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In planta side-chain glucosinolate modification in Arabidopsis by introduction of dioxygenase Brassica homolog BoGSL-ALK

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Abstract Aliphatic glucosinolates and their derived isothiocyanates are important secondary metabolites in crucifers. Some of these compounds have beneficial activities such as carcinogen detoxification, pesticidal and antifungal properties, but others are anti-nutritional; the differences are largely due to side chain modifications. We report the cloning and in planta functionality analysis of *BoGSL-ALK*, a gene whose protein product influences side-chain modifications in the glucosinolate pathway. Expression of this Brassica gene was demonstrated in *Arabidopsis thaliana* by assaying RNA activity and monitoring changes in the glucosinolate profiles in leaves and seeds of transformed plants. Dependent on the proposed uses of the crops under development, the ability to regulate *BoGSL-ALK* expression is a key step towards engineering Brassica crops with specific glucosinolate content.

Keywords. Secondary metabolites · Glucosinolates · Transformation · Nutraceuticals

Introduction

It has been known for some time that glucosinolates (GSL) and their breakdown products that result from the action of the enzyme myrosinase have important biological activity. The range of activities of these compounds is wide – some being beneficial, others detrimental for human and animal consumption (Mithen et al. 2000; Rosa et al. 1997). Antinutrional GSL have been studied most extensively in rapeseed, a crop that has been subjected to intense breeding efforts to reduce the concentration of these compounds (Rosa et al. 1997). This goal has been partially achieved, resulting in the development of ''Double zero'' (zero erucic acid and zero GSL) rapeseed or Canola varieties (Rosa et al. 1997). More recently however, some GSL and their derived products have been reported to have beneficial properties as potent inducers of phase II enzymes involved in carcinogen detoxification in mammals (Zhang et al. 1994) and antibiotic effect on bacteria *Heliobacter pylori* causing gastric infection (Fahey et al. 2002). Furthermore, other GSL have been reported to be effective agents for the biological control of pathogenic fungi, nematodes and weeds (Mithen et al. 2000; Subbarao et al. 1999). Consequently, there is renewed interest in studying the biosynthesis and inheritance of GSL for the possible genetic manipulation and engineering of plants with specific contents of these compounds.

Although approximately 120 kinds of GSL have been identified in plants, each plant species has only a few major GSL (Fahey et al. 2001). *Brassica* species contain mostly aliphatic (derived from methionine) and indolyl (derived from tryptophan) GSL. Indolyl GSL are omnipresent in Brassica crops, although their levels are subject to environmental fluctuations. No null mutants for indolyl GSL have been reported (Rosa et al. 1997). On the other hand, aliphatic glucosinolate content is highly heritable, and the specific kinds and content of these compounds vary in Brassica crops and varieties of the same crop (Kushad et al. 1999).

Aliphatic GSL are classified by side-chain size as 3-carbon (3C), 4-carbon (4C) and 5-carbon (5C) GSL. They can also be classified by side-chain structure into methylthioalkyl, methylsulfinylalkyl, alkenyl and hydroxyalkenyl glucosinolates. Five major loci have been inferred from intense genetic analysis of these compounds in *Arabidopsis thaliana*, including *GS-ELONG, GS-OX, GS-ALK, GS-OH* and *GSOHP* (Campos de Quiros et al. 2000; Hall et al. 2001; Kliebenstein et al. 2001a; Mithen et al. 1995). *GS-ELONG* presumably determines the length of the C side-chains in this species by the addition of methylene groups, which is catalyzed by isopropylmalate synthases-like enzymes (Ipms) that

