Theor Appl Genet (2003) 106:727–734
DOI 10.1007/s00122-002-1123-x

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Development of isothiocyanate-enriched broccoli, and its enhanced ability to induce phase 2 detoxification enzymes in mammalian cells

Received: 12 April 2002 / Accepted: 27 June 2002 / Published online: 24 October 2002
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Abstract Broccoli florets contain low levels of 3-methylsuphinylpropyl and 4-methylsulphinylbutyl glucosinolates. Following tissue disruption, these glucosinolates are hydrolysed to the corresponding isothiocyanates (ITCs), which have been associated with anticarcinogenic activity through a number of physiological mechanisms including the induction of phase II detoxification enzymes and apoptosis. In this paper, we describe the development of ITC-enriched broccoli through the introgression of three small segments of the genome of *Brassica villosa*, a wild relative of broccoli, each containing a quantitative trait locus (QTL), into a broccoli genetic background, via marker-assisted selection and analysis of glucosinolates in the florets of backcross populations. Epistatic and heterotic effects of these QTLs are described. The ITC-enriched broccoli had 80-times the ability to induce quinone reductase (a standard assay of phase II induction potential) when compared to standard commercial broccoli, due both to an increase in the precursor glucosinolates and a greater conversion of these into ITCs.

Keywords Isothiocyanates · Glucosinolates · Nitriles · Broccoli · Anticarcinogenesis · Phase 2 enzymes · Marker-assisted selection

Communicated by H. Nybom

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Introduction

Epidemiological studies suggest that the consumption of cruciferous vegetables reduces the risk of cancer, particularly of the gastro-intestinal tract (Block et al. 1992; Verhoeven et al. 1996). Physiological experiments have demonstrated that 3-methylsulfinylpropyl (3-MSP) and 4-methylsulfinylbutyl (4-MSB) isothiocyanates (ITCs), derived from the corresponding glucosinolates in broccoli (Fig. 1), are potent inducers of phase II detoxification enzymes in mammalian cell cultures and rodents (Zhang et al. 1992; Faulkner et al. 1998). These enzymes, such as glutathione-S-transferase and quinone reductase, promote the metabolism and excretion of potential carcinogens, and thus provide a mechanism to explain the anticarcinogenic activity of crucifers (Johnson et al. 1994; Musk et al. 1995; Jongen 1996; Maheo et al. 1997; Mithen et al. 2000). 4-MSB ITC has also been shown to induce apoptosis (Chen et al. 1998; Huang et al. 1998).

In comparison to the amount of glucosinolates found in tissues of several wild forms of *Brassica oleracea*, the levels in commercial broccoli (*B. oleracea* var. *italica*) are low. We wish to develop broccoli lines which produce enhanced levels of 3-MSP and 4-MSB ITCs to further explore the role of these compounds in the metabo-

