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(54) Title: METHOD FOR SELECTIVE INCREASE OF THE ANTICARCINOGENIC GLUCOSINOLATES IN *BRASSICA SP.*

(57) Abstract

The invention relates to a method for producing *Brassica oleracea* with elevated anticarcinogenic glucosinolate derivatives. The elevated levels are obtained by crossing wild *Brassica oleracea* species with *Brassica oleracea* breeding lines, and subsequently selecting hybrids with levels of 4–methylsulfinylbutyl and/or 3–methylsulfinylpropyl glucosinolates elevated above that initially found in *Brassica oleracea* breeding lines. The invention also relates to edible *Brassica* plants, such as broccoli plants, with elevated levels of 4–methylsulfinylbutyl glucosinolate and/or 3–methylsulfinylpropyl glucosinolates, and to seeds of such plants.

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Method for Selective Increase of the Anticarcinogenic Glucosinolates in *Brassica sp.*

FIELD OF INVENTION

The present invention relates to methods for the selective increase of anticarcinogenic glucosinolate derivatives in *Brassica* species, and to *Brassica* species with enhanced levels of anticarcinogenic glucosinolate derivatives and in particular edible *Brassica* vegetables with elevated levels of the anticarcinogenic glucosinolate derivatives 4-methylsulfinylbutyl isothiocyanate and/or 3-methylsulfinylpropyl isothiocyanate. The present invention also provides methods for selection of genetic combinations of broccoli containing high levels of anticarcinogenic glucosinolate derivatives and methods to evaluate the anticarcinogenic properties of these genetic combinations. The invention further relates to compositions of matter comprising *Brassica* vegetables with concentrations of 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolate between 10 and 100 μmoles/g dry weight.

BACKGROUND ART

The present invention provides methods for the production of *Brassica* vegetables with elevated levels of specific glucosinolates and derivatives thereof. In particular the invention provides methods for the production and selection of *Brassica* vegetables with elevated levels of 3-methylsulfinylpropyl and/or 4-methylsulfinylbutyl glucosinolates. These glucosinolates are converted by the activity of the enzyme myrosinase into isothiocyanate derivatives which have been demonstrated to be potent inducers of phase II detoxification enzymes, elevated activity of which is associated with reduced susceptibility to the neoplastic effects of carcinogens. The invention provides genetic combinations which 1.) exhibit elevated levels of 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolate and 2.) exhibit low activity of the GSL-ALK allele which encodes an activity capable of converting these

glucosinolates into the alkenyl derivatives, which do not posses the anti-carcinogenic properties of the isothiocyanate derivatives of these glucosinolates and 3.) suitable myrosinase activity capable of producing isothiocyanate derivatives of said glucosinolates. Accordingly these genetic combinations provide elevated levels of specific glucosinolates, reduced production of alkenyl derivatives of these glucosinolates and favoured production of isothiocyanate derivatives of said glucosinolates. The invention further relates to the use of genetic markers to select the genetic combinations described above.

It is known that a diet high in vegetables is associated with a reduction in the risk of certain types of cancer and hence it is desirable to include a significant amount of vegetables in the human diet. The anticarcinogenic activity of vegetables has been associated with the presence of several classes of secondary metabolites. Evidence is growing that some of these secondary metabolites are involved in lowering the risk of certain types of cancer and hence are considered anticarcinogenic. Accordingly, enhancing the level of anticarcinogenic metabolites provides a useful strategy for the reduction of cancer risk, in complementation with dietary advice to increase the consumption of vegetables.

The precise mechanism by which vegetables provide a decreased risk of many types of cancer is not known with certainty, but there are many lines of evidence which support the involvement of vegetables in the prevention of cancer. In particular, the role of cruciferous vegetables in the prevention of cancer is widely supported through epidemiological studies and more recently biochemical studies. One class of secondary metabolites that is implicated in the beneficial effects of cruciferous vegetables is the isothiocyanate derivatives of certain glucosinolates. Four complementary pieces of evidence suggest that isothiocyanates derived from the hydrolysis of methylsulfinylalkyl glucosinolates found in crucifers may be important in the human diet in reducing the risk of cancer. (1.) Dietary provision of

cruciferous vegetables protects rodents against chemically induced cancer (Wattenberg, L. W. (1985) *Cancer Res.* 45, 1-8.). (2.) Methylsulfinylalkyl isothiocyanates are known to be potent inducers of phase II detoxification enzymes in murine hepatoma Hepa 1c1c7 cells in culture (Zhang, Y., Talalay, P., Cho, C.-G., & Posner, G. H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 2399-2403 and Tawfiq, N., Heaney, R. K., Plumb, J. A., Fenwick, G. R., Musk, S. R. R., & Williamson, G. (1995) *Carcinogenesis* 16, 1191-1194.), which are associated with reduced susceptibility of mammals and mammalian cell cultures to the toxic and neoplastic effects of carcinogens. (3.) Sulforaphane (4-methylsulfinylbutyl isothiocyanate) blocks the formation of mammary tumors in Sprague-Dawley rats treated with 9,10-dimethyl-1,2-benzanthracene (Zhang, Y., Kensler, T. W., Cho, C.-G., Posner, G. H., & Talalay, P. (1994) *Proc. Natl. Acad. Sci. USA* 91, 3147-3150.). (4.) Epidemiological studies show that people with high levels of vegetables in their diet are less susceptible to cancer (Block, G., Patterson, B., & Suber, A. (1992) *Nutr. and Cancer* 18, 1-19.). Thus the beneficial effects of a diet high in certain glucosinolates may include a reduction in the risk of cancer. However, it appears that only certain glucosinolates and more accurately, certain derivatives of specific glucosinolates may be primarily responsible for the beneficial effect.