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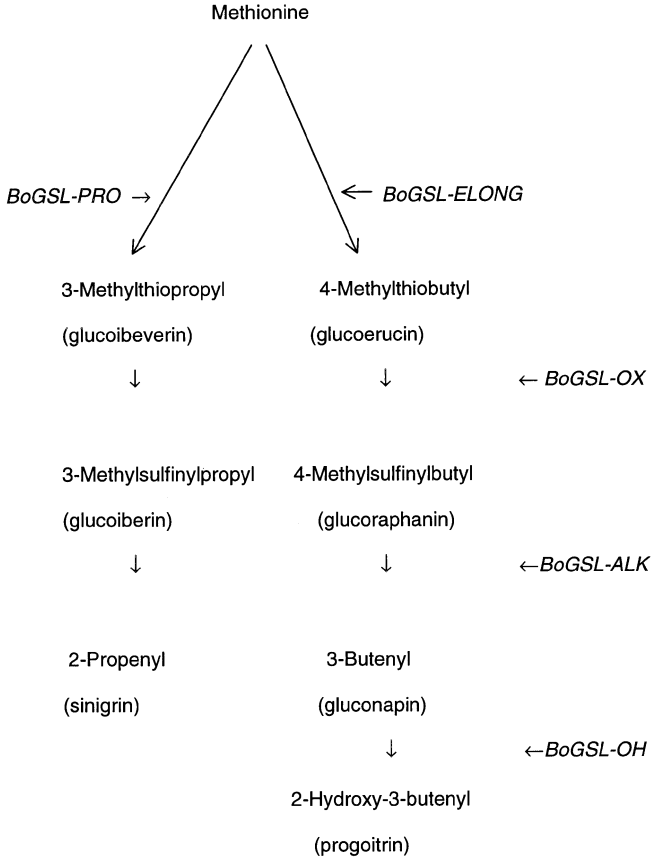

Fig. 1 Model for aliphatic glucosinolate biosynthesis in *Brassica oleracea*, including the inferred major genes controlling this process

are coded by multiple gene members organized in a gene family (Campos de Quiros et al. 2000). The rest of the genes are side-chain modification genes. For example, *GS-OX* changes methylthioalkyl into methylsulfinylalkyl; *GS-ALK* induces alkenyl GSL by desaturation and loss of the methylsulfenyl moiety, which can then be transformed next by *GS-OH* into hydroxyalkenyl GSL. The production of 3-hydroxypropyl GSL is mediated by *GS-OHP* (Kliebenstein et al. 2001a; Hall et al. 2001).

Homologs to most of these genes have been reported in *Brassica* species (Giamoustaris and Mithen 1996; Li et al. 2001), although in *B. oleracea* at least two Ipms genes seem to be involved the C-side chain size: *BoGSL-PRO*, which determines GSL with 3C side chains, whereas *BoGSL-ELONG* determines 4C side-chains (Fig. 1) (Campos de Quiros et al. 2000; Li et al. 2001). Due to the lack of natural variation for 3C glucosinolates in *A. thaliana*, the *GS-PRO* homolog has not been reported in this species. Combinations of functional or null alleles at each locus determine the GSL composition of each genotype in *A. thaliana* as well as in *Brassica* species (Campos de Quiros et al. 2000; Giamoustaris and Mithen 1996; Kliebenstein et al. 2001a; Li et al. 2001). Recently, candidate genes for *GS-ELONG* (isopropylmalate synthase-like homolog) and *GS-ALK* (oxoglutarate-dependent dioxygenase homolog) in *Arabidopsis* have been identified and analyzed by fine mapping (Campos de Quiros et al. 2000; Giamoustaris and Mithen 1996, Hall et al. 2001). However, the functionality of *GSL-ELONG* remains to be demonstrated, whereas that of *GSL-ALK* and *GSL-OHP* has been inferred by co-segregation analysis, natural variation and heterologous expression in *Escherichia coli* (Kliebenstein et al. 2001a, b).Therefore, it has not been established in planta that any of these genes are really involved in GSL regulation, and the feasibility of modifying GSL profiles in specific plant genotypes is unknown. In this paper, we report for the first time that a functional allele of the *B. oleracea* homolog for *GS-ALK*, (*BoGSL-ALK*$^+$) indeed regulates alkenyl GSL production. The function of this gene was demonstrated by complementary transformation of *A. thaliana* ecotype Columbia, in which the corresponding allele for *GS-ALK* is non-functional.

Materials and methods

The following populations were used in our mapping and gene tagging experiments. Two recombinant inbred line populations, developed by crossing collard Georgia (*B. oleracea*) to cauliflower Purple (120 lines) and collard to broccoli Topper 43–70 (90 lines) (Kianian and Quiros 1992). A doubled haploid population of broccoli Lu-Ling (60 lines) was also used for this purpose.

