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Genetics of aliphatic glucosinolates. IV. Side-chain modification in *Brassica oleracea*

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Abstract The biochemical and genetical relationship between aliphatic glucosinolates which have methylthioalkyl, methylsulphinylalkyl and alkenyl side chains has not been resolved by biochemical studies. In this study, two hypothetical models are tested by the genetic analysis of a backcross population between *Brassica drepanensis* and *B. atlantica*. The results support one of the models in which 3-methylthiopropyl glucosinolate is sequentially converted to 3-methylsulphinylpropyl, and then to 2-propenyl glucosinolate, by the action of dominant alleles at two loci. RFLP mapping positioned both loci on the same linkage group homologous to the *B. napus* N19 linkage group. The implication of the results for the genetic manipulation of glucosinolates in *Brassica* to improve flavour and nutritional properties, and in order to investigate plant-insect interactions, is discussed.

Key words Aliphatic glucosinolates · *B. drepanensis* · *B. atlantica* · *B. oleracea* · Sulfurophane · Gene mapping

Introduction

Glucosinolates are sulphur-containing glycosides which occur in cruciferous crops. The glucosinolate (GSL) molecule consist of a common glycone moiety and a variable aglycone side chain which is derived from an α-amino acid. Glucosinolates which have aliphatic side chains, derived from methionine, are the most prominent type of glucosinolates in leaves of *Brassica* vegetable and salad crops. The length and structure of the aliphatic side chain is variable (Table 1), and is important in determining many of the biological activities of glucosinolates. The majority of *Brassica* vegetables and salad crops have a predominance of alkenyl glucosinolates, although *B. oleracea* var. *italica* (broccoli and calabrese) has mainly 3-methylsulphinylpropyl and 4-methylsulphinylbutyl glucosinolates. Following tissue disruption, glucosinolates undergo hydrolysis, catalysed by a thioglucosidase ('myrosinase'), to release an array of products of which isothiocyanates are the most prominent under ambient conditions of temperature and pH (Fig. 1). Isothiocyanates are partially volatile and are the major flavour compounds of cruciferous crops. The flavour of these crops is partially determined by the total amount of glucosinolates, the glucosinolate side-chain structures, and myrosinase activity. The presence of certain isothiocyanates, such as those with methylthioalkyl side chains, results in bitterness which may make particular cultivars unacceptable to the consumer.

Methylsulphinylalkyl isothiocyanates, and to a lesser extent methylthioalkyl isothiocyanates, induce phase-2 detoxification enzymes, such as quinone reductase and glutathione transferase, in both cell cultures and mice (Zhang et al. 1992, 1994; Tawfiq et al. 1995). These enzymes are associated with reduced susceptibility to the toxic and neoplastic effects of carcinogens. The anticancer activity of these isothiocyanates may contribute to the reduced susceptibility of people with high levels of fresh vegetable in their diet to certain types of cancer (Block et al. 1992). In addition to the flavour and anticancer activity of glucosinolates and isothiocyanates, these metabolites also mediate herbivore interactions with crucifers (Chew 1982; Giamoustaris and Mithen 1995).

Magrath et al. (1994) and Mithen et al. (1995) proposed a biochemical genetical model of aliphatic glucosinolate biosynthesis in which a small number of genes regulate side-chain elongation and side-chain modifications (Fig. 2). The length of the side chain is regulated by a product(s) from the *Gsl-elong* genes. For example, a functional copy of this gene converts 2-propenyl into 3-butenyl GSL, and 3-methylsulphinylpropyl into 4-methylsulphinylbutyl GSL (Mithen et al. 1995). However, the hypothetical relationship between methylthioalkyl, methylsulphinylalkyl and alkenyl glucosinolates proposed in this model remains to be tested. Two alternative models for the interconversions of these glucosinolates have been proposed (Fig. 3).