There are numerous individual glucosinolates in cruciferous plants. Glucosinolates have a common glycone moiety and a variable aglycone side chain. The structure of the glucosinolate side chain varies in length and chemical composition.

Glucosinolates are formed by the action of a number of enzymes, encoded by a small number of glucosinolate biosynthetic alleles (GSL alleles). In the glucosinolate pathway, methionine is converted to homo-methionine and dihomo-methionine by the activity of the GSL-ELONG allele. Homo-methionine is eventually converted to 3-methylthiopropyl glucosinolate followed by conversion to 3-methylsulfinylpropyl glucosinolate by the activity of GSL-OXID allele and finally 2-

propenyl glucosinolate by the activity of GSL-ALK allele. Dihomo-methionine is converted to 4-methylthiobutyl glucosinolate, then to 4 methylsulfinylbutyl glucosinolate by the activity of GSL-OXID allele, then to 3-butenyl glucosinolate by the activity of GSL-ALK allele and finally converted to 2-hydroxy-3-butenyl glucosinolate by the activity of GSL-OH allele.

In general, the 3-methylsulfinylpropyl glucosinolates and 4-methylthiobutyl glucosinolates produce non-volatile isothiocyanates and hence these particular glucosinolates contribute little to flavour. In contrast, the volatile alkenyl derivatives can contribute to flavour, both positively and negatively, dependant on the plant species and particular glucosinolate derivative.

In *B. oleracea* vegetables, glucosinolates have either a three or four carbon side chain. Glucosinolates can be hydrolysed by the action of myrosinase which is often induced upon tissue damage. Many vegetables have alkenyl (2-propenyl and 3-butenyl) glucosinolates which result in the production of volatile products upon hydrolysis through the action of myrosinase. Some vegetables contain a 2-hydroxy-3-butenyl glucosinolate called progoitrin. This glucosinolate produces an unstable isothiocyanate that spontaneously cyclizes to produce oxazolidone-2-thiones, which are undesirable in diets due to their goitrogenic properties. Isothiocyanates derived from alkenyl and hydroxyalkenyl glucosinolates can have both positive and negative effects on flavour.

Broccoli accumulates low levels of glucosinolates with 4-metylsulfinylbutyl and 3-methylsulfinylpropyl side chains since broccoli has a greatly reduced activity of the GSL-ALK allele, responsible for the conversion of glucosinolates into alkenyl derivatives. It is believed that the popularity of broccoli as a vegetable is due in part to the relatively modest contribution to taste made by 4-metylsulfinylbutyl and 3-methylsulfinylpropyl glucosinolate derivatives in contrast to the strong flavour imparted by other glucosinolates, particularly the volatile derivatives of

glucosinolates.

Thus, methods to increase the dietary amount of specific isothiocyanate derivatives of certain glucosinolates may provide vegetables with enhanced anticarcinogenic properties without altering the taste and/or palatability of the vegetable. However, the art does not provide a means to conveniently increase the levels of the specific glucosinolates in cruciferous vegetables. Moreover, the art does not provide a convenient means to assure that these glucosinolates are not converted to the alkenyl derivatives, but rather the isothiocyanate derivatives which have anticarcinogenic properties. Of the numerous glucosinolates that may be produced by *Brassica* vegetables, 4-methylsulfinylbutyl glucosinolate and 3-methylsulfinylpropyl glucosinolate have been identified as being the precursors to the most potent anticarcinogenic isothiocyanate derivatives. The art does not provide a convenient means to increase these specific glucosinolates in a specific fashion while preventing the formation of other glucosinolate or glucosinolate derivatives that may have undesirable flavour characteristics.

4-Methylsulfinylbutyl glucosinolate and 3-methyl-sulfinylpropyl glucosinolate glucosinolates are found in several cruciferous vegetables, but are most abundant in broccoli varieties (syn. calabrese: *Brassica oleracea* L. var. *italica*) which lack a functional allele at the GSL-ALK locus. The presence of a functional GSL-ALK allele converts these glucosinolates to their alkenyl homologues, which are poor inducers of phase II enzymes (Tawfiq, N., Heaney, R. K., Plumb, J. A., Fenwick, G. R., Musk, S. R. R., & Williamson, G. (1995) *Carcinogenesis* 16, 1191-1194.). Therefore the presence of a functional GSL-ALK allele precludes the possibility of producing a variety with high levels of these anticarcinogenic isothiocyanates since the glucosinolates will be converted to alkenyl derivatives. Additionally the production of isothiocyanates from glucosinolates requires the activity of the enzyme myrosinase. Hence enhanced production of these specific isothiocyanates depends on both the levels of

glucosinolate precursors (which are influenced by the activity encoded by the GSL-ALK allele) and the levels or activity of myrosinase which produces the isothiocyanate derivatives of glucosinolates.