For the gene tagging experiments we extracted plant DNA by a simplified CTAB procedure method (Saghai-Maroof et al. 1984) in 2× buffer: 100 m*M* Tris–HCl, 1.4 *M* NaCl, 20 m*M* EDTA, 2% CTAB, pH 8.0. Sequence-related amplified polymorphism (SRAP) markers were used for tagging gene *BoGSL-ALK*, which segregated in the populations described above as described by Li and Quiros (2001). The amplified DNA fragments were assayed with a LI-COR sequencer IR2 (LI-COR, Neb.).

For the bacteria artificial chromosome (BAC) clone screening and sequencing experiments, we used a BAC library constructed with the broccoli doubled haploid line Early Big-10. We screened the library for clones harboring a marker (SRAP133) associated to the *BoGSL-ALK* gene. Two rounds of polymerase chain reaction (PCR) were used for the BAC library screening by three-dimensional pooling of the clones following the strategy of Koes et al. (1995). For this purpose we designed the following primers: FC1, 5′-TCAGGGCAGGTAAGAACAATT-3′; FC2, 5′GAGGTAGGTTACCTTGAT-3′. These were designed on the basis of the sequence of marker SRAP133. All DNA sequencing was done by the dideoxynucleotide chain termination method using a DNA sequencer (ABI377; Applied Biosystems, Foster City, Calif.).

The functional allele for the *BoGSL-ALK* allele present in collard Georgia (Li et al. 2001) was used to make the construct for the transformation experiments. After positional cloning of *BoGSL-ALK* from broccoli as described in the Results and discussion, we designed the following two primers to amplify the functional allele of this gene from collard: 5′-TTCCATCATTTACTTTCTCAG-3′ and 5′-AAGCCGGTCCTCCTAATTTGTA-3′. The PCR conditions for amplification were: 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min. The amplified product was first cloned into the TA cloning vector (InVitro Gene, Calif.) and then inserted into binary vector pCB302 (Xiang et al. 1999) to create a construct containing this sequence and the *bar* gene as a selectable marker, thereby conferring resistance to the herbicide Finale (Farnam, Arizona). The construct was electroporated into *Agrobacterium tumefaciens* strain GV3101. For *Agrobacterium* transformation, the flower dip method was applied to transform wild-type *A. thaliana* ecotype Columbia (Clough and Bent 1998).

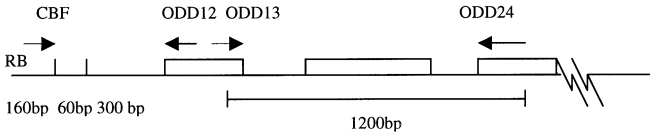

Fig. 2 Schematic representation of primer locations (*arrows*) in the vector and *BoGSL-ALK*+ allele. *RB* Right border in T-DNA from vector; *boxes* indicate the first three exons of *BoGSL-ALK*+

Approximately 7,000 seeds from the treated plans were sown in flats. Transformants were selected by spraying seedlings at the first-true leaf stage with 0.1% Finale. These were confirmed by PCR using a pair of primers, one located in the pCB302 vector and the other one on exon1 of the functional *BoGSL-ALK*+ homolog (Fig. 2). The sequences of the primers used were as follows: CBF, 5′-AACTGAAGGCGGGAAACG-3′; ODD12, 5′-TTGAATATCCAGTGTAAGGT-3′. The PCR conditions for amplification were: 35 cycle of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min.

For the gene expression experiments we used reverse transcription (RT)-PCR to confirm *BoGSL-ALK* expression in the transformants. Total RNA was prepared as described in Sambrook et al. (1989). A 10-µg aliquot of RNA from each plant was used to synthesize cDNA using Themoscript RT-PCR Systems (Life Technologies, Gaithersburg, Md.) and following the manufacturer protocol. The primers used were as follows: ODD13, 5′-AACAGCGAAACGATCCAGAAGA-3′, located on exon 2; ODD24, 5′-GTGCTTCTCGTCCACAA-3′, on exon 3 of *BoGSL-ALK* (Fig. 2). Amplification conditions were the same as those specified above.