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Table 1 Aliphatic glucosinolates occuring in *Brassica* vegetable and salad crops. 4-Methylsuphinylbutyl isothiocyanate is the most potent inducer of phase-2 detoxification enzymes. Glucosinolates with propyl and propenyl side-chains are found only in *B. oleracea* genotypes, and those with pentyl and pentenyl side-chains are found only in *B. rapa* genotypes (Fenwick et al. 1983)

Systematic name	Trivial name
3-Methylthiopropyl	Glucoibeverin
3-Methylsulphinylpropyl	Glucoiberin
2-Propenyl	Sinigrin
4-Methylthiobutyl	Glucoerucin
4-Methylsulphinylbutyl	Glucoraphanin
3-Butenyl	Gluconapin
2-Hydroxy-3-butenyl	Progoitrin
5-Methylthiopentyl	Glucoberteroin
5-Methylsulphinylpentyl	Glucoalyssin
4-Pentenyl	Glucobrassicanapin
2-Hydroxy-4-pentenyl	Gluconapoeiferin

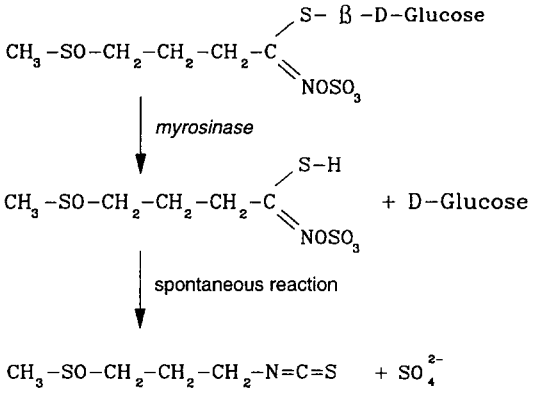

Fig. 1 Conversion of 3-methylsulphinylpropyl GSL to 3-methylsulphinylpropyl isothiocyanate ('iberin')

Firstly, both alkenyl and methylsulphinylalkyl glucosinolates may be derived directly from a methylthioalkyl precursor by either elimination of a methanethiol group, to produce an alkenyl glucosinolate, or oxidation to produce a methylsulphinylalkyl glucosinolate (Underhill et al. 1973; Kräling et al. 1990). Alternatively, alkenyl glucosinolates may be derived from methylthioalkyl glucosinolates via a methylsulphinylalkyl intermediatory (Magrath et al. 1994; Mithen et al. 1995). Resolving which of these pathways is correct is important in developing strategies to manipulate the aliphatic glucosinolate content of *Brassica* crops in order to improve flavour and nutritional effects, as well as to study pest interactions.

Attempts to resolve this pathway through a biochemical approach have not been successful. Chisholm and Matsuo (1972) found that in *Armoracea lapathifolia* (horseradish) both 3-methylthiopropyl and 3-methylsulphinylpropyl were converted into 2-propenyl glucosinolate with equal efficiency and it was not possible to distinguish the immediate *in planta* precursor. In the present paper, we report the result of a genetic approach to resolving this part of aliphatic glucosinolate biosynthesis by studying a segregating population developed from a cross between wild species of the *Brassica oleracea* n=9 C-genome species complex. These taxa have been shown to be interfertile amongst themselves and with cultivated forms of *B. oleracea* (von Bothmer et al. 1995), and to have high levels of individual aliphatic glucosinolates (Mithen et al. 1987). While the total level of glucosinolate is influenced by environmental factors and the age of the leaves, the ratio of individual aliphatic glucosinolates is constant. These taxa have a perennial growth habit with a juvenile phase before flowering. Changes in day length or temperature have no effect on the length of this juvenile period, which may last several years (personal observation). Once flowering has been initiated, they flower for several weeks each year between phases of vegetative growth. Individual plants can be maintained for several years.