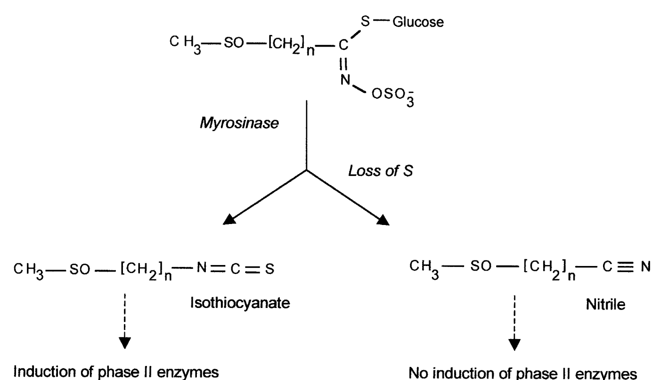

Fig. 1 Hydrolysis of glucosinolates

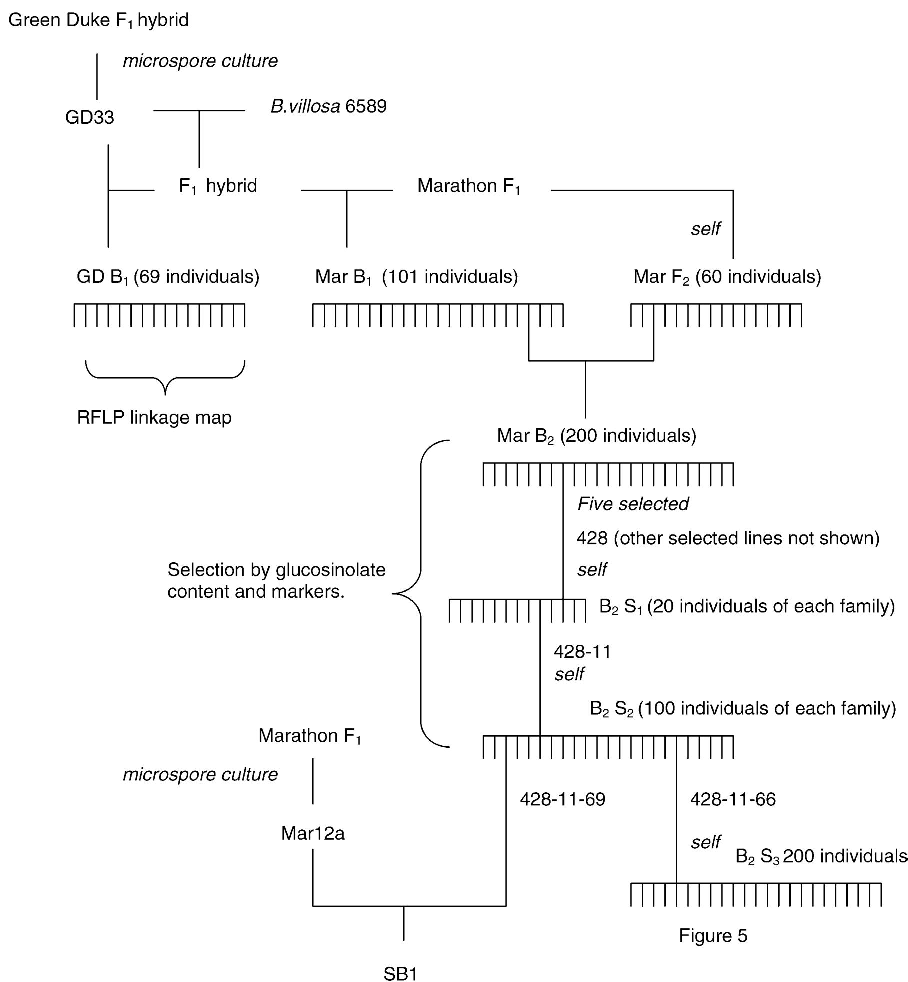

Fig. 2 Summary of the breeding programme to derive the 428-11-69 and 428-11-66 individuals, and the subsequent hybrid (SB1) and B_2S_3 segregating population. 428-11-69 is homozygous for *B. villosa* alleles at each of the three QTLs on LGs 2, 5 and 9, whereas 428-11-66 is homozygous for *B. villosa* alleles at QTL-2 on LG5, but heterozygous for alleles at LG2 and LG9

lism of potential carcinogens. In a previous paper (Faulkner et al. 1998), we reported the glucosinolate content of *Brassica villosa*, a wild species of the *B. oleracea* n = 9 complex, and commercial broccoli cultivars, and demonstrated that F_1 hybrids between broccoli and *B. villosa*, expressed higher levels of 3-MSP and 4-MSB glucosinolate than broccoli. Extracts of these hybrids were potent inducers of quinone reductase (QR) in murine hepa1c1c7 cells, a marker enzyme for the induction of phase II detoxification enzymes in mammalian systems (Prochaska and Santamaria 1988). Moreover, the extent of induction was greater than expected; thus while there was a ten-fold increase in glucosinolate content, there was a 100-fold increase in enzyme induction. It was speculated that this may have arisen from greater conversion of the glucosinolate to ITC.

In this paper, we report the development of advanced backcross lines and experimental F_1 hybrids of broccoli which comprise small segments of the *B. villosa* genome introgressed into a standard broccoli background, and identify three genome segments on linkage groups 2, 5 and 9 which contain *B. villosa* alleles which are responsible for enhancing glucosinolate content. We further show that genotypes which are potent inducers of QR, show greater conversion of glucosinolates to isothiocyanates as opposed to nitriles.

Materials and methods

Parental material for crosses and breeding programme

The glucosinolate content of *B. villosa*, the doubled-haploid breeding line GD33 derived from the commercial F_1 hybrid Green Duke (*B. oleracea* var. *italica*), and the F_1 hybrid between these two lines has previously been described (Faulkner et al. 1998). Briefly, GD33 contains low levels of 4-MSB glucosinolate, whereas, *B. villosa* contains high levels of 3-MSP glucosinolate. The F_1 broccoli cultivar Marathon and the double-haploid line Mar 12a contains low levels of 4-MSB and 3-MSP. The absolute levels of glucosinolates in all material depends upon growth conditions. In general, cultivated broccoli lines have between 3 and 10 μmol g^{-1} dry weight of methylsulphinylalkyl glucosinolates in their florets, whereas wild species have between 50 and 100 μmol g^{-1} of glucosinolates. A precise comparison of wild and cultivated accessions is not possible due to differences in floret morphology. Mithen (2001) provides further details of the range of glucosinolate concentrations found within wild and cultivated *Brassica* species, and environmental factors that influence expression of glucosinolates.