Accordingly a genetic combination which specifies the production of high levels of 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolates is desirable, but the production of the anticarcinogenic isothiocyanate derivatives of these glucosinolates requires additional genetic combinations. Thus methods to achieve these genetic compositions provides novel compositions of matter not presently found in commercially grown cruciferous vegetables. The present invention recites methods for achieving these genetic combinations.

The levels of glucosinolates in commercially grown broccoli are relatively low compared to those found in salad crops such as rocket (*Eruca sativa*), which accumulates 4-methylthiobutyl glucosinolate, and watercress (*Rorippa nasturtium-aquaticum*), which accumulates phenethyl glucosinolate (Fenwick, G. R., Heaney, R. K., & Mullin, W. J. (1983) *Crit. Rev. Food Sci. Nutr.* 18, 123-201). Exposure to enhanced levels of 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolate in broccoli would be expected to enhance the potency of induction of phase II enzymes when ingested. Thus broccoli with increased levels of the anticarcinogenic 4-methylsulfinylbutyl isothiocyanate and/or 3-methylsulfinylpropyl isothiocyanate would be a valuable addition to a diet that is designed to lower the risk of cancer. Additionally, such changes would be unlikely to lead to reduced palatability as methylsulfinylalkyl glucosinolates are non-volatile and have a relatively small contribution to flavour, in contrast to the majority of other isothiocyanates found in vegetables and salad crops (Fenwick, G. R., Heaney, R. K., & Mullin, W. J. (1983) *Crit. Rev. Food Sci. Nutr.* 18, 123-201). Thus altering the levels of these specific glucosinolates would not change the taste of the cruciferous vegetables which carry the genetic combinations

encoding the trait.

Many wild members of the *Brassica oleracea* species complex (chromosome number, n = 9) have high levels of individual aliphatic glucosinolates (Mithen, R., Lewis, B. G., & Fenwick, G. R. (1987) *Phytochemistry* 26, 1969-1973. and Giamoustaris, A. & Mithen, R. (1996) *Theor. Appl. Genet.* 93, 1006-1010.). Studies on the genetics of glucosinolates in these taxa has been instrumental in elucidating the genetic pathway for glucosinolate biosynthesis. It is evident that certain species in this taxa could be valuable in *Brassica* breeding programs designed to specifically enhance 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolate and, by so doing, the anticarcinogenic potential of the plant. However, the art does not provide methods to increase the concentration of 4-methylsulfinylbutyl glucosinolate and/or 3-methylsulfinylpropyl glucosinolate through genetic combinations nor does it provide a convenient means by which the anticarcinogenic properties of the vegetables containing said genetic combinations can be assessed. The present invention provides these methods and genetic combinations.

Foremost amongst these are genetic combinations which incorporate the genes from members of the *B. villosa-rupestris* complex from Sicily, which possess a non-functional GSL-ALK allele, and may be the wild progenitors of cultivated broccoli. Thus the present invention utilises wild relatives and progenitors of commercial broccoli as a source of the genes needed to derive a genetic combination capable of producing high levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and the genetic combination that favours the production of isothiocyanate derivatives of these glucosinolates rather than alkenyl derivatives.

4-Methylsulfinylbutyl isothiocyanate (also referred to as sulforaphane), derived from the corresponding glucosinolate found in some *Brassica* species, has previously been identified as a potent inducer of phase II detoxification enzymes (e.g. QR; quinone reductase [NADP(H):quinone-acceptor]

oxidoreductase) in murine hepatoma Hepa 1c1c7 cells. Similarly, 3-methylsulfinylpropyl isothiocyanate is a strong inducer of phase II enzymes. Measurement of the induction of QR in murine hepatoma Hepa 1c1c7 cells provides a rapid and reliable indicator of the ability of vegetable extracts to induce phase II enzymes in mammalian cells (Prochaska, H. J., Santamaria, A. B., & Talalay, P. (1992) *Proc. Natl. Acad. Sci. USA* 89, 2394-2398.), and hence of putative anticarcinogenic activity. This assay has been used to assess the potential of synthetic isothiocyanates (Zhang, Y., Talalay, P., Cho, C.-G., & Posner, G. H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 2399-2403 and Talalay, P., De Long, M. J., & Prochaska, H. J. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8261-8265.), extracts from cruciferous vegetables (Tawfiq, N., Wanigatunga, S., Heaney, R. K., Musk, S. R. R., Williamson, G., & Fenwick, G. R. (1994) *Exp. J. Cancer Prev.* 3, 285-292.) and myrosinase-treated glucosinolates (Tawfiq, N., Heaney, R. K., Plumb, J. A., Fenwick, G. R., Musk, S. R. R., & Williamson, G. (1995) *Carcinogenesis* 16, 1191-1194). However, the glucosinolate/isothiocyanate content of the vegetable extracts has generally not been reported nor has the relative anticarcinogenic potential of various cruciferous vegetables been reported.