GSL profiles in leaves and seeds were determined by high performance liquid chromatography (HPLC) using the method described by Kraling et al. (1990) with some modifications. Ground tissue was extracted twice with 70% methanol at 80 °C for 10 min. After applying the supernatant to a DEAE-Sephadex A-25 (Sigma, St. Louis, Mo.) column, the GSL were converted into desulfoglucosinolates with 0.5% sulfatase H-1 (Sigma) in water for 16 h at room temperature. The desulfoglucosinolates were then eluted by adding 1.5 ml water. The resulting desulfoglucosinolates were separated by HPLC in a gradient of acetonitrile. Using 2-propenyl glucosinolate (Sigma) and the HPLC, we compared a chromatograph from an unknown to the chromatograph of Linetta, a rapeseed variety widely used as a standard for glucosinolate identification, to match the unknown peaks with the corresponding known glucosinolates. For the Arabidopsis seeds, the HPLC procedure, glucosinolate identification and quantification were done as described by Kliebenstein et al. (2001b).

Results and discussion

We isolated *BoGSL-ALK* by positional cloning after identifying a closely linked SRAP marker to this candidate gene. For this purpose, we screened more than 3,000 polymorphic SRAP bands in our mapping populations, finding several markers linked to *BoGSL-ALK*. One of these, SRAP133, completely co-segregated with *BoGSL-ALK* in the three populations, which have different genetic backgrounds as described in the Materials and methods. BLAST analysis of the sequence of the isolated SRAP133 DNA band revealed a homologous sequence located on Arabidopsis BAC clone F4C21. The pair of primers based on the SRAP133 (FC1 and FC2) located a single broccoli BAC clone, B13H21, harboring this marker. Partial sequencing of B13H21 disclosed 11 sequences on this clone that matched the same number of genes on *A. thaliana* contiguous BAC clones F4C21 and T4I9. Among these 11 sequences, one corresponded to *A. thaliana* candidate gene *GS-ALK*, which was inferred by genetic and biochemical analyses (Hall et al. 2001; Kliebenstein et al. 2001a). *GS-ALK* is a single gene in a group of three tandem duplicates of 2-oxoglutarate-dependent dioxygenase homologs (ODD), *ODD1*, *ODD2* and *ODD3*. *ODD2* corresponds to *GS-ALK* (Hall et al. 2001; Kliebenstein et al. 2001a).

We carried out comparative sequence analysis to highlight the function of the *BoGSL-ALK* gene and to gain additional information on the structural changes in its functional and non-functional alleles. We first compared the sequences of both the non-functional broccoli allele *BoGSL-ALK*– (GenBank no. AY044424) and the functional collard allele *BoGSL-ALK*+ (GenBank no. AY044425). GenScan (Burge and Karlin 1998) detected three exons in *BoGSL-ALK*+, producing a predicted coding sequence of 1,317 nucleotides and a protein of 439 amino acids. On the other hand, allele *BoGSL-ALK*– has only two exons with 957 nucleotides of predicted coding sequence, encoding a protein of 319 amino acids. After CLUSTALW (Thompson et al. 1994) alignment of the genomic DNA sequences for these two alleles, we observed a 2-bp deletion in exon 2 in allele *BoGSL-ALK*– causing a frameshift that results in the observed shorter coding sequence (data not shown). We also sequenced the region corresponding to exon 2 in Chinese cabbage (*B. rapa*), a related species of *B. oleracea* in which *GSL-ALK* is functional judged by the existence of alkenyl GSL in this crop. The sequence of Chinese cabbage did not have the small deletion observed in *BoGSL-ALK*– and had virtually the same sequence as that observed in collard allele *BoGSL-ALK*+ (data not shown). These results indicate that most likely the non-functional allele *BoGSL-ALK*– present in broccoli originated from the 2-bp deletion in exon 2. Moreover, we compared the sequence of functional *BoGSL-ALK*+ allele with the sequences available in *Arabidopsis*, including an *ODD* homolog corresponding to *ODD1 (AOP1)* (Mithen et al. 1995) from the Columbia wild type (GenBank no. AF069442), *AOP2* (*ODD2*, *GS-ALK)* (Mithen et al. 1995) from the wild type of Cape Verde Islands (CVI, GenBank no. AF417858) and two others corresponding to *AOP3* (*ODD3*, *GS-OHP*) from the wild type of CVI and Landsberg (GenBank nos. AF418274, AF417859) (Fig. 3). The alignments of the deduced sequences of amino acids of these five coding sequences showed that in *ODD1* the sequence corresponding to exon 2 in *BoGSL-ALK*+ was split by the presence of an extra intron, resulting in a gene with four exons producing a smaller predicted protein that may not be functional (Hall et al. 2001). Although this event was not present in *AOP3*, a large deletion (Fig. 3) occurred in exon 2 of this gene. In spite of this deletion, this allele might be functional (Hall et al. 2001). Only the *AOP2* sequence from the CVI ecotype, which has a functional *GL-ALK* allele, maintained a similar size to that of *BoGSL-ALK*+ except for a few base changes. It has also been