Production of the GD33 × *B. villosa* RFLP map

A single plant of the wild species *B. villosa* acc. 6589 Biv. (referred to as *B. villosa*) was hybridized to the doubled-haploid breeding line GD33, derived from the commercial F_1 hybrid Green Duke (*B. oleracea* var. *italica*). An F_1 individual (GD × *B. villosa*) was backcrossed to the recurrent parent GD33 and seed obtained (GD B_1 population, Fig. 2). Sixty nine B_1 plants were grown in a glasshouse under standard conditions and florets harvested. The florets were immersed immediately in liquid nitrogen and freeze dried. DNA was extracted, followed by restriction enzyme digestion with *Eco*R1, gel electrophoresis, alkaline transfer and Southern hybridization (Sharpe et al. 1995). RFLP loci were identified following hybridization with small *Pst*1 fragments of genomic DNA clones (Teutonico and Osborn 1994) from two *Brassica napus* libraries ('pW' and 'pN' prefix), a *B. oleracea* library ('pO' prefix) and a *Brassica rapa* library ('pR' prefix). Other loci were detected using genomic DNA clones from *Arabidopsis*. Microsatellite loci were detected using primers from the AAFC *Brassica* microsatellite consortium ('s' prefix, personal communication Dr. M. Trick, John Innes Centre), and methods described by Sebastian et al. (2000). Linkage groups 2 and 5 were also constructed for the Mar B_1 and Mar S_1 populations and were comparable to those of GD B_1.

Linkage analysis and map construction was performed using MAPMAKER v 3.0 (Lander et al. 1987). Linkage groups were assembled using a minimum LOD score of 3.0 and recombination frequencies converted to map distances (cM) using the Kosambi mapping function (Kosambi 1944). QTLs were identified using the computer program MAPMAKER/QTL v 1.1 (Lander and Botstein 1989). Linkage groups were named following the classification used previously for *B. oleracea* (Parkin et al. 1995). Single-marker analysis was carried out by the *t*-test (Magrath et al. 1994) to identify RFLP markers, which were putatively linked to quantitative trait loci. Analysis of variance was then used to compare glucosinolate levels at each RFLP locus, and threshold values of significance calculated as described previously (Churchill and Doerge 1994; Ray et al. 1996).

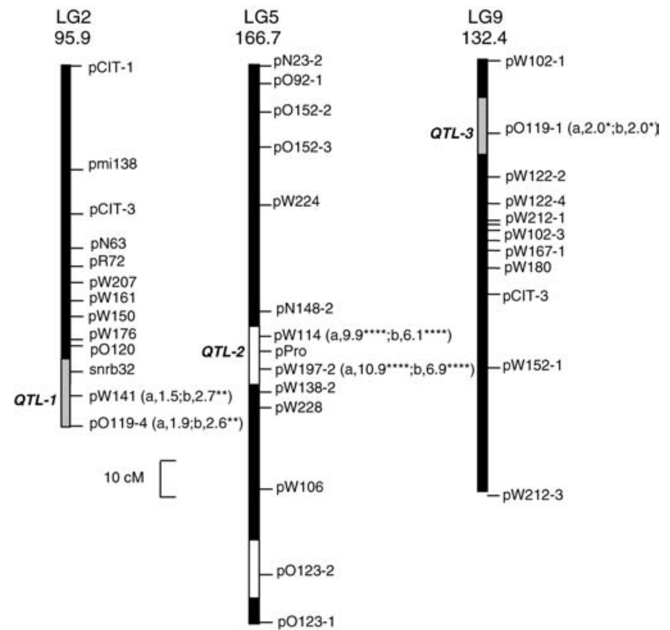

Fig. 3 RFLP maps of LGs 2, 5 and 9 of individual 428-11-66 showing the three introgressed segments of *B. villosa* which contain QTLs 1, 2 and 3. *Black areas* represent regions homozygous for cultivar alleles. *Grey areas* represent heterozygous segments. *White areas* represent segments homozygous for *B. villosa* alleles (note: individual 428-11-69 is homozygous for *B. villosa* alleles at QTLs 1, 2 and 3). Linkage groups 1, 3, 4, 6, 7 and 8 (data not shown) are homozygous for cultivar alleles in both 428-11-66 and 428-11-69. The association between marker genotype for the level of *a* 3-MSP and *b* total aliphatic glucosinolate (calculated using ANOVA) is also indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