In the present invention, this assay has been used to determine the relationship between the ability to induce QR activity (anticarcinogenic potential) and the glucosinolate content of three wild members of the *B. oleracea* complex, which have high levels of 3-methylthiopropyl (*B. drepanensis*) 3-methylsulfinylpropyl (*B. villosa*) and 2-propenyl (*B. atlantica*) glucosinolates respectively, when combined with commercial broccoli cultivars through conventional crosses and hybrids between the wild accessions and a commercial double haploid broccoli breeding lines. Accordingly methods and compositions have been derived which identify the genetic compositions required for the stable production of specific glucosinolates (e.g. of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates) and the production of the corresponding

isothiocyanate derivatives. These genetic combinations provide useful breeding lines for the production of commercial varieties of broccoli and other cruciferous vegetables that have 10 - 100 times the levels of these anticarcinogenic compounds than currently found in commercially grown varieties.

It has been found for example, that a ten-fold increase in the level of 4-methylsulfinylbutyl glucosinolate is obtained by crossing broccoli cultivars with selected wild taxa of the *Brassica oleracea* (chromosome number, n = 9) complex. Similarly increases in levels of 3-methylsulfinylpropyl glucosinolate is observed. Tissue from these hybrids exhibited a 100-fold increase in the ability to induce quinone reductase in Hepa 1c1c7 cells over commercially grown broccoli cultivars due to both an increase in 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and the increased conversion of 4-methylsulfinylbutyl glucosinolate to sulforaphane. Accordingly the invention provides methods and genetic compositions for the production of commercially valuable cruciferous vegetables containing high levels of anticarcinogenic secondary metabolites. The invention further contemplates the development of broccoli breeding lines with enhanced anticarcinogenic activity.

The selection of breeding lines with high levels of anticarcinogenic compounds is further facilitated by the use of molecular markers to establish the chromosomal location of the glucosinolate biosynthetic genes and to assist in selection of backcross lines which contain the genetic composition of greatest utility for the purposes of enhancing the levels of anticarcinogenic compounds in broccoli.

SUMMARY OF THE INVENTION

The present invention provides methods to increase levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates in *Brassica* vegetables by genetic means. These means include crossing of wild *Brassica* species to broccoli species, selection of lines with elevated 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and evaluating the anticarcinogenic properties of said genetic combinations by

measuring the potency of plant cell extracts to induce phase II enzymes. RFLP markers can be used to select lines in crosses which have a high proportion of broccoli genetic background and to establish the position of the relevant glucosinolate biosynthesis genes on the map of the *Brassica* genome.

Hybrids between commercial broccoli cultivars and the two wild species *B. villosa* and *B. drepanensis* are fully fertile and backcross populations are made. Broccoli lines with enhanced levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and associated anticarcinogenic activity are developed from these populations. The efficiency of the development of these lines is considerably enhanced by the availability of molecular markers to select for both glucosinolate content and the desired genetic background.

BRIEF DESCRIPTIONS OF THE DRAWINGS

Fig. 1. Genetic model of aliphatic glucosinolate biosynthesis in *Brassica*.

Fig. 2. Induction of QR activity (phase II enzymes) in murine hepatoma Hepa 1c1c7 cells using extracts from the cultivar Marathon and synthetic sulforaphane (4-methyl-sulfinylbutyl isothiocyanate).

Fig. 3. Effect of extracts of *B. drepanensis* ; *B. villosa* ; and *B. atlantica* on induction of QR (phase II enzymes) in murine hepatoma Hepa 1c1c7 cells.

Fig. 4. The effect of extracts of the cultivar GD DH and the hybrids recovered from crosses with wild species [GD DH x *B. drepanensis*] ; [GD DH x *B. villosa*] ; and [GD DH x *B. atlantica*] ; on the induction of QR (phase II enzymes) in murine hepatoma Hepa 1c1c7 cells.

Fig. 5. Gas chromatograph profile of the glucosinolates from an extract from a hybrid [GD DH x *B. drepanensis*].

DETAILED DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide an edible cruciferous vegetable with high levels of anticarcinogenic compounds, namely 4-methylsulfinylbutyl

isothiocyanate and/or 3-methylsulfinylpropyl isothiocyanate.

It is another object of the present invention to provide methods for the selective increase of anticarcinogenic glucosinolate derivatives in *Brassica* species, and to provide *Brassica* species with enhanced levels of anticarcinogenic glucosinolate derivatives and in particular edible *Brassica* vegetables with elevated levels of the anticarcinogenic glucosinolate derivatives 4-methylsulfinylbutyl isothiocyanate and/or 3-methylsulfinylpropyl isothiocyanate.

It is yet another object of the present invention to provide methods for selection of genetic combinations of broccoli containing high levels of anticarcinogenic glucosinolate derivatives and methods to evaluate the anticarcinogenic properties of these genetic combinations.

