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RFLP mapping of quantitative trait loci controlling seed aliphatic-glucosinolate content in oilseed rape (*Brassica napus* L.)

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Abstract We report the RFLP mapping of quantitative trait loci (QTLs) which regulate the total seed aliphatic-glucosinolate content in *Brassica napus* L. A population of 99 F_1-derived doubled-haploid (DH) recombinant lines from a cross between the cultivars Stellar (low-glucosinolate) and Major (high-glucosinolate) was used for single-marker analysis and the interval mapping of QTLs associated with total seed glucosinolates. Two major loci, *GSL-1* and *GSL-2*, with the largest influence on total seed aliphatic-glucosinolates, were mapped onto LG 20 and LG 1, respectively. Three loci with smaller effects, *GSL-3*, *GSL-4* and *GSL-5*, were tentatively mapped to LG 18, LG 4 and LG 13, respectively. The QTLs acted in an additive manner and accounted for 71% of the variation in total seed glucosinolates, with *GSL-1* and *GSL-2* accounting for 33% and 17%, respectively. The recombinant population had aliphatic-glucosinolate levels of between 6 and 160 μmoles per g^{-1} dry wt of seed. Transgressive segregation for high seed glucosinolate content was apparent in 25 individuals. These phenotypes possessed Stellar alleles at *GSL-3* and Major alleles at the four other *GSL* loci demonstrating that low-glucosinolate genotypes (i.e. Stellar) may possess alleles for high glucosinolates which are only expressed in particular genetic backgrounds. *Gsl-elong* and *Gsl-alk*, loci which regulate the ratio of individual aliphatic glucosinolates, were also mapped. *Gsl-elong-1* and *Gsl-elong-2*, which control elongation of the α-amino-acid precursors, mapped to LG 18 and LG 20 and were coincident with *GSL* loci which regulate total seed aliphatic glucosinolates. A third tentative QTL, which regulates side-chain elongation, was tentatively mapped to LG 12. *Gsl-alk*, which regulates H_3CS– removal and side-chain de-saturation, mapped to LG 20.

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Introduction

Oilseed rape (*Brassica napus* L.) is one of the most important crops in the world. It is grown primarily for its oil which is used both for nutritional and industrial purposes (Fenwick et al. 1983). After oil extraction, the residual seed-meal is limited in its usefulness as a protein source by a group of secondary metabolites known as glucosinolates (Fenwick 1984; Duncan 1991). Various biological functions have been ascribed to glucosinolates and their break-down products (e.g. see Andersen and Muir 1966; Larsen 1981; Larsen et al. 1992). Enzymatic hydrolysis of glucosinolates leads to a complex mixture of products (Benn 1977). Some of these are cytotoxic (e.g. isothiocyanates and their derivatives; Horáková 1966), others cause severe nutritional problems in livestock (e.g. *l*-5-vinyl-2-thiooxazolidone; Fenwick et al. 1983). The elimination of glucosinolates could potentially make rapeseed-meal as valuable as the oil. To this end, plant breeders have selected against high seed glucosinolate content in oilseed rape (Kondra and Stefansson 1970; Röbbelen and Thies 1980; Gland et al. 1981) and, through the introgression of alleles from the Polish cultivar "Bronowski", have reduced the seed aliphatic-glucosinolate content from above 100 μmoles g^{-1} to less than 20 μmoles g^{-1}. The reduction in seed glucosinolate content has been entirely due to a reduction in aliphatic glucosinolates, derived from methionine; the levels of indolyl glucosinolates, derived from tryptophan, have remained approximately constant. Despite initial concerns, the reduction in seed aliphatic-glucosinolates has had no discernable effects on the interaction between oilseed rape and pests and pathogens. This is partly because the total level of leaf aliphatic glucosinolates in low seed-glucosinolate cultivars is similar to that in high seed-glucosinolate cultivars for the majority of the

growing season (Mithen 1992). Manipulation of leaf-glucosinolate content does, however, have significant effects on herbivore interactions (Giamoustaris and Mithen 1995).