Development of advanced backcross lines

The same F_1 individual GD × *B. villosa* used for the RFLP mapping, was crossed to the F_1 hybrid broccoli cultivar Marathon and seed obtained. One hundred and one progeny of this cross, referred to as 'Mar B_1' plants, were grown in a glasshouse under standard conditions. The Marathon F_1 plant was also self-pollinated and 30 Mar F_2 plants grown. One 'Mar B_1' plant and one Mar F_2 plant were selected on the basis of high glucosinolate content and floret morphology, and crossed together to generate Mar B_2 seed. A Mar B_2 population of 200 plants was grown in a field trial (1998) and plants were selected for high glucosinolate content and agronomic characters. Plants were grown in rows of 50, with 0.5 m between plants and 1 m between rows. Plants were initially raised in modules in a glasshouse and planted out into a field plot after 4 weeks growth. Plots were netted to protect against pigeon damage. Of the 200 plants, 60 which expressed high levels of 3-MSP and 4-MSB glucosinolates were genotyped with RFLP markers identified from the GD B_1 RFLP linkage map, which were associated with increased levels of aliphatic glucosinolates, and of these 30 were selfed. The extent of the *B. villosa* genome within these 30 selected Mar B_2 individuals was determined across the nine RFLP linkage groups. Twenty self-progeny (B_2S_1) of five individuals, (including plants 428 and 465, Fig. 2), were grown in a glasshouse and genotyped with RFLP markers. Individuals were selected on the basis of glucosinolate content, floret morphology and heading time, being heterozygous at RFLP loci pW141, pO119-4 (LG2), homozygous for the *B. villosa* allele at pW114 and pW197-2 (LG 5, Fig. 3), and homozygous for Marathon or Green Duke alleles at as many other RFLP loci as possible. These plants were self-pollinated to produce B_2S_2 seed families. One hundred plants of five selected B_2S_2 family (including 428-11) were grown in a second field trial (1999). RFLP analysis was carried out to determine the extent of *B. villosa* segments on linkage groups 2, 5 and 9 of individuals within the B_2S_2 family 428-11 and their effects on glucosinolate content. Ten plants from B_2S_2 family 428-11 that had contrasting RFLP genotypes at pW141, pO119-4 (LG2) and pO119-1 (LG9, Fig. 3), including 428-11-66, 428-11-69 and 428-11-70 (Fig. 2), were self-pollinated to generate B_2S_3 seed. 200 plants of each B_2S_3 line were grown in a further field trial (2000) to confirm the proposed genetic model and the effect of RFLP genotype at loci on LG2, 5 and 9 on glucosinolate content within the introgressed segments determined.

Experimental hybrids (SB1) were produced by crossing plant 428-11-69 [homozygous for *B. villosa* alleles at pW114, pO119-4 (LG2), pW114 and pW197-1 (LG5), and pO119-1 (LG9)] to a doubled-haploid line (Mar 12A) derived from the F_1 hybrid Marathon (provided by Dr. Graham King, Horticultural Research International, Wellesbourne). The experimental hybrids were grown in a field trial, as previously described in 2000, in two plots at different levels of elemental nitrogen. The nitrogen was applied at 250 kg (standard) and 350 kg/nitrogen/hectare (high) in the form of ammonium sulfate as a fertilizer to the seed bed and as a top dressing prior to heading.

Glucosinolate analysis

Glucosinolates were extracted from freeze-dried florets, converted to desulfoglucosinolates and analyzed by HPLC (Magrath et al. 1993). Benzyl glucosinolate (not found in *Brassica*) was used as an internal standard. Analysis was undertaken on florets collected from field and glasshouse trials. Over 2,000 analyses were undertaken.

Analysis of glucosinolate hydrolysis products by LC-MS

Approximately 0.1 g of milled freeze-dried floret tissue was moistened by the addition of deionized water (2 ml), homogenized and left at room temperature for 1 h with occasional mixing. Hot 70% (v/v) methanol (3 ml) was added, mixed thoroughly and incubated for a further 15 min at +70 °C. The mixture was cooled to room temperature and centrifuged for 5 min at 3,000 g. Supernatants were removed and the volume decreased in a vacuum centrifuge to 200 µl volumes to produce an equivalent concentration of 100 mgml^{-1} of extract. LC-MS was used to detect isothiocyanates and nitriles, in a manner similar to that described previously (Rose et al. 2000). An aliquot (20 µl) of hydrolyzed extracts of florets was injected onto a Spherisorb 5 µM ODS2 (4.6 × 250 mm) column. Samples were eluted at a flow rate of 0.750 ml/min with a gradient of 1% acetonitrile: 99% water +0.1% trifluoroacetic acid increasing to 90% acetonitrile over 8 min and then returning to 1% acetonitrile over the subsequent 4 min, with UV absorbance at 235 and 270 nm. Mass spectral data of compounds in the eluent were analyzed via atmospheric pressure electrospray ionization (gas temperature 350 °C, drying gas 10.0 l/min, nebulizer pressure 60 psig, fragmentor voltage 60 V and scanned over the range of 50–200 m/v). The occurrence of glucosinolate degradation products in the mass spectral data was sought by extraction for predicted [M+H$^+$] ions of 3-MSP nitrile, 4-MSB-nitrile, 3-MSP ITC and 4-MSB ITC (132, 146, 164 and 178 respectively). This was confirmed by the analysis of fragmentation patterns while varying the fragmentor voltage, and the ratio of [M+H$^+$]+1 and [M+H$^+$]+2 ions. The mass for 3-MSP and 4-MSB nitriles and ITCs was similar to that obtained previously by GC-MS (Faulkner et al. 1998). The presence of 3-MSP and 4-MSB ITCs were confirmed by the analysis of commercial standards. Subsequently, single-ion monitoring on the molecular ion was used. Through the use of standards, the amount of 3-MSP and 4-MSB ITC was shown to be linearly correlated with the amount of 164 and 178 ions (r^2 = 0.93 and r^2 = 0.95 respectively) enabling the amounts of these ITCs in the plant extracts to be quantified.