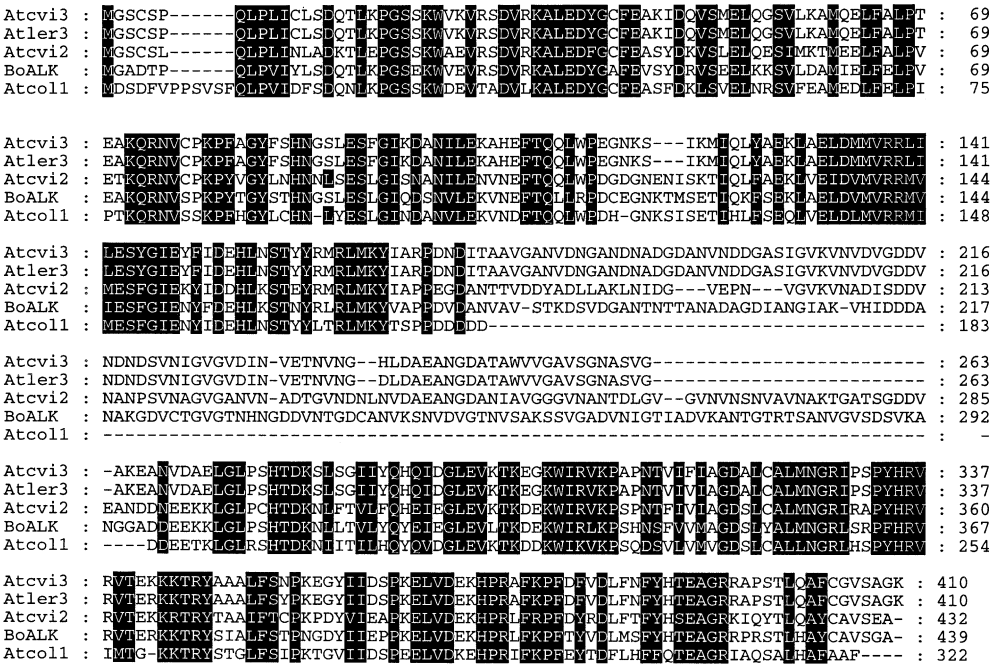

Fig. 3 CLUSTALW alignment of *BoGSL-ALK* (*BoALK*) amino acid sequences from collard with corresponding functional *Arabidopsis thaliana* homolog from ecotype Cape Verde Islands (*Atcvi2*), and to predicted products of duplicate Arabidopsis 2-oxyglutarate-dependent-dioxygenase genes *ODD1* from ecotype Columbia (*Atcol1*) and *ODD3* from ecotypes Landsberg (*Atler3*) and Cape Verde Islands (*Atcvi3*). *Shading* includes conserved amino acids

shown by heterologous expression in *E. coli* that of the *ODD2* and *ODD3* genes in *Arabidopsis*, only *ODD2* has the same function as *BoGSL-ALK*. Interestingly, *ODD3* has been inferred to confer GS-OHP function in the heterologous expression analysis mentioned above, resulting in hydroxyalkyl GSL (Hall et al. 2001). However, this activity has yet to be confirmed in planta.