It is still another object of the present invention to provide compositions of matter comprising *Brassica* vegetables with concentrations of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates between 10 and 100 μmoles/g dry weight.

These and other objects of the invention are provided by one or more of the embodiments described below.

The selection of broccoli with elevated levels of anticarcinogenic glucosinolates is not possible in the present commercial genetic background used to develop commercial broccoli cultivars. Based on the genetic model of glucosinolate biosynthesis shown in Figure 1, the production of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates is dependant on a number of genetic factors. These include low activity of the GSL-ALK allele and proper levels of activity encoded by the GSL-OXID alleles and other alleles responsible for the production of glucosinolate precursors. It is believed that the relatively mild taste of broccoli is associated with relatively low levels of glucosinolates in general and low levels of volatile glucosinolates in particular. This is evident when the total glucosinolate profiles of commercial broccoli and wild relatives are compared.

Thus methods to derive broccoli with desirable flavour characteristics and elevated levels of anticarcinogenic glucosinolates must encompass selection of lines with the proper genetic combination which do not produce strong flavoured alkenyl glucosinolates. It is also desirable to maintain low levels of other glucosinolates in order to avoid off-flavour production. Thus methods to achieve the specific elevation of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates must not lead to an overall production of glucosinolates or production of alkeynl glucosinolates. The present invention provides methods to accomplish these goals.

Under normal field conditions, it is believed that wild type *B. oleracea* would not likely cross-fertilize with cultivated *B. oleracea*. It is thought that compatibility traits are turned off when the cultivars flowers.

The present invention also contemplates the use of genetic markers to facilitate the selection of lines which contain the desired genetic combination. The use of RFLP markers, or DNA probes which segregate with specific traits is well known in the art, however the present invention describes specific DNA probes that have been shown to be useful for the selection of genetic combinations that lead to elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates in *Brassica oleracea*. Furthermore, RFLP analysis provides a useful means to assess the portion of the genome of a hybrid plant which is derived from broccoli or wild species. Thus the use of RFLP or DNA probes finds utility in the rapid selection of plants which contain the desired proportion of wild species and broccoli genomes. Thus selection of broccoli with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates comprising a high percentage of the desired commercial genome is greatly facilitated by the use of DNA markers to analyse hybrid plants following crossing of broccoli with wild species.

The present invention also describes methods for the assessment of the anticarcinogenic properties of broccoli containing elevated levels of 4-methylsulfinylbutyl and/or 3-

methylsulfinylpropyl glucosinolates through assays for the induction of phase II enzymes. Although the long-term anticarcinogenic effects of specific derivatives of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates can not be precisely determined and will be further dependant on many additional factors, dietary and otherwise, the use of the induction assay provides compelling evidence for the anticarcinogenic effects of the isothiocyanate derivatives of the specific glucosinolates.

Thus the present invention describes methods that permit the selection of *Brassica sp.* with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, methods to evaluate the anticarcinogenic effects of said plant species, and methods and compositions that permit the derivation of broccoli lines with anti-carcinogenic properties.

In one embodiment of the present invention, a method is used to select broccoli lines with elevated levels of specific glucosinolates which comprises:

I.) Crossing wild species with broccoli double haploid breeding lines;

II.) Analyzing F1 hybrids, selecting the hybrids with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinyl-propyl glucosinolates and backcrossing to broccoli breeding lines;

III.) Analysis of glucosinolates in individual plants of the B1 (Backcross 1) generation;

IV.) One or two further rounds of backcrossing (B2, B3) with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

V.) Analysis of B3 (Backcross 3) population with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

VI.) Selection of a broccoli line with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates which carries the anticarcinogenic trait capable of causing a strong induction of phase II enzymes.

Thus the method allows for the selection of broccoli lines with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates. The elevated levels of these specific glucosinolates is correlated with the anticarcinogenic property by evaluation of the induction of phase II enzymes. By employing backcrossing, genetic combinations are derived which comprise the anticarcinogenic trait in a genetic background found in commercial broccoli. Accordingly the production of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates in commercial cultivars of broccoli is accomplished, giving rise to a new and valuable broccoli composition.

In another embodiment of the present invention, the anticarcinogenic capability of the lines is further combined with specific alleles for self incompatibility which is useful in seed production strategies. It is known that certain cruciferous species carry various alleles for self-incompatibility and these alleles are often employed for hybrid seed production. Thus hybrid broccoli may be produced which carries the genetic combination that produces elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates. It is another object of the present invention to select broccoli lines with high levels of 4-methylsulfinyl-butyl and/or 3-methylsulfinylpropyl glucosinolates and a specific combination of self-incompatibility (SI) alleles. In some instances, it may be possible to use a molecular probe for identification of SI alleles, such as the probe pW150 (available from Dr. Tom Osborne, Department of Agronomy, University of Wisconson, Madison, WI, 53706 and described in Toroser et al., Theoretical and Applied Genetics, 91:802-808.1995), or analysis of the actual SI protein can provide selection of the desired SI allele.