Fractionation of hydrolysed extracts

Aliquots (25 µl) of extracts Mar B_2S_2 428-11-69 and of Mar B_2S_2 465-8-94 were injected onto the column and separated with a similar gradient to that described above, except that trifluoroacetic acid was omitted. Fractions were collected for each min over the 12 min separation period. The procedure was repeated eight times producing eight fractions for each 1 min of sample collected. Fractions which contained isothiocyanates (peaks 3 and 4, see Fig. 8) from extract Mar B_2S_2 428-11-69 and fractions which contained nitriles from extract Mar B_2S_2 465-8-94 (peaks 1 and 2, see Fig. 8) were warmed to +40 °C and dried under a stream of air. Once dried, each fraction was resuspended in deionized water (200 µl), pooled, vacuum condensed to produce a final volume of 200 µl (the original starting volume) and then analyzed by LC-MS to confirm the presence of isothiocyanates or nitriles respectively.

Induction of quinone reductase

Hydrolyzed extracts of two lines (Mar B_2S_2 428-11-69 and Mar B_2S_2 465-8-94), which expressed 39.0 µmol/g and 38.1 µmol/g dry weight of methylsulfinylalkyl glucosinolates respectively, and an extract from Marathon, were tested for their ability to induce QR in murine hepa1c1c7 cells, as described previously (Tawfiq et al. 1994; Faulkner et al. 1998). The isothiocyanate fraction of extract 428-11-69 and the nitrile fraction of extract 465-8-94 were also analyzed for their ability to induce QR. Each extract (whole or fractionated) was prepared twice and analyzed on two occasions at four concentrations using four replicates for each concentration.

Results

An RFLP map derived from broccoli × *B. villosa*

A doubled-haploid broccoli line derived from the cultivar Green Duke (GD33) was crossed to *B. villosa* (Faulkner et al. 1998), and the F_1 backcrossed to GD33. Sixty nine B_1 plants were used to construct a framework RFLP linkage map, part of which is shown in Fig. 3. The map comprised 75 loci arranged in nine linkage groups. The linkage groups ranged in size from 57.7 to 166.9 cM and covered a total distance of 976 cM. Comparison with the RFLP genetic linkage map of *B. oleracea* (Bohuon et al. 1996) and to the integrated linkage map of *B. oleracea* (Sebastian et al. 2000), indicated that the total map and the individual linkage groups were of comparable size, and the linkage relationship between the majority of markers was similar. Seven microsatellite loci were added to the map.

Analysis of advanced backcross lines

Preliminary genetic analysis of glucosinolate content in the GD B_1 and also the Mar B_1 progeny identified putative QTLs on linkage groups 2 and 5 which regulate both the amount of total aliphatic glucosinolate content, and the ratio of 3-MSP to 4-MSB. The QTL on LG5 was most apparent in glasshouse-grown plants; *villosa* alleles at RFLP locus pW114 resulted in enhanced levels of 3-MSP (Faulkner 2000). The effect of *villosa* alleles at a QTL on LG2, defined by *villosa* alleles at RFLP loci pO119-4 and pW141 was most apparent in field-grown plants in which there were enhanced levels of both 3-MSP and 4-MSB. To provide further evidence for the role of these putative QTLs, advanced backcross lines were developed in which selected individuals were selfed and backcrossed to an inbred line derived from Marathon. RFLP and glucosinolate analysis of several field-grown plant families confirmed that introgressed segments on LGs 2, 5 and 9 had a significant effect on glucosinolate content, whereas other introgressed segments present in these families had no effect. These segments are designated QTL-1, QTL-2 and QTL-3, respectively (Fig. 3). Family 428-11 contained several individuals which were all homozygous for *villosa* alleles at QTL-2 (i.e. RFLP pW114 and pW197-2) but varied at their genotype at QTL-1 and QTL-3, and selfed progeny from these individuals were used to provide more-detailed analysis of the effect of these introgressed segments.