The seed-glucosinolate content of oilseed rape is a quantitative trait under complex genetic regulation (Kondra and Stefansson 1970; Rücker and Rudloff 1991). Recent advances in the construction of RFLP linkage maps have enabled researchers to detect and map quantitative trait loci (QTLs) (Weller 1986; Paterson et al. 1988). Estimates of QTL effects on phenotype can provide information on the genotypic control of complex traits and DNA markers closely linked to QTLs can be used to select plants with desirable phenotypes (Edwards et al. 1992). Detailed genetic maps based on RFLPs have been constructed for *B. napus* (Landry et al. 1991; Ferreira et al. 1994). A *B.napus* map constructed by Ferreira et al. (1994) was based on a recombinant doubled-haploid (DH) population from a cross between high- and low-glucosinolate parents. These DH lines represent a permanent population for mapping and in the present study they were used to identify QTLs which regulate the aliphatic seed-glucosinolate content of *B. napus*.

Materials and methods

Plant material

A single plant from the *B. napus* cv Major (high in glucosinolate) was crossed with a doubled-haploid line derived from cv Stellar (low in glucosinolate) and a segregating population was obtained by microspore culture of a single F_1 hybrid plant (Ferreira et al. 1994). Self-pollinated seeds from 105 F_1-derived doubled-haploid (DH) lines were harvested between August and September 1992 from a field trial held in Madison, Wis. Open-pollinated seeds from these lines were obtained during August 1993 from a field trial held in Griffin Ga.

Glucosinolate analyses

Seed samples from self- and open-pollinated plants were analyzed for total and individual glucosinolate content using the extraction and HPLC procedures described by Heaney et al. (1986). All individuals from the selfed population were analyzed for total glucosinolate content. For comparative purposes, 30 corresponding genotypes, ten individuals from the lower extreme of the frequency distribution, ten from the higher and ten having intermediate total aliphatic glucosinolates, were analyzed from the open-pollinated population. Glucosinolate identification was based on a comparison of retention times with authentic glucosinolate standards. Benzyl glucosinolate (0.8 m*M*; 50 µl) was used as the internal standard and was included at the beginning of each extraction. Published relative response factors were used for the quantification of individual glucosinolates (Heaney et al. 1986).

The calculation of flux through reactions for side-chain modifications giving rise to an alteration in the content of individual glucosinolates were as described by Magrath et al. (1993). The following formulae were used to obtain the phenotypic data used to map the loci controlling the percentage butyls (*Gsl-elong*) and butenyls (*Gsl-alk*):

Percentage butyls = 100 × (butenyl + hydroxybutenyl + methylthiobutyl + methylsulphinylbutyl)/ total aliphatic glucosinolates

Percentage pentyls = 100 × (pentenyl + hydroxypentenyl + methylthiopentyl + methylsulphinylpentyl)/total aliphatic glucosinolates

Percentage butenyls = 100 × (butenyl + hydroxybutenyl)/ total butyl glucosinolates

RFLP linkage analysis

One-hundred-and-five DH lines were used previously to construct a linkage map of 138 RFLP loci (Ferreira et al. 1994). A revised map containing 21 linkage groups plus five pairs of loci covering 1343 cM was used for the present study. The map contained a total of 196 RFLP loci: 131 loci were detected by 92 genomic DNA clones from either a *B. napus* cv Westar library ("wg" prefix) or a *B. rapa* cv Tobin library ("tg" prefix) and 59 loci were detected by cDNA clones from a *B. napus* cv Westar library ("ec" prefix) (Ferreira et al. 1994; Thormann et al. 1994). The revised map was constructed as described previously (Ferreira et al. 1994). The linkage group numbers in the present paper correspond to those previously published by Ferreira et al. (1994). Subsequent analysis has combined LG16 and LG18 into a single linkage group.

QTL analysis

Phenotypic data was analysed by a single-marker QTL analysis, as described by Magrath et al. (1994), and by interval-mapping analysis (Lander and Botstein 1989). Interval-mapping was carried out using the MAPMAKER/QTL computer program (Lincoln and Lander 1990). Multipoint linkage map distances were calculated using the Kosambi (1944) transformation function of MAPMAKER. A LOD ($\log_{10}$ of the likelihood odds ratio) score of 3 was taken to indicate QTLs which had a significant effect on the phenotype (Lander and Botstein 1989), and a LOD score of 2 was used as the lower threshold for QTLs which had tentative effects on the phenotype.