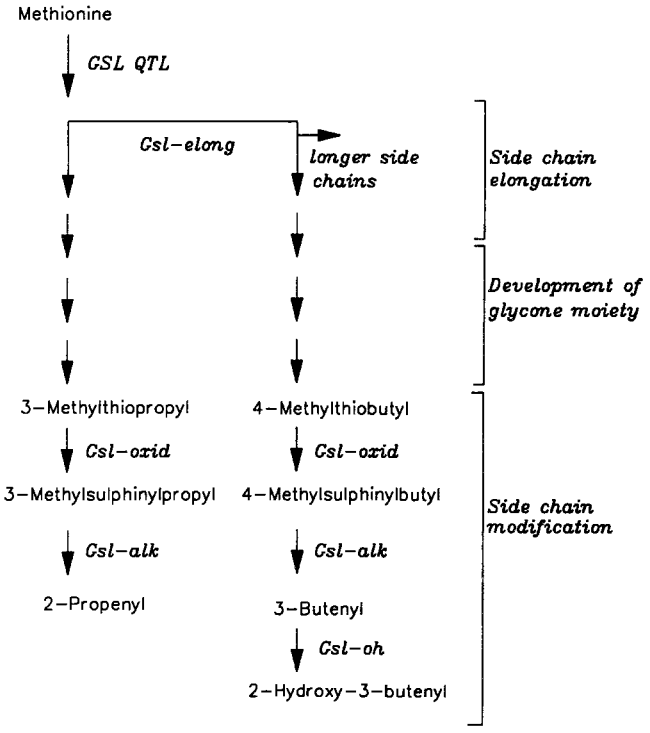

Fig. 2 Hypothetical pathway of aliphatic glucosinolate biosynthesis. See Fig. 3 for further details of side-chain modification

Materials and methods

Preliminary studies had shown that leaves of *B. drepanensis* Caruel (syn. *B. villosa* Biv. subsp *drepanensis*) from Mt. Erice, Sicily, had predominantly 3-methylthiopropyl glucosinolate while those of *B. atlantica* (Coss.) Schultz (syn *B. insularis* Moris) from El Haouria, Tunisia, had predominantly 2-propenyl glucosinolate (Giamoustaris 1996; Mithen et al. 1987). Seeds of these two taxa were sown in John Innes no 1 compost in March 1989. Plants were grown in a glasshouse without any supplementary lighting with a minimum temperature of 8°C. Plants of *B. atlantica* flowered for the first time in February 1991, and those of *B. drepanensis* in January 1993. In subsequent years, both taxa started flowering in January for a period of 2–3 months and then returned to a vegetative state. Crosses were made between the two taxa and F_1 hybrid plants grown in identical

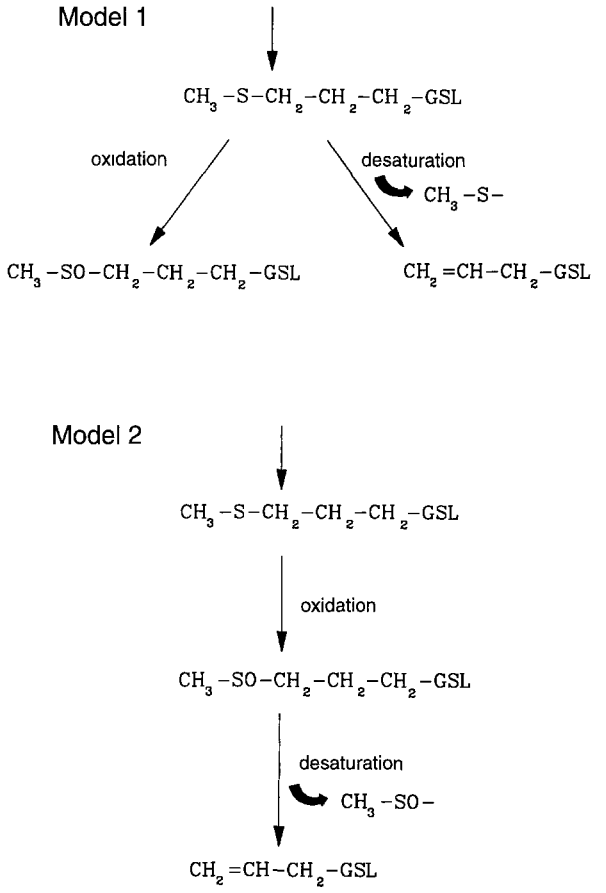

Fig. 3 Two hypothetical models for the conversion of 3-methylthiopropyl GSL to 2-propenyl GSL

Table 2 Aliphatic glucosinolate content of leaves of the parent plants and the F_1 hybrid. The levels were measured in young, fully expanded leaves. The total level of glucosinolates is influenced by growth conditions, but the proportion of individual aliphatic glucosinolates is constant