Thus in another embodiment of the present invention, a method is used to select broccoli lines with high levels of specific glucosinolates and SI alleles which comprises:

I.) Crossing wild species with broccoli double haploid breeding lines containing specific SI alleles;

II.) Analyzing F1 hybrids, selecting the hybrids with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and backcrossing to broccoli breeding lines, screening for SI alleles with RFLP markers, selection of individuals with desired combination of contrasting SI alleles;

III.) Analysis of glucosinolates in individual plants of the B1 (Backcross 1) generation;

IV.) One or two further rounds of backcrossing with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, screening for proper SI alleles with RFLP markers, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

V.) Analysis of B3 (Backcross 3) population with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

VI.) Selection of a broccoli line with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and proper SI alleles which carries the anticarcinogenic trait capable of causing a strong induction of phase II enzymes.

Thus broccoli lines are derived which carry specific SI alleles useful for crossing in a hybrid seed production scheme. Accordingly hybrid broccoli seed that carries the genetic combination for elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates can be produced by crossing with the appropriate parent. Said seed is valuable since hybrid broccoli also carries many genetic combinations important for agronomic performance.

As another embodiment of the present invention, a method is described which employs the use of DNA markers that segregate with specific glucosinolate profiles. In this method, the use of DNA markers, or more specifically markers known as QTLs (quantitative trait loci) are used to select the genetic combination in broccoli that leads to elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates. In particular the use of the markers known as pW176, pW207 and pW141 located on chromosome 2 and the markers known as pW224, pW114, pW145, pW123, pW138, pW197, pW228, pW106 located on chromosome 5 is described (available from Dr. Tom Osborne, Department of Agronomy, University of Wisconson, Madison, WI, 53706 and described in Toroser et al., Theoretical and Applied Genetics, 91:802-808, 1995 and described in Ferreira et al., Theoretical and Applied Genetics 89:615-621, 1994.)

It has been found that two regions of the genome (found on chromosome 2 and 5) of wild *Brassica oleracea* are required for the expression of elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates. The use of the markers greatly facilitates the selection of lines from hybrids and backcrosses between broccoli and wild species which contain the genetic combination responsible for the production of elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates. Furthermore, it has been found that the QTL located on chromosome 5 specifically regulates the levels of 3-methysulfinylpropyl glucosinolate and has little effect on the levels of 4-methylsulfinylbutyl glucosinolate. Thus it is possible to manipulate the levels of 3-methysulfinylpropyl glucosinolate and 4-methylsulfinylbutyl glucosinolate independently by the use of molecular probes in addition to simple selection of lines.

Accordingly in this embodiment of the present invention a method to produce broccoli with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates is described which comprises:

I.) Crossing wild species with broccoli double haploid breeding lines;

II.) Analyzing F1 hybrids and selecting the hybrids with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates by screening with RFLP probes associated with the production of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and backcrossing selected lines to broccoli breeding lines;

III.) Analysis of glucosinolates in individual plants of the B1 (Backcross 1) generation;

IV.) One or two further rounds of backcrossing with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates by screening with RFLP probes associated with the production of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

V.) Analysis of B3 (Backcross 3) population with selection of plants with the highest level of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates by screening with RFLP probes associated with the production of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates and analysis of glucosinolate profiles, anticarcinogenesis screening of selected individuals by induction of phase II enzymes;

VI.) Selection of a broccoli line with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates which carries the anticarcinogenic trait capable of causing a strong induction of phase II enzymes.

Thus the use of RFLP probes for identifying specific regions of the wild *Brassica oleracea* genome (e.g. so-called QTLs) responsible for the production of elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates greatly facilitates the production of edible broccoli with anticarcinogenic properties.

The foregoing embodiments allow for the selection of

broccoli lines with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates, preferably broccoli with a composition comprising concentrations of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates between 10 and 100 μmoles/g dry weight. Furthermore, the foregoing embodiments allow for the selection of broccoli lines with elevated levels of 4-methylsulfinylbutyl and/or 3-methylsulfinylpropyl glucosinolates capable of causing a 10 - 100 fold increase in the induction of phase II enzymes when compared to broccoli cultivars commonly found in commerce.

The skilled artisan would contemplate that the methods described herein may also be applied to obtain cruciferous *B. oleracea* vegetables other than broccoli, including cabbage such as white cabbage, green cabbage such as Savoy, cauliflower, Brussels sprouts, kale, kohlrabi and the like. It is further contemplated that rutabaga (*B. napus*) and turnip (*B. rapa*) may also be manipulated according to the method and genetic combinations of the present invention.

The following examples illustrate the method but in no way limit the scope of the invention.

Example 1. Methods for the measurement of glucosinolate content in wild and commercial *Brassica* species and hybrids thereof.