Results

Phenotype and frequency distribution

Total aliphatic glucosinolates

Seeds from the parental lines Major and Stellar had 109 µmoles g^{-1} and 18 µmoles g^{-1} of aliphatic glucosinolates, respectively. The phenotypic distribution of total aliphatic glucosinolates in seeds from self pollination of DH lines is shown in Fig. 1. Six lines had equivalent or lower lev-

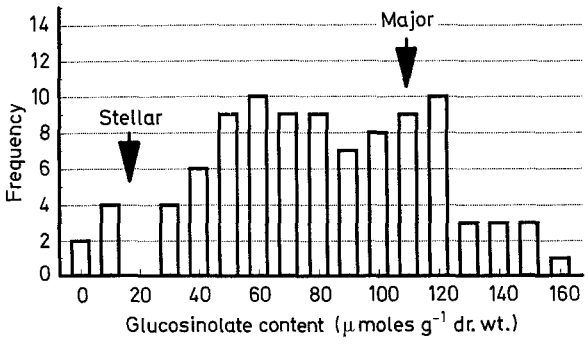

Fig. 1 Phenotypic distribution of recombinant DH lines of *B. napus* for total seed aliphatic-glucosinolates. The parental phenotypes are indicated by *arrows*

Table 1 Associations between RFLP markers and total seed aliphatic-glucosinolate content

LG[a]	Locus	Segragation ratio[b]	t-Value[d]
20	*tg1h12*	3.89 : 1.00[c]	4.02***
	wg3f7a	2.77 : 1.00[c]	6.48***
1	*wg2d11a*	1.00 : 1.04	3.67***
	wg7a8a	1.10 : 1.00	2.94**
	wg5a6	1.00 : 1.09	3.18**
18	*wg6c6a*	1.00 : 1.53	2.62**
4	*ec2b3*	1.00 : 1.27	2.84**
	wg9e9	1.00 : 1.10	3.15**
13	*wg8h5*	1.33 : 1.00	2.76**

[a] LG, linkage group
[b] Segregation ratios of DH lines for marker genotypes Major:Stellar
[c] χ^2 test, significantly different from 1:1 ratio ($P<0.001$)
[d] t-Values were obtained by comparing phenotypic values of the two classes with either Stellar or Major alleles at the RFLP loci with the use of Student's t-test, ** =$P<0.01$; *** =$P<0.001$

els of glucosinolates than Stellar, and 25 lines had higher levels of glucosinolates than Major. The distribution suggested that levels of aliphatic glucosinolates were determined by alleles at several loci. The levels within the seeds from the open-pollinated lines were highly correlated with those from the self-pollinated lines ($r^2=0.78$). Single-marker QTL analyses indicated that Stellar alleles at RFLP markers *tg1h12* and *wg3f7a* on LG 20, and markers *wg2d11a* and *wg3h8a* on LG 1, were significantly associated with low levels of glucosinolates ($P<0.001$). At less-stringent levels of significance ($P<0.01$), alleles at other RFLP markers on LG 20, LG 1 and also on LG 18, LG 4 and LG 13 were significantly associated with variation in glucosinolate content (Table 1). Contrary to expectation, genotypes possessing alleles from Major at RFLP loci on LG 18 had significantly lower levels of aliphatic glucosinolates than those which had alleles from Stellar. Examination of the genotypes of the tails of the distribution showed that all genotypes with less than 20 μmoles g^{-1} had Stellar alleles at the presumed glucosinolate loci on LG 20 (*GSL-1*) and LG 1 (*GSL-2*), whereas the 22 out of the 25 transgressive genotypes which had glucosinolate levels higher than those in Major (109 μmoles g^{-1}) had Major alleles at the *GSL-1* and *GSL-2* loci on LG 20 and LG 1 and Stellar alleles at the *GSL-3* locus on LG 18 (Table 2). Thus, results from single-marker analyses suggested that the level of aliphatic seed glucosinolates was determined largely by alleles at two loci, on LG 20 and LG 1, with minor effects of alleles at three additional loci on LG 4, LG 18 and LG 13. Alleles at the five loci accounted for 71% of the total variation in seed aliphatic-glucosinolates, with the two major loci, *GSL-1* and *GSL-2*, accounting for 32.8 and 17.2% of the variation respectively (Table 3).