Item	*B. drepanensis*	*B. atlantica*	F_1 hybrid
Total μMol/g dry wt	88.3	29.4	51.6
Proportion of individual aliphatic glucosinolates (see Fig. 4)			
3-Methylthiopropyl GSL	0.90	0.01	0.06
3-Methylsulphinylpropyl GSL	0.10	0.02	0.02
2-Propenyl GSL	0.00	0.98	0.92

Table 3 Phenotype and putative genotype of 83 B_1 plants. A '+' indicates the presence of a dominant functional allele at the *Gsl* locus *B. drepanensis* has phenotype A and *B. atlantica* has phenotype B (see Fig. 4). Phenotypes C and D are novel phenotypes due to genetic recombination between the *Gsl-alk* and *Gsl-oxid* loci

Phenotype (see Fig. 4)			Genotype	
			Gsl-oxid-C	*Gsl-alk-C*
A	3-Methylthiopropyl + 3-Methylsulphinylpropyl (trace)	26	–	–
B	2-Propenyl	27	+	+
C	3-Methylsulphinylpropyl	14	+	–
D	3-Methylthiopropyl + 2-propenyl (trace)	20	–	+

conditions to the parental plants. F_1 plants flowered in January 1995 and one individual was backcrossed to the parental *B. drepanensis* plant. The B_1 progeny were grown in a glasshouse under standard conditions and leaves were harvested from plants when they were 6–8 weeks old, along with leaves from the parental and F_1 plants. Leaves were immediately frozen in liquid nitrogen and freeze dried, prior to glucosinolate and DNA extraction. Glucosinolates were extracted, converted to desulphoglucosinolates, and analysed by HPLC as described by Magrath et al. (1993). DNA was extracted from the parental plants, the F_1 hybrid plant and 61 B_1 individuals. The DNA was restricted with *Eco*RI, the fragments separated by electrophoresis and blotted onto nylon filters as described by Sharpe et al. (1995). The filters were probed with 29 anonymous genomic probes. An initial set of probes was selected with reference to the *Brassica* RFLP maps as described by Sharpe et al. (1995) and Parkin et al. (1995) so that it would be probable that RFLP loci were detected on each of the nine linkage groups. After linkage of the glucosinolate genes to RFLP loci had been detected, additional probes were used which identified loci on the relevant linkage group. Linkage analyses was performed initially by two-point mapping and subsequently with the MAPMAKER version 3.0 software. Linkage groups were assembled using a minimum LOD score of 3.0. The Kosambi function was used to convert recombination fractions into centiMorgans.

Results and discussion

B. drepanensis had high levels of 3-methylthiopropyl glucosinolate, with smaller amounts of 3-methylsulphinylpropyl glucosinolate, while *B. atlantica* had high levels of 2-propenyl glucosinolate (Table 2). These profiles were consistent with that found in a previous study (Mithen et al. 1987). The F_1 hybrid had high levels of 2-propenyl GSL and lower levels of both 3-methylthiopropyl and 3-methylsulphinylpropyl GSL (Table 2). In the B_1 progeny, the total level of glucosinolates varied from 26.2 μMol/g dry wt to 118.5 μMol/g dry wt in a continuous manner. However, individual plants had one of four distinct glucosinolate profiles. Two of these phenotypes corresponded to the parental phenotypes. The other two were novel phenotypes which occurred as a result of genetic recombination. One of these had high levels of 3-methylsulphinylpropyl GSL, while the other combined high levels of 3-methylthiopropyl GSL with lower levels of 2-propenyl GSL (Table 3, Fig. 4). The ratio of these four phenotypes did not differ significantly from 1:1:1:1 (χ^2=4.22, P>0.1), although there was a significant reduction in the expected number of recombinant genotypes compared to the parental genotypes (see below). These results are consistent with the second hypothetical model in which alleles at two loci regulate the conversion of 3-methylthiopropyl to 2-propenyl glucosinolate via a 3-methylsulphinylpropyl glucosinolate intermediary. A dominant allele at the first locus (*Gsl-oxid-C*) converts 3-methylthiopropyl to 3-methylsulphinylpropyl by