A double haploid broccoli breeding line derived from the cultivar Green Duke (referred to as GD DH, Bouhuon, E. J. R., Keith, D. J., Parkin, I. A. P., Sharpe, A. G., & Lydiate, D. J. (1996) *Theor. Appl. Genet.* 93, 833-839.), three commercial cultivars (Trixie, Green Comet and Marathon) and three wild *Brassica* species: *B. drepanensis* Caruel (syn. *B. villosa* Biv. subsp *drepanensis*), *B. villosa* Biv. and *B. atlantica* (Coss.) O. E. Schultz, were grown in a glasshouse under standard conditions as previously described (Magrath, R., Herron, C., Giamoustaris, A., & Mithen, R. (1993) *Plant Breed.* 111, 55-72). Each of the wild species was crossed to the GD DH breeding line, F_1 seeds were obtained and grown under standard conditions. A deposit of seeds of the cultivar derivative GD DH was made on February 11, 1999 with the

National Collection of Industrial and Marine Bacteria Limited (NCIMB) in Aberdeen, Scotland, and was assigned the deposit No. NCIMB 41008. Inflorescences were harvested from the cultivars after 8-12 weeks and from the hybrids after 12-16 weeks, immediately frozen in liquid nitrogen and freeze-dried. Synthetic sulforaphane was kindly supplied by Professor P. Talalay, The John Hopkins University School of Medicine, Baltimore, MD.

Glucosinolates were extracted from the freeze-dried material, converted to desulfoglucosinolates and analysed by HPLC as previously described (Magrath, R., Herron, C., Giamoustaris, A., & Mithen, R. (1993) *Plant Breed.* 111, 55-72) using benzyl glucosinolate as an internal standard.

Extracts from the freeze-dried material were assessed for their induction activity in murine hepatoma Hepa 1c1c7 cells. Approximately 0.1 g of milled freeze-dried material was moistened by addition of water (2 ml), homogenised and left at room temperature for 1 hr with occasional mixing. Hot 70% (v/v) methanol (3 ml) was added and mixed thoroughly prior to incubation for 15 min at +70°C. The homogenates were cooled to room temperature and centrifuged for 5 min at 3000 r.p.m. Supernatants were removed and the volume decreased in a vacuum centrifuge to approximately one fifth of the initial volume. The resulting concentrates were filtered through sterile non-pyrogenic filters (0.22 mm) and stored at -70°C prior to testing. The concentrations of each extract were expressed as dry weight of original material to each ml of culture medium.

Induction was measured according to published methods (Tawfiq, N., Heaney, R. K., Plumb, J. A., Fenwick, G. R., Musk, S. R. R., & Williamson, G. (1995) *Carcinogenesis* 16, 1191-1194., Prochaska, H. J., & Santamaria, A. B. (1988) *Anal. Biochem.* 169, 328-336., Williamson, G., Plumb, G. W., Uda, Y., Price, K. R., & Rhodes, M. J. C. (1996) *Carcinogenesis* 17, 2385-2387.) with the following modifications. Each sample was analysed at eight concentrations using four replicates for each concentration. b-Napthoflavone was used as a positive control at a concentration of 0.2 mM. This typically produced a three-

fold induction (CD; 0.02 mM) and was comparable to previous determinations. Each cultivar/hybrid was extracted on three occasions and analysed separately.

Non-volatile and volatile components of the hydrolytic breakdown products of the cultivars, wild species and the hybrids were analysed by GC-MS using a HP Chemstation GP800A equipped with a 30m x 0.25mm HP1 crosslinked methylsilane column (Hewlett Packard Co. Palo Alto, CA. USA). Typically, the column was heated from +60°C to +250°C at 20°C/min and mass spectra scanned from 35 to 250 m/z. Approximately 0.1 g of freeze-dried material was moistened with water, mixed thoroughly and incubated for 1 hr with occasional mixing. Non-volatile hydrolytic products were extracted from the samples with methylene chloride and filtered prior to analysis. In order to analyse volatile products, freeze-dried material (0.1 g) was moistened with water (0.5 ml), the glass vial sealed immediately and volatile products collected from the vial headspace with a solid phase matrix extraction (SPME) probe (Supelco Inc., Bellefonte, PA, USA).

Example 2. Glucosinolate content of the *Brassica* lines.

Table 1 below shows the glucosinolate content of commercial broccoli cultivars Green Comet, Marathon and Trixie, wild *B.oleracea* species and hybrids between a doubled haploid line of the commercial cultivar Green Duke and wild *Brassica* species.

TABLE 1

Individual and total aliphatic glucosinolate content (μmoles/g dry weight ± 1 standard error) of broccoli cultivars, wild *Brassica* species and hybrids produced from crosses between GD DH and the wild *Brasssica* species.