In order to demonstrate the functionality of the *ODD2* homolog in *B. oleracea*, we introduced by transformation the collard functional allele *BoGSL-ALK*$^+$ into *A. thaliana* ecotype Columbia. The aliphatic GSL profile of this ecotype consists of 4-methylsulfinylbutyl and 3-methylsulfinylpropyl GSL, indicating that Columbia is homozygous recessive for the null allele of *GS-ALK*. Following spraying with the herbicide, approximately 3% of the seedlings resulting from the transformation survived. We screened 200 putative transformants by PCR for the presence of a 520-bp band corresponding to a portion of the vector and the exon 1 of *BoGSL-ALK*$^+$ allele (Fig. 2). All of these plants generated a band of the expected size, whereas no such band was amplified from untransformed control plants of the *Arabidopsis* wild-type ecotype. To investigate *BoGSL-ALK* expression, we used a pair of primers located inside the collard *BoGSL-ALK*$^+$ allele to amplify cDNAs generated by the same putative allele introduced to the *Arabidopsis* transformants, using as control untransformed wild-type individuals. Positive bands of the expected size (840 bp) were obtained from only transformed plants (Fig. 4). These bands were further confirmed to correspond to the *BoGSL-ALK*$^+$ by collecting DNA from the gels and subsequent sequencing. After sequencing, we found that the cDNA sequence from the *Arabidopsis* transformants was identical to the collard cDNA sequence. This test established that the *ODD2 Brassica* homolog from collard expressed in the *Arabidopsis* transformants.

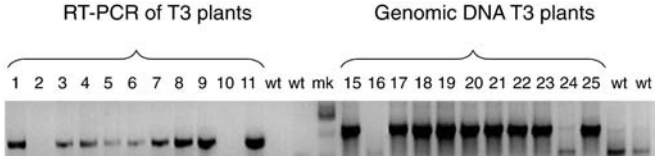

Fig. 4 **Left panel** *Lanes 1–11* RT-PCR from cDNA amplified from transformed T3 Arabidopsis plants produced a single 840-bp band. Wild type Columbia (*wt*) and non-transformed plants (*lanes 2*, *10*) failed to amplify this band. **Right panel** Genomic DNA, from the same plants (*lanes 15–25*). Untransformed plants lack single band of 1,200 bp

As final confirmation, we carried out a functional analysis of the *Arabidopsis* transformants by inspection of their GSL phenotype. In order to avoid possible uncertain changes for GSL in the transformants due to transient expression or the loss of expression of the foreign gene, we used T3 lines subjected to two cycles of selfing to perform the GSL analysis (Kraling et al. 1990). GSL were extracted from segregating T3 lines and from wild-type Columbia controls for HPLC analysis. In 23 T3 lines containing the *BoGSL-ALK*$^+$ allele, the glucosinolate profile in their leaves changed by the presence of three additional peaks, which were absent in nine of the T3 lines lacking *BoGSL-ALK*. The profiles of the latter were the same as that observed for the wild-type, untransformed ecotype. The three new peaks in the transformed plants carrying *BoGSL-ALK*$^+$ correspond to 2-hydroxy-3-butenyl, 2-propenyl glucosinolate and 3-butenyl glucosinolate (Fig. 5). Therefore, it can be concluded from these profiles that *BoGSL-ALK* converted in planta more than 80% of the 4-methylsulfinylbutyl GSL precursor into 3-