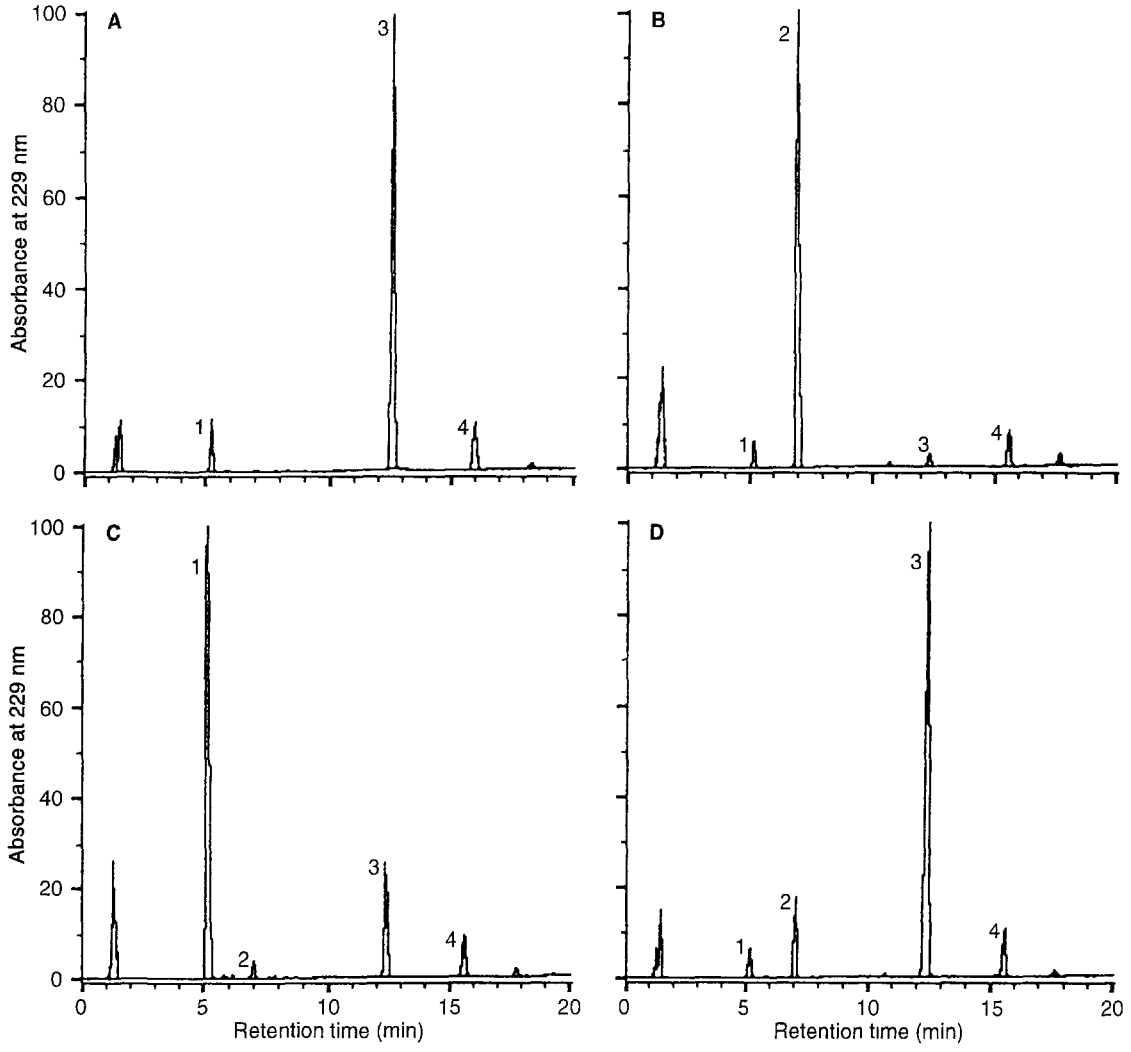

Fig. 4A–D HPLC chromatograms of desulphoglucosinolates from the four phenotypes observed in the B_1 population. HPLC conditions are as described by Magrath et al. (1993). Absorbance is a relative measure with that of the largest peak standardised to 100. *Peak 4* is an internal standard of known and identical concentration in all samples, which enables the levels of individual glucosinolates to be quantified **A** and **B** correspond to the two parental phenotypes, having predominantly 3-methylthiopropyl and 2-propenyl GSL respectively, and **C** and **D** to the two recombinant phenotypes. *Peak 1* 3-methylsulphinylpropyl GSL; *peak 2* 2-propenyl GSL; *peak 3* 3-methylthiopropyl GSL; *peak 4* benzyl GSL (internal standard)