	MSP*	MSB	PROP	BUT	MTP	OH-BUT	TOTAL
Green Comet	0.1±0.1	0.8±0.5	0.2±0.1	0.0	2.7±0.5	0.5±0.3	4.3±0.5
GD DH	0.2±0.2	4.6±1.1	0.0	0.0	2.3±0.6	0.0	7.1±1.1
Marathon	1.0±0.3	5.4±1.1	0.2±0.1	0.0	4.1±0.7	0.0	10.7±1.8
Trixie	0.4±0.2	11.1±2.1	0.2±0.1	0.0	4.9±1.2	0.0	16.6±2.6
B. atlantica	0.9±0.7	0.0	92.8±25.4	0.5±0.3	1.1±1.0	0.0	95.3±26.6
B. drepanensis	11.0±1.7	0.0	0.0	0.0	51.6±9.3	0.0	62.6±10.9
B. villosa	119±18	1.4±0.2	0.0	0.1±0.1	3.4±0.9	0.1±0.1	124±19
GD† *x B.atlantica*	2.2±0.8	5.3±1.4	76.9±20.8	23.6±6.3	2.0±0.7	43.7±6.7	154±30
GD x B. drepanensis	26.2±2.9	76.5±8.9	0.0	0.0	1.9±0.4	0.0	105±12
GD x B. villosa	26.4±2.7	81.8±5.0	0.0	0.0	1.0±0.3	0.0	109±7

*MSP:3-methylsulfinylpropyl, MSB: 4-methylsufinylbutyl, PROP: 2-propenyl, BUT: 3-butenyl, MTP: 3-methylthiopropyl, OH-BUT: 2-hydroxy-3-butenyl. † GD:GD DH.

The level of 4-methylsulfinylbutyl glucosinolate in the broccoli cultivars were similar to those previously reported (Carlson, D. G., Daxenbichler, M. E., van Etten, C. H., Kwolek, W. F., & Williams, P. H. (1987) *J. Amer. Soc. Hort. Sci.* 112, 173-178.). Wild species had approximately a ten-fold greater level of total aliphatic glucosinolates than the cultivars. *B. villosa, B. drepanensis* and *B. atlantica* had predominantly 3-methylsulfinylpropyl, 3-methylthiopropyl and 2-propenyl glucosinolates. The differences in the glucosinolate profiles were attributed to differences in alleles at the GSL-OXID and GSL-ALK loci (Giamoustaris, A. & Mithen, R. (1996) *Theor. Appl. Genet.* 93, 1006-1010.). In the hybrids of [GD DH x *B. drepanensis*] and [GD DH x *B. villosa*] 4-methylsulfinylbutyl was the most abundant glucosinolate due to the dominant nature of the GSL-ELONG and GSL-OXID alleles found in GD DH and to the null GSL-ALK alleles in both parents. In the [GD DH x *B. atlantica*] hybrid, 3-butenyl glucosinolate was the predominant glucosinolate. 2-Hydroxy-3-butenyl glucosinolate was also present due to the action of a functional GSL-OH allele in the GD DH line (unpublished), which is not usually evident due to the null GSL-ALK allele preventing the biosynthesis of 3-butenyl glucosinolate.

Example 3. Induction of phase II enzymes.

Synthetic sulforaphane was used as a positive control to quantify induction of QR in Hepa 1c1c7 cells. It was a potent inducer and produced a three-fold induction at 1.6 mM (CD; 0.4 mM), comparable to previous determinations. No cytotoxicity was observed for any sample at any of the concentrations tested. Extracts from all the cultivars were poor inducers over the concentration range 0.001 mg/ml to 0.125 mg/ml. However, the induction was less than expected if 100% of the glucosinolates had been converted to isothiocyanates, and not to other products such as thiocyanate or nitrile derivatives (Fenwick, G. R., Heaney, R. K., & Mullin, W. J. (1983) *Crit. Rev. Food Sci. Nutr.* 18, 123-201).

For example, Marathon contained 5.4 mmoles of 4-methylsulfinylbutyl glucosinolate/g dry weight (Table 1).

Therefore, an extract containing 75 mg/ml of Marathon would be expected to have a 4-methylsulfinylbutyl isothiocyanate concentration of 0.4 mM which would result in a two-fold induction. However, no significant induction was observed at this concentration. Indeed, 2.5 mg/ml of Marathon extract was required for a two-fold induction, which if 100% of the glucosinolate is converted to isothiocyanate, is equivalent to 13.5 mM 4-methylsulfinylbutyl isothiocyanate. Thus, either a small proportion of the glucosinolate had been converted to 4-methylsulfinylbutyl isothiocyanate, or the induction of QR activity was reduced by other components in the vegetable extract.

To test for the presence of an inhibitor, Marathon extract (0.125 mg/ml) was spiked with synthetic sulforaphane prior to application to the Hepa 1c1c7 cells. The induction observed was similar to that of pure sulforaphane alone (Fig. 2), which demonstrated that there had been no inhibitory effect by other components in the extracts. In Figure 2, the induction of QR in Hepa 1c1c7 cells using Sulforaphane (▵); Marathon extract (0.125 mg/ml) with added sulforaphane (■) or Marathon extract (0.001 mg/ml to 0.125 mg/ml), (○), is shown. The estimated isothiocyanate concentration of the Marathon extract has been based on an assumption that 100% of the parent glucosinolate is converted to the isothiocyanate. Thus, Marathon extract (1 mg/ml) is equivalent to 5.4 uM 4-methylsulfinylbutyl isothiocyanate. Results for Marathon (0.125 mg/ml) with added sulforaphane have been plotted with respect to the added synthetic sulforaphane concentration only, