH12075; B, a RNAi transgenic plant; C, a F1 plant (*B. napus*
512 rapeseed DH241×*B. napus* canola DH12075); D, a RNAi transgenic F1 plant
513 (DH241×RNAi transgenic DH12075); E, 2-Propenyl glucosinolate (sinigrin, standard);
514 arrows in B, D and E indicate the position of the 2-Propenyl glucosinolate peak. The

515 numbers on the peaks correspond to the following glucosinolates: 1, 2-hydroxy-3-butenyl
516 (progoitrin); 2, 2-Propenyl (Sinigrin); 3, 2-hydroxy-4-pentenyl (napoleiferin); 4, 3-
517 Butenyl (gluconapin); 5, 4-hydroxy-3-idolylmethyl (4-hydroxy-glucobrassicin); 6, 4-
518 Pentenyl (glucobrassicanapin); 7, 3-idolylmethyl (glucobrassicin); peak 8, 1-Methoxy-3-
519 indolylmethyl glucosinolate (neoglucobrassicin).

520 Figure 2. Semi-quantitative analysis of *Elong* gene expression showing down-regulated
521 expression in the RNAi transgenic lines. A, panel 1, amplified RNAi inserts with
522 genomic DNA of *B. napus* canola DH12075 and RNAi lines (Elong2-52, Elong2-49,
523 Elong2-47, Elong2-14, Elong2-12, Elong1-14, Elong1-1); panel 2, RT-PCR products of
524 *Elong* gene cDNA in the same set of samples as in panel 1; panel 3, RT-PCR products of
525 ubiquitin cDNA as a check. B, Qantification of *Elong* gene transcripts in the RNAi
526 transgenic lines was performed using ALPHAEASE FC software with error bars
527 indicating SE from four repeats. C, panel 1, Amplified RNAi inserts in non transgenic
528 cross of *B, napus* rapeseed and canola (DH241×DH12075) and crosses of rapeseed and
529 canola RNAi lines (DH241×Elong2-49, DH241×Elong1-14, DH241x Elong1-1); panel 2
530 and 3, RT-PCR products of Elong and ubiquitin cDNA showing the down-expression of
531 *Elong* genes in transgenic crosses of *B. napus* rapeseed and transgenic canola. D,
532 Qantification of *Elong* gene transcripts in the crosses of *B. napus* rapeseed and transgenic
533 canola line was performed using ALPHAEASE FC software with error bars indicating
534 SE from three repeats.

535 Figure 3. Mean GSL content in T2 seeds of *B. napus* transgenic canola lines and the
536 original canola line DH12075. A, 3C (2-Propenyl), 4C (2-hydroxy-3-butenyl and 3-
537 Butenyl), and 5C (2-hydroxy-4-pentenyl and 4-Pentenyl) GSL content in T2 seeds of six
538 transgenic lines (Elong1-1, Elong1-14, Elong2-14, Elong2-47, Elong2-49, Elong2-52),
539 non-transgenic plants in segregating T1 transgenic lines and the original canola DH line
540 DH12075. B, Aliphatic, indole and total GSL content in T2 seeds of the same samples in
541 A. SE bars are presented for each sample ($n>4$); * Indicates changes that are significantly
542 different ($p<0.05$) compared to the DH canola line DH 12075; ** Indicates changes that
543 are highly significantly different ($p<0.01$) compared to the DH canola line DH 12075.

Figure 4. Mean GSL content in F_2 seeds of *B. napus* rapeseed and non-transgenic *B. napus* canola and transgenic canola. A, 3C (2-Propenyl), 4C (2-hydroxy-3-butenyl and 3-Butenyl), and 5C (2-hydroxy-4-pentenyl and 4-Pentenyl) GSL content in F_2 seeds of five transgenic crosses (DH241×Elong1-1, DH241×Elong1-14, DH241×Elong2-12, DH241×Elong2-14, and DH241×Elong2-49), non-transgenic cross (DH241×DH12075) and non transgenic parents (DH12075 and DH241). B, aliphatic, indole and total GSLs content in the same set of samples mentioned above. SE bars are present for each sample ($n>4$); * Indicates changes that are significantly different ($p<0.05$) compared to the F_2 progeny from non transgenic cross (DH241×DH12075); ** Indicates changes that are highly significantly difference ($p<0.01$) compared to the F_2 progeny from non transgenic cross (DH241×DH12075).