oxidation, and a dominant allele at the second locus (*Gsl-alk-C*) converts the 3-methylsulphinylpropyl GSL to 2-propenyl glucosinolate by removal of the terminal methylsulphinyl group and insertion of a double bond. In the B_1 progeny, 53 plants had either of the two parental phenotypes (Fig. 4 A and B) while 34 plants had either one of the other two recombinant phenotypes (Fig. 4 C and D). This ratio is different from the 1:1 expected ratio if the two loci are unlinked (χ^2=4.15, $0.01<P<0.05$), and suggests a recombination fraction of 39.1% between the two loci, which, if converted to genetic distance with the use of the Kosambi function, is equivalent to 52.5 cM.

The results do not support the alternative model in which 2-propenyl GSL is derived directly from 3-methylthiopropyl GSL; a model in which 50% of the B1 progeny would be expected to have 2-propenyl GSL. These results are, however, consistent with studies on glucosinolate biosynthesis in *Arabidopsis thaliana* in which alleles at a single locus (*Gsl-alk-Ar*) on chromosome 4 convert methylsulphinylalkyl GSLs into alkenyl GSLs (Mithen et al. 1995). It is likely that *Gsl-alk-C* and *Gsl-alk-Ar* loci are homologues of each other.

In many crucifers which have alkenyl GSLs, methylsulphinylalkyl GSLs are not apparent. This is likely to be due to an efficient mechanism for the conversion of methylsulphinylalkyl into alkenyl GSLs catalysed by the gene products of the *Gsl-alk* locus. The results of the present study suggest that methylsulphinylalkyl GSLs are the immediate precursors of alkenyl GSLs and, therefore, the levels of these anticancer glucosinolates could be enhanced by inhibiting the removal of the methylsulphinyl group and desaturation.

The genotype of each of the B_1 progeny could be ascertained at each of the two *Gsl* loci (Table 3). The 29 genomic probes identified 65 polymorphic loci. The *B. atlantica* allele at the *Gsl-oxid* locus co-segregated with an

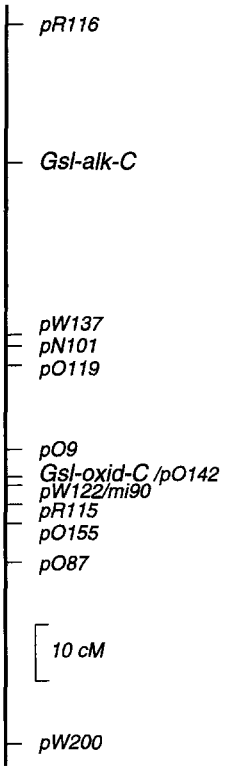

Fig. 5 RFLP map of the position of the *Gsl-oxid-C* and the *Gsl-alk-C* loci. The linkage group is homologous to the N19 group of *B. napus sensu* Sharpe et al. (1995)

allele at the RFLP locus *pO142* (Fig. 5), and was closely linked to several other RFLP loci. The *Gsl-alk* locus was positioned on the same linkage group, 7.0 cM from *pW157* and 17.5 cM from *pO125* (Fig. 5). The genetic distance between the two *Gsl* loci positioned on the RFLP linkage map, calculated with MAPMAKER (52.0 cM), is similar to the estimate from the phenotypic analysis (52.5 cM). The linkage group corresponds to the *B. napus* group N19, *sensu* Parkin et al. (1995) which is part of the *B. napus* C genome (Parkin et al. 1995). The order of the RFLP loci on these two linkage groups is identical, but the genetic distances between loci are different, particularly between *pR116* and *pW137*. While this suggests differences in the frequency of recombination in the two genomes, the consistency of the order of the loci provides additional evidence that there has not been any major genomic rearrangements either within the *Brassica* C-genome complex or between the C genome of diploid species and the corresponding parts of the *B. napus* genome.

4-Methylsulphinylbutyl isothiocyanate ('sulforaphane') is the most efficient isothiocyanate at inducing phase-2 detoxification enzymes. To optimise the level of this glucosinolate in cruciferous vegetables and salad crops, a combination of a null allele at the *Gsl-alk* locus and a functional allele at the *Gsl-elong* locus (Magrath et al. 1994), which produces butyl glucosinolate derivatives, is required. There are two strategies by which this could be achieved. Firstly, marker-assisted selection could be used to introduce the correct alleles into *Brassica* crops from either wild or cultivated forms of *B. oleracea*, and, secondly, the *Gsl-elong* and *Gsl-alk* genes could be cloned from *Arabidopsis thaliana* (Mithen et al. 1995) and used to manipulate glucosinolates in *Brassica*.

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References

Block G, Patterson B, Subar A (1992) Fruits, vegetables and cancer prevention. A review of the epidemiological evidence. Nutrition and Cancer 18:1–19

Bothmer R von, Gustafsson M, Snogerup S (1995) *Brassica* sect. *Brassica* (Brassicaceae). II. Inter- and intraspecific crosses with cultivars of *B. oleracea*. Genet Res Crop Evol 42:165–178

Chew FS (1988) Biological effects of glucosinolates. In: Cutler HG (ed) Biologically active natural products – potential use in agriculture. American Chemical Society Symposium, Washington DC, pp 155–181

Chisholm M, Matsuo M (1972) Biosynthesis of allylglucosinolate and 3-methylthiopropylglucosinolate in horseradish, *Amoracea lapathifolia*. Phytochemistry 11:203–207

Giamoustaris A (1996) Glucosinolates in *Brassica* and their role in mediating pest and pathogen interactions. PhD thesis, University of East Anglia, Norwich, UK

Giamoustaris A, Mithen R (1995) Modifying the leaf glucosinolate content of oilseed rape and its effect on its interaction with specialist and generalist pests. Ann Appl Biol 126:347–63

Fenwick G, Heaney R, Mullin W (1983) Glucosinolates and their breakdown products in food and food plants. CRC Crit Rev Food Sci Nutrit 18:123–301

Kraling K, Röbbelen G, Theis W, Herrmann M, Ahmadi M (1990) Variation of seed glucosinolates in lines of *Brassica napus*. Plant Breed 105:33–39

Magrath R, Herron C, Giamoustaris A, Mithen R (1993) The inheritance of aliphatic glucosinolates in *Brassica napus*. Plant Breed 111:55–72

Magrath R, Morgen M, Bano F, Parkin I, Sharpe A, Lister C, Turner J, Dean C, Lydiate D, Mithen R (1994) Genetics of aliphatic glucosinolates I. Side-chain elongation in *Brassica napus* and *Arabidopsis thaliana*. Heredity 72:290–299

Mithen R, Lewis BG, Fenwick GR (1987) Glucosinolates in wild and cultivated *Brassica* species. Phytochemistry 26:1969–1973

Mithen R, Carke J, Lister C, Dean C (1995) Genetics of aliphatic glucosinolates. III. Side-chain structure of aliphatic glucosinolates in *Arabidopsis thaliana*. Heredity 74:210–215

Parkin I, Sharpe A, Keith D, Lydiate D (1995) Identifications of the A and C genomes of amhidiploid *Brassica napus* (oilseed rape). Genome 38:1122–1131

Sharpe A, Parkin I, Keith D, Lydiate D (1995) Frequent non-reciprocal translocations in the amphidiploid genome of *Brassica napus* (oilseed rape). Genome 38:1112–1121

Tawfiq N, Heaney R, Plumb J, Fenwick G, Musk S, Williamson G (1995) Dietary glucosinolates as blocking agents against carcinogenesis: glucosinolate breakdown products assessed by induction of quinone reductase activity in murine hepa1c1c7 cells. Carcinogenesis 16:1191–1194

Underhill E, Wetter L, Chisholm M (1973) Biosynthesis of glucosinolates. Biochem Soc Symp 38:303–326

Zhang Y, Talalay P, Cho C-G, Posner G (1992) A major inducer of anticancer protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 89:2399–2403

Zhang Y, Kensler T, Cho C-G, Posner G, Talahay P (1994) Anticancinogenic activities of sulfurophane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci USA 91: 3147–3150