Glucosinolate and RFLP analysis of selfed individuals (B_2S_3) from 428-11-66, 428-11-69 and 428-11-70

Line 428-11-66 was heterozygous at QTL-1 (i.e. heterozygous at RFLP loci pO119-4 and pW141) and QTL-3 (pO119-1), and homozygous for *villosa* alleles at QTL-2. Fig. 4 shows the effect of the introgressed segments on

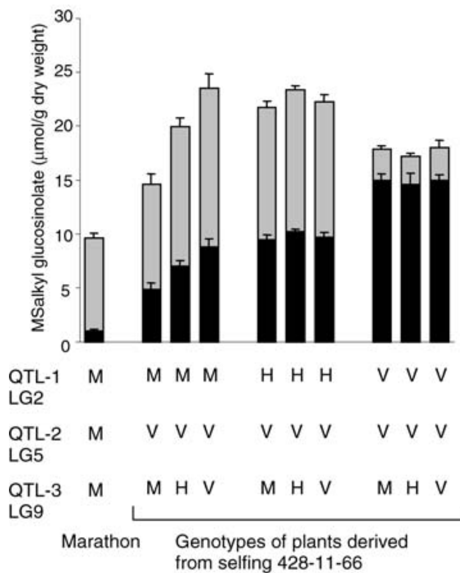

Fig. 4 Effect of genotype at QTLs 1, 2 and 3 on the level of total MSalkyl glucosinolates and the individual levels and ratios of 3-MSP (*shaded in black*) and 4-MSB (*shaded in grey*) in a plant derived from selfing 428-11-66. Results are shown as means ± SE. *M* = homozygous for Marathon alleles, *V* = homozygous for *B. villosa* alleles, *H* = heterozygous

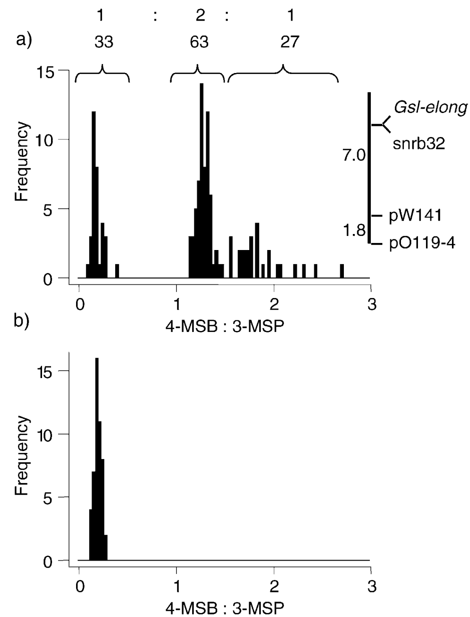

Fig. 5a, b Segregation of relative 4-MSB glucosinolate to 3-MSP glucosinolate in 123 selfed individuals of 428-11-66 (**a**) and 60 selfed individuals of 428-11-69 (**b**). The ratio of the three classes in selfed individuals of 428-11-66 is not significantly different from 1:2:1 (χ^2 = 0.66, 2 *df*, P < 0.05) and indicates a Mendelian elongation gene. The location of the elongation gene '*Gsl-elong*' on LG2 is shown

the levels of 3-MSP and 4-MSB glucosinolate which comprised >95% of the total methonine-derived glucosinolates and on the total levels. In the absence of *villosa* alleles at QTL-1 and QTL-3, *villosa* alleles at QTL-2 result in a significant increase in the levels of 3-MSP glucosinolate (*F* value = 54.7, P < 0.0005), but with no effect on levels of 4-MSB. Introducing *villosa* alleles at QTL-3, whilst maintaining cultivar alleles at QTL-1, resulted in an increase in both 3-MSP and 4-MSB, without altering the ratio between the two glucosinolates; heterozygous plants expressed intermediate levels of both glucosinolates. In contrast, introducing *villosa* alleles at QTL-1, whilst maintaining cultivar alleles at QTL-3, increases glucosinolate content but also increases the ratio of 3-MSP to 4-MSB. In this case, plants which were heterozygous had significantly higher total levels than either of the two homozygous genotypes. However, the ratio of 3-MSP to 4-MSB was greatest in plants which were homozygous for *villosa*. This suggests that the segment on LG2 may contain two distinct genes. One, which varies the ratio of side chain length and the other, which varies the total amount. There was a significant interaction between alleles at QTL-1 and QTL-3; *villosa* alleles at QTL-3 only have an effect if there are no *villosa* alleles at QTL-1. This interaction is also apparent from a two-way analysis of variance (QTL-1: *F* value = 11.3, P < 0.018; QTL-3: *F* value = 4.3, P < 0.0005; QTL-3*QTL-1: *F* value = 3.1, P < 0.020), although this does not reveal the intricacies of the interactions.