If the presence of Stellar alleles at two loci is largely responsible for low levels of glucosinolates, one would expect approximately 25% of the DH lines to have low levels of glucosinolates (<20 μmoles g^{-1}). The lack of lines with low levels was due to the excess of lines with alleles from Major at RFLP loci on LG 20 (Table 1; Ferreira et al. 1994).

The putative positions of *GSL* loci were confirmed through interval mapping which suggested that there were two significant QTLs which had major effects on linkage groups 20 and 1, and a tentative QTL which had smaller

Table 2 Genotypes of transgressive lines (i.e. lines with higher amounts of glucosinolates than Major, Fig. 1) at individual *GSL* loci controlling total seed aliphatic-glucosinolate content

DH line	[GSL][b] (μmoles g^{-1})	Genotype at individual *GSL* loci				
		GSL-1 (LG 20)	*GSL-2* (LG 1)	*GSL-3* (LG 18)	*GSL-4* (LG 4)	*GSL-5* (LG 13)
347	110	M	M/S	S	M	S[c]
449	110	M	M	M	M/S	M
433	111	M	M	S	M	S
409	114	M	M	S	S	S
440	114	M	M	S	M	M
398	117	M	M	S	S	M
311	118	M	M[c]	S[c]	M[c]	M[c]
319	118	M	M	S	M/S	S
321	118	M	M[c]	S	M	S[c]
357	119	M	S[c]	S[c]	–	M[c]
392	120	M	M	S	M	M
368	122	M	M[c]	S	M	–
450	122	M	M/S	S	S	M
397	124	M	M	S	S	S
424	125	M	S	S	M[c]	–
317	129	M	M	S	M	M
405	132	M	M	S	M	S
401	134	M	S	M	M	M
337	138	M	M[c]	S	S	M
455	142	M	M	M	M	M/S
302	145	M	M	S[c]	M	M
386	147	M	M	S	M	M
310	148	M	M	S	S[c]	M[c]
391	148	M	M	S	M	M
308	159	M	M/S	S[c]	M	M/S

[a] Closest linked RFLP markers *tg1h12* and *wg3f7a* for *GSL-1*, *wg2d11a* and *wg7a8a* for *GSL-2*, *wg7a11* and *wg6c6* for *GSL-3*, *wg9e9* and *ec2b3* for *GSL-4*, *wg8h5* and *wg3h4* for *GSL-5*
[b] Total glucosinolate content of transgressive lines with glucosinolates in excess of the Major parent (109 μmoles g^{-1})
[c] Information available only for the first of the RFLP-marker pair at the *GSL* locus; M/S=Major allele at one of the flanking markers and Stellar allele at the other flanking marker; – = data not available

Table 3 ANOVA and coefficients of variation for five putative QTLs which regulate total seed aliphatic-glucosinolate content. The five QTLs explain 71% of the variation in glucosinolate content

Locus	LG marker	Nearest RFLP	*df*	MS	Coefficient of variation	
GSL-1	20	*wg3f7a*	1	26679	700.4	32.8%
GSL-2	1	*wg2d11a*	1	12623	367.0	17.2%
GSL-3	18	*wg6c6a*	1	2446	55.5	2.6%
GSL-4	4	*wg9e9*	1	6060	161.9	7.5%
GSL-5	13	*wg8h5*	1	8368	231.3	10.8%
Error			92	621		

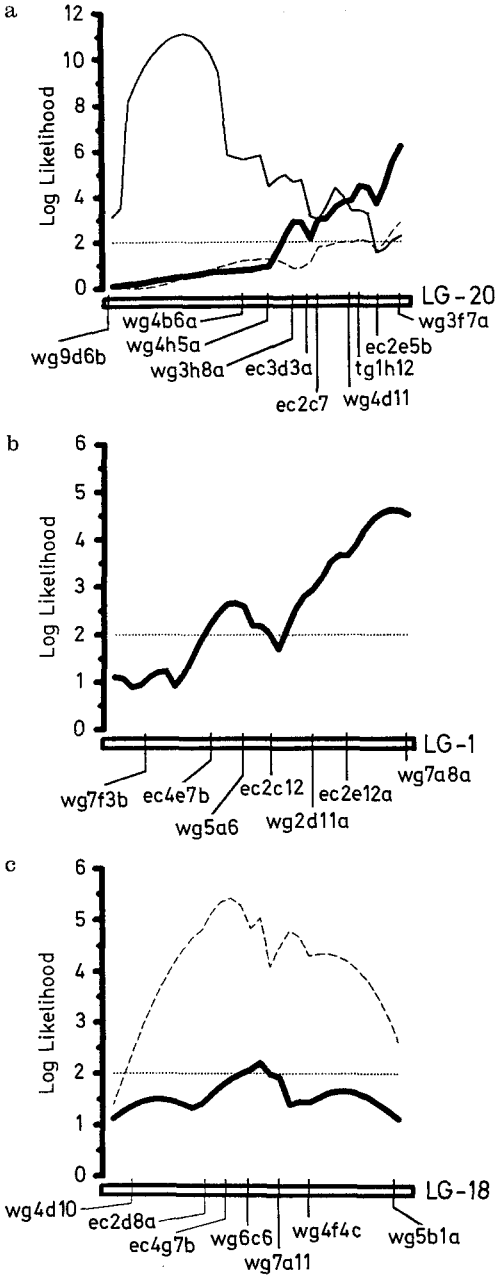

Fig. 2a–c Log-likelihood odds ratios (LOD) for QTLs controlling total seed aliphatic-glucosinolate content (*GSL*), side-chain de-saturation (*Gsl-alk*) and side-chain elongation (*Gsl-elong*). LG18 has been combined with LG16, *sensu* Ferreira et al. (1994). ——— *GSL*; ——— = *Gsl-alk*; – – – – = *Gsl-elong*. **a** Linkage Group 20. **b** Linkage Group 1. **c** Linkage Group 18

effects on linkage groups 18 and 13. (Fig. 2). Thus these results confirmed those found by single-marker analysis, i.e. that Stellar alleles at loci *GSL-1* on LG 20 and *GSL-2* on LG 1 are responsible for lines having less than 20 μmoles g^{-1} of glucosinolates, whereas alleles at these, and possibly at least three other, loci (on LG 18, 13 and 4) are responsible for determining the level of glucosinolates in genotypes which have higher amounts of glucosinolates.

Individual glucosinolates

Although 2-hydroxy-3-butenyl glucosinolate was the most abundant glucosinolate in both Stellar and Major (Fig. 3) there were differences between the two parents in the ratio of different classes of glucosinolates which were attributable to different fluxes through the aliphatic-glucosinolate biosynthetic pathway (Fig. 4). There was a difference in the ratio of the two classes of glucosinolates with different side-chain lengths. The α-amino-acid precursors of glucosinolates are thought to be elongated by successive carbon additions from an undetermined methyl donor. The flux between homomethionine, 2-amino-6-methylhexanoic acid and 2-amino-7-methylheptanoic acid is regulated by alleles at the *Gsl-elong* loci (Magrath et al. 1994) which determine the ratio of the different classes of glucosinolates with different side-chain lengths (Fig. 4). Ninety-four percent of glucosinolates in Stellar were derived from 2-amino-6-methylthiohexanoic acid (i.e. methylthiobutyl, methylsulphinylbutyl, butenyl and hydroxybutenyl) whereas the remaining 6% were glucosinolates derived from the chain-elongated methionine-homologue 2-amino-7-methylheptanoic acid (i.e. methylthiopentyl, methylsulphinylpentyl, pentenyl and hydroxypentenyl). In contrast, 84% of glucosinolates in Major were derived from 2-amino-6-methylhexanoic acid and 16% from its elongated homologue. In the homozygous DH lines, the percentage of glucosinolates derived from 2-amino-6-methylthiohexanoic acid segregated in a continuous manner. Single-marker and interval mapping identified significant QTLs on LG 18 and LG 20 which regulated this flux. These were coincident with the QTLs which regulate total aliphatic-glucosinolate content (Figs. 2a, c). A third QTL was tentatively mapped to LG12.

In addition to differences in side-chain length, Stellar possessed significant quantities of 4-methylsulphinylbutyl glucosinolate which was only present in trace amounts in Major (Fig. 3). This difference can be attributed to a difference in the extent of conversion of 4-methylsulphinylbutyl into butenyl glucosinolate regulated by alleles at the *Gsl-alk* locus (Magrath et al. 1994; Mithen et al. 1995). In Major, 99% of 4-methylsulphinylbutyl glucosinolates were converted into butenyl (and hydroxybutenyl glucosinolate), whereas in Stellar only 79% was converted, resulting in significant amounts of methylsulphinylbutyl glucosinolates in the seeds. There was a continuous distribution for this trait in the DH lines. Interval mapping and single-marker analysis identified a locus on LG 20 which had a major effect on this flux and another tentative locus, on a linkage group containing RFLP markers *tg4d2b* and *wg7b6b* (Ferreira et al. 1994), which regulated this flux (Fig. 2a).

Discussion

The levels of aliphatic glucosinolates in the recombinant DH lines derived from an F_1 hybrid of Stellar×Major were

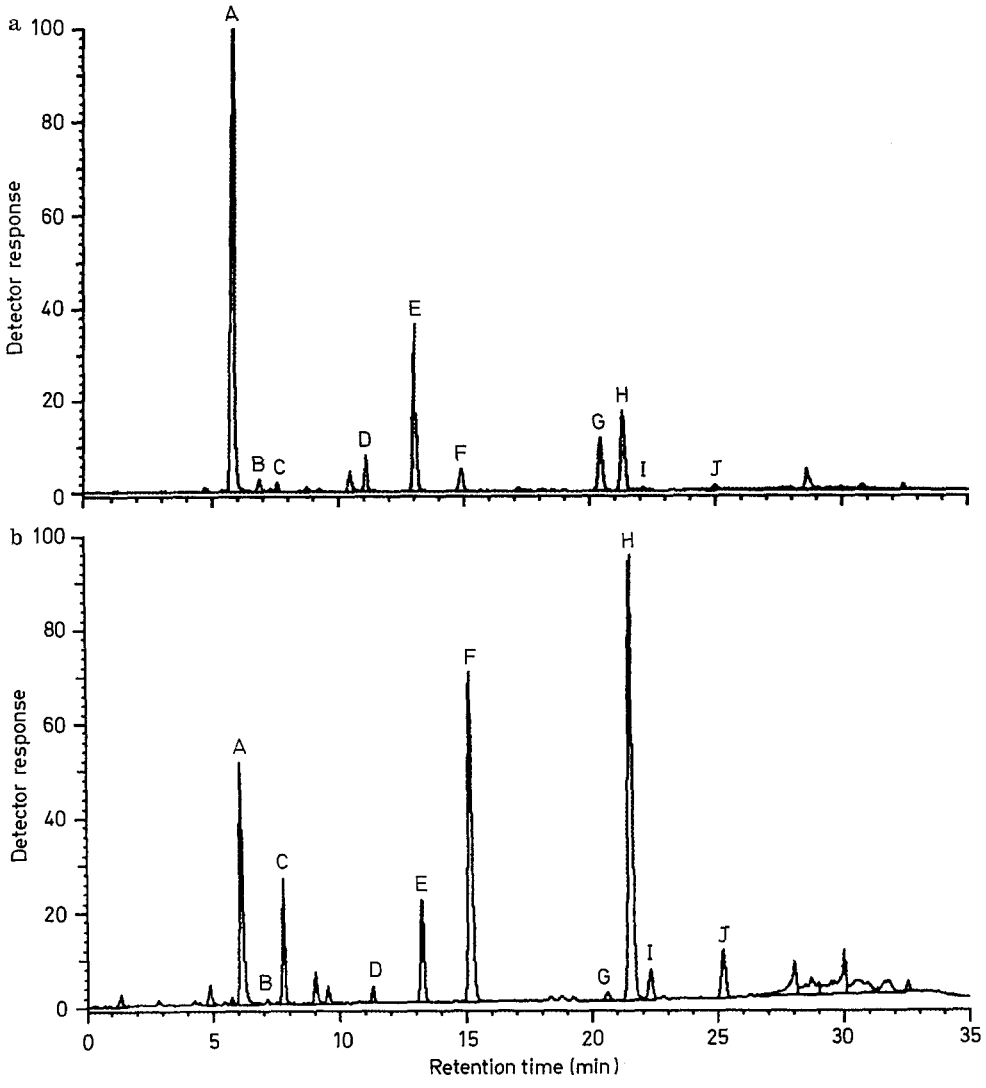

Fig. 3a, b HPLC profiles of glucosinolates from seeds of mapping population parents, Major (**a**) and Stellar (**b**). Letters *A to J* denote specific glucosinolates: *A* 2-hydroxybut-3-enylglucosinolate; *B* prop-2-enylglucosinolate; *C* 4-methylsulphinylbutylglucosinolate; *D* 2-hydroxypent-4-enylglucosinolate; *E* but-3-enylglucosinolate; *F* 4-hydroxyindol-3-ylmethylglucosinolate; *G* pent-4-enylglucosinolate; *H* benzylglucosinolate (internal standard); *I* 4-methylthiobutylglucosinolate; *J* indol-3-ylmethylglucosinolate

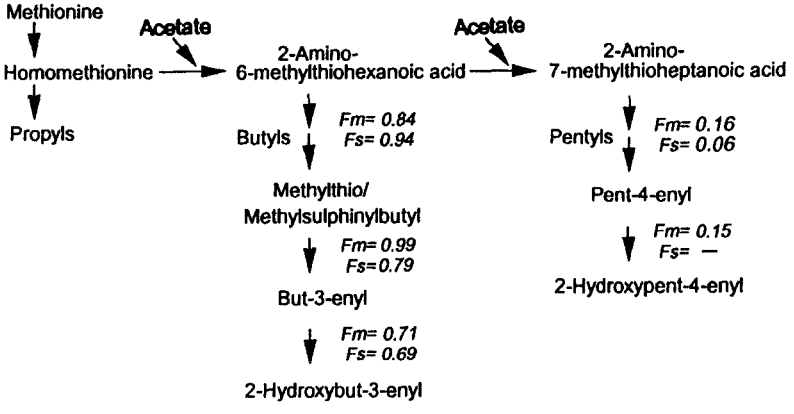

Fig. 4 Hypothetical pathway of aliphatic-glucosinolate biosynthesis from α-amino-acid homologues (see Larsen 1981 and Magrath et al. 1994). *Fm* = flux in Major; *Fs* = flux in Stellar

regulated by alleles at two loci on LG 20 and LG 1 which had major effects, and three tentative loci on LG 18, LG 4 and LG 13 which had smaller effects. Only lines containing Stellar alleles at *GSL-1* and *GSL-2* had seed with less than 20 μmoles g^{-1}, suggesting that these genes are needed for low glucosinolates. Other loci had only minor effects; however, the full range of glucosinolate levels was regulated by alleles at all of the *GSL* loci. The lines with higher levels of aliphatic glucosinolates than Major had Major alleles at each of the *GSL* loci, with the exception of *GSL-3*, at which they almost invariably had Stellar alleles (Table 2). This indicates that lines with low levels of gluco-

sinolates may possess alleles for high glucosinolates which are expressed only in particular genetic backgrounds.

Genes from the Polish spring-rape variety Bronowski have been introgressed into both spring and winter oilseed rape cultivars to obtain the low-glucosinolate phenotype. However, a yield penalty has been incurred in winter oilseed rape cultivars as a result of the introgression of the low-glucosinolate phenotype. A possible reason for this is that parts of the Bronowski genome, which may be linked to *GSL* loci, have caused a reduction in winter hardiness (Röbbelen and Thies 1980). The identification and RFLP mapping of loci which regulate seed glucosinolates will enable the introgression of alleles for low seed-glucosinolate content with simultaneous selection for the background genotype of the recurrent parent using marker loci throughout the genome. Additionally, it will enable identification of the specific low-glucosinolate alleles from Bronowski that have been transferred into winter and spring oilseed rape cultivars. It is possible that other breeding material, not derived from Bronowski, has also contributed alleles for low glucosinolates in modern oilseed rape varieties.

The genetic regulation of side-chain elongation of aliphatic glucosinolates in *B. napus* and *Arabidopsis thaliana* was reported by Magrath et al. (1994). Synthetic *B. napus* lines with large differences in the ratio of different classes of side-chain lengths were used to map two loci, *Gsl-elong-A* and *Gsl-elong-C*, in *B. napus* which regulate the elongation of the amino-acid precursors of aliphatic glucosinolates. In the present study, three loci have been identified which are responsible for the smaller differences in the ratio of side-chain lengths observed between Stellar and Major. The RFLP probes used in the present study and those in the study described by Magrath et al. (1994) are different and, at present, the RFLP maps developed with the different probes have not been integrated. Thus, it is not possible to say whether the *Gsl-elong* loci mapped in the two studies are at the same position in the *Brassica* genome. The map positions of two of the *Gsl-elong* loci identified in this study are coincident with *GSL* loci which regulate total aliphatic-glucosinolate content. It is likely that alleles at the *Gsl-elong* loci may also regulate the initial entry of methionine into aliphatic-glucosinolate biosynthesis and hence regulate the total levels of aliphatic glucosinolates (Magrath et al. 1994). The results presented in this study are consistent with this hypothesis. A putative homologous gene has been mapped to chromosome 5 in *A. thaliana* (Magrath et al. 1994). Cloning of this gene and its use in antisense constructs may enable the total level of glucosinolates to be manipulated by transformation, as an alternative to the introgression of alleles at several loci from low-glucosinolate genotypes.

The presence of 4-methylsulphinylbutyl glucosinolate in Stellar was attributed to allelic variation between the parents at a significant *Gsl-alk* locus on LG 20 which had a major effect, and a tentative locus linked to the RFLP markers *tg4d2b* and *wg7b6b* which had a smaller effect. The proposed consequence of Stellar alleles at these loci is to reduce the removal of the terminal H_3CS- radical from the methylsulphinyl side-chain and hence reduce the proportion and absolute levels of 3-butenyl and 2-hydroxy-3-butenyl glucosinolate. This locus was linked to one of the *Gsl-elong* loci mapped in this study which also regulates the side-chain structure of glucosinolates. Parkin et al. (1994) mapped loci which regulate the hydroxylation of alkenyl glucosinolates (which could not be mapped in the present study since both parents have similar levels of hydroxylation, see Fig. 4) to the same linkage groups as those that regulate side-chain elongation. Likewise, Mithen et al. (1995) have reported that the genes in *A. thaliana* which regulate the conversion of methylsulphinyalkyl glucosinolates to alkenyl glucosinolates, and those which convert methylsulphinylpropyl glucosinolate to hydroxypropyl glucosinolate, are closely linked (or are alleles at the same locus) on chromosome 4. It is possible that several loci which regulate different parts of the aliphatic-glucosinolate biosynthetic pathway are linked on the same chromosomes in these species.

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Note added in proof

Uzunova et al. (1995) report the development of a *B. napus* RFLP linkage map and the localization of four QTLs for seed glucosinolate content. It is possible to partially integrate this map with the RFLP map used in the present study as the two maps have RFLP loci in common. The two most important QTLs identified by Uzunova et al. are likely to correspond to the two significant QTLs identified in the present study; *gsl-1* on group 16 (*sensu* Uzonova et al.) is equivalent to *GSL-1* on LG 20 (this study) and *gsl-3* on group 18 is equivalent to *GSL-2* on LG 1.

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