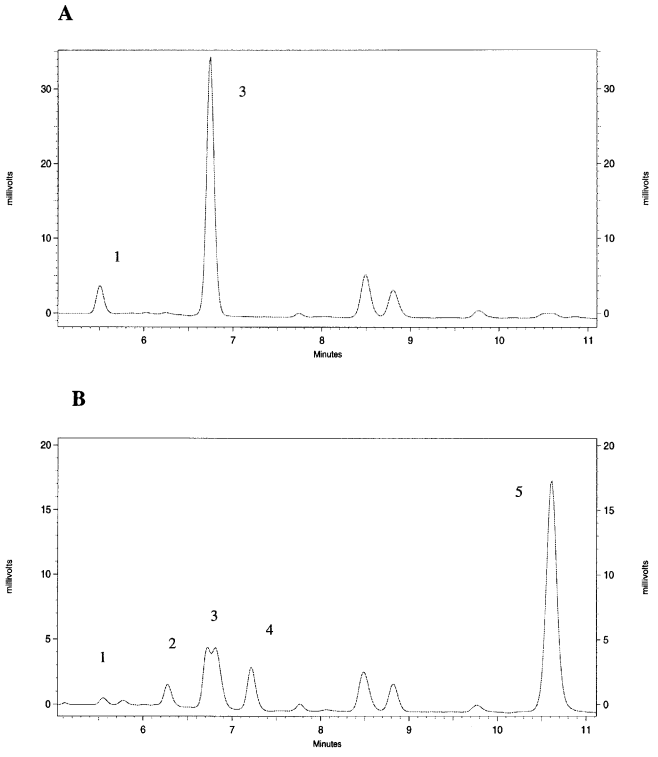

Fig. 5.A, B Glucosinolate (GSL) profiles for transformed and wild-type *A. thaliana* ecotype Columbia. **A** GSL profile of wild-type ecotype lacking a functional GS-ALK allele displays two main aliphatic GSL: 3-carbon 3-methylsulfinylpropyl (*peak 1*) and 4-carbon 4-methylsulfinylbutyl (*peak 3*). **B** Transformed Columbia plants with functional *BoGSL-ALK* allele display reduced concentration of precursor 3-methylsulfinylpropyl (*peak 1*), which is converted to 2-propenyl (*peak 4*), and reduced concentration of precursor 4-methylsulfinylbutyl (*peak 3*), which is converted into 3-butenyl (*peak 5*). Presence of 2-hydroxy-3-butenyl (*peak 2*) indicates activity of allele at *GS-OH* locus (see Fig. 1). *Peak 3*, which shows a double peak, includes 4-methylsulfinylbutyl and 2 (*S*)-hydroxy-3-butenyl, a derivative from 3-butenyl (*peak 5*) (Mithen et al. 1995)

butenyl glucosinolate, and the 3-methylsulfinylpropyl GSL precursor into 2-propenyl glucosinolate (Fig. 5). The GSL profiles of seeds from the transformed plants displayed a fourth new peak, probably corresponding to 2-benzoyloxy-3-butenyl as reported by Kliebenstein et al. (2001b). Additionally, the content of 4-methylsulfinylbutyl increased, whereas the content of 4-methylthiobutyl, 3-benzyoloxypropyl, and 4-benzoyloxyl-3-butenyl glucosinolates dramatically decreased. This is shown in relative amounts in the histogram depicted in Fig. 6. In conclusion, the cloning of *BoGSL-ALK* provides the opportunity to engineer *Brassica* crops with specific glucosinolate content by modifying their GSL side-chains. This manipulation can have several possible applications. For example, down-regulation or silencing of *BoGSL-ALK* could produce rapeseed varieties lacking the antinutrient progoitrin (2-hydroxy-3-butenyl GSL) and would simultaneously produce plants accumulating glucoraphanin (4-methylsulphinylbutyl GSL) as a source of anticarcinogens. On the other hand, if the objective was to accumulate sinigrin (2-propenyl GSL) for biological control (for example, incorporating crop residue in the soil to control pathogenic fungi, weeds or nematodes), then *BoGSL-ALK* could be introduced to Brassica crops with high biomass capacity. In the future the use of the genes determining the length of the side chains of the aliphatic glucosinolates, mainly 3C or 4C, in conjunction with *BoGSL-ALK* will allow a higher accumulation of specific 3C or 4C glucosinolates. Our demonstration of the functionality in planta of *BoGSL-ALK* is the first step, but a key step, in achieving this goal.

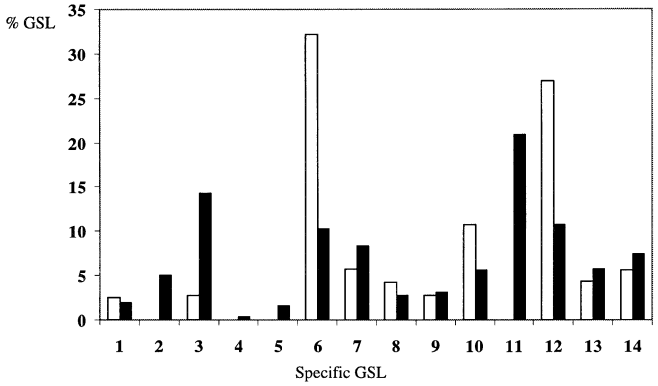

Fig. 6 Relative amounts (expressed in percentage) of main GSL for seeds of transformed (*shaded bars*) and wild-type *A. thaliana* ecotype Columbia (*clear bars*). *Bars: 1–5* Same as Fig. 5, *6* 4-methylthiobutyl, *7* 8-methylsulfinyloctyl, *8* indolyl-3-methyl, *9* 5-methylthiopentyl, *10* 3-benzoyloxypropyl, *11* 2-benzoyloxy-3-butenyl, *12* 4-benzoyloxybutyl, *13* 7-methylthioheptyl, *14* 8-methylthioctyl

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