Recombination within the introgressed segment on the bottom of LG2 enabled a Mendelian gene, which affects the ratio of 3-MSP to 4-MSB, to be located. The ratio of the two side-chain classes varied in a discrete 1:2:1 ratio (χ^2 = 0.66, 2 *df*, P < 0.05) in 123 selfed plants of 428-11-66 (Fig. 5a), and a gene was positioned 7.0 cM from pW141, and coincident with the microsatellite marker snrb32. It is likely that this elongation gene is homologous to the isopropylmalate synthase-like genes, which have been cloned from *Arabidopsis* (Campos et al. 2000). At the level of resolution possible with the density of markers and the size of population, it was not possible to further define the position of the QTL, but it is likely that there are other genes in this region which effect glucosinolate content. It is of possible significance that QTL-1 and QTL-3 are both linked closely to RFLP alleles identified by probe pO119. This may suggest that this part of LG2 and 9 are duplicates, and the two QTLs represent copies of the same gene or genes.

Individual 428-11-69 contrasted to 428-11-66 as it possessed homozygous *villosa* alleles at each of the three QTLs. Analysis of 60 selfed individuals of 428-11-69 showed no discrete segregation for glucosinolates (Fig. 5b). Likewise, analysis of selfed progeny from 428-11-70, which was also homozygous for *villosa* alleles at each of the three QTLs, gave consistent results with the genetic model.

Expression of glucosinolates in experimental hybrids

Plant 428-11-69 was crossed to plants of a doubled-haploid line derived from Marathon (Mar12A). Five experi-

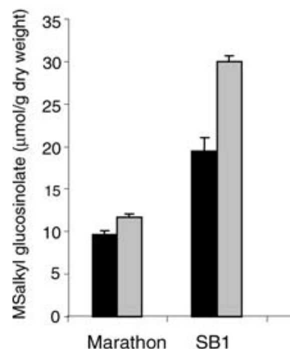

Fig. 6 Expression of MSalkyl glucosinolates in Marathon and the experimental F_1 hybrid Mar12A × 428-11-69 (SB1) grown at two levels of elemental nitrogen. Plants grown at standard levels of nitrogen are shown in *black* and plants grown at high levels in *grey*. Values shown are means ± SE

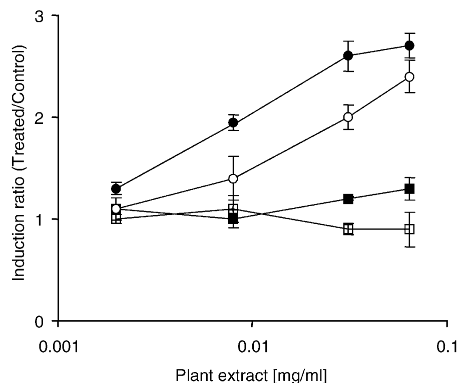

Fig. 7 Effect of whole and fractionated extracts of 428-69-11 and 465-8-94 on the induction of QR. Whole extract of 428-11-69 (●); isothiocyanate fraction from extract of 428-11-69 (○); whole extract of 465-8-94 (■); nitrile fraction from extract of 465-8-94 (□). Values at each concentration are means ± SE from one analysis

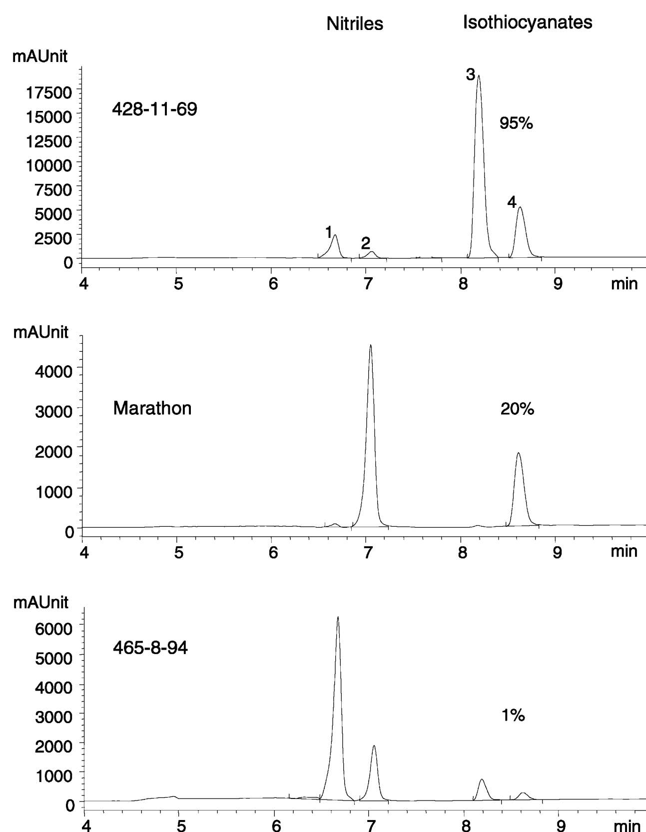

Fig. 8 Analysis of glucosinolate degradation products by LC-MS. *Peak 1* 3-MSP nitrile; *peak 2* 4-MSB nitrile; *peak 3* 3-MSP isothiocyanate; *peak 4* 4-MSB isothiocyanate. The percentage conversion of glucosinolate to isothiocyanate, determined by quantitative LC-MS analysis, is shown

mental hybrid (SB1) and 20 Marathon individuals were grown in two field plots, one having an additional fertilizer top dressing. Experimental F_1 hybrids possessed significantly more aliphatic glucosinolates than the standard cultivar Marathon. Levels were significantly higher in the higher fertility plot (Fig. 6).

Correlation between glucosinolate content and QR induction, and the analysis of glucosinolate degradation products

Two individuals, 428-11-69 and 465-8-94, were selected to explore the relationship between glucosinolates and QR induction. The latter plant was chosen as it contained similar levels of glucosinolates to 428-11-69, but in a different genetic background. The ability of these lines to induce QR was compared to that of Marathon, using standard assays. Extracts from 428-11-69 were approximately 80-times more potent at inducing QR than Marathon. The CD value (amount required to double the activity) of extract 428-11-69 was 0.01 ± 0.01 mg/ml compared to 0.84 ± 0.42 mg/ml for Marathon. Surprisingly, extracts from 465-8-94 showed no induction at all (Fig. 7).

LC-MS analysis of the hydrolyzed extracts used for the QR induction experiments revealed that 3-MSP and 4-MSB nitriles were major products from the hydrolysis of glucosinolates in Marathon and 465-8-94, but were minor constituents in hydrolyzed extracts of 428-11-69. Quantitative analysis suggested that 95% of the glucosinolates in 428-11-69 were hydrolyzed to ITCs, but only 20% in Marathon and 1% in 465-8-94 (Fig. 8). Quantification of nitriles was not possible due to lack of appropriate standards. The QR induction was repeated, using both whole extracts and the ITC containing fraction of 428-11-69 and the nitrile fraction of 465-8-94. This confirmed that the ITC containing fraction was responsible for the induction of QR; the nitrile-containing fraction exhibited no induction (Fig. 7).

Discussion

The introgression of three segments of the *B. villosa* genome into broccoli has been shown to enhance the glucosinolate content of broccoli. A segment of LG5 specifically increases 3-MSP, while segments on LG2 and LG9 increase both 3-MSP and 4-MSB. Epistatic interaction

between the QTLs on LG2 and LG9, and the heterotic effects of the QTL on LG2, has been demonstrated. The nature of the gene or genes within these QTLs is not known. Current comparative genetic studies between *Brassica* and *Arabidopsis* have enabled a series of candidate genes to be identified, which we will report presently. These may enable a genetic modification approach to be adopted to enhancing anticarcinogenic activity, as opposed to breeding strategy.

High levels of glucosinolates were expressed in the experimental hybrid SB1. This clearly demonstrates that the alleles at each of the QTLs only need to be in a heterozygous state. Thus, for the development of commercial F_1 hybrid cultivars, high glucosinolate alleles only need to be introgressed into one of the two parental inbred lines, considerably increasing the ease of the breeding programme.

Enhancing glucosinolates does not necessarily lead to an increased ability to induce QR in mammalian cells. Analysis of degradation products suggest that there are additional genetic factors which result in the conversion of the glucosinolates to either isothiocyanates, which are QR inducers, or to nitriles, which do not induce QR, and are potentially toxic. Preliminary analysis suggests that this trait (i.e. the ratio between isothiocyanates and nitrile) is highly heritable. It is likely that the ratio of ITCs to nitriles is determined by the expression or activity of a protein similar to the epithiospecifier protein (Foo et al. 2000). In *B. napus*, which contains alkenyl glucosinolates, this protein results in the removal of the sulphur from the isothiocyanate group, and its rearrangement across the double bond of the alkenyl side chain, to result in the generation of epithionitriles as opposed to isothiocyanates. In broccoli, in which the glucosinolate side chain lacks a double bond, the sulphur may be lost, to result in nitrile production. In the current study we show that nitriles do not induce QR in murine hepa1c1c7 cells. However, these compounds may have other effects upon gene expression in mammalian cells. These are currently being explored.

In conclusion, we have developed advanced broccoli breeding lines with enhanced ITC production through the introgression of three genomic segments of *B. villosa* into a standard broccoli genetic background. These lines will now enable dietary studies that will further explore the role of ITCs and broccoli in reducing cancer risk.

Acknowledgements The authors thanks BBSRC, The University of Nottingham and Seminis Seeds for funding, and the glasshouse and field staff of the John Innes Centre and the University of Nottingham for assistance with plant cultivation. The seeds of the Mar12a broccoli line were provided by Dr. Graham King (Horticultural Research Institute International). Richard Mithen would also like to express his gratitude to Professor César Gómez-Campos (Crucifer Germplasm Collection, Universida Politécnica, Madrid) for providing seeds of *B. villosa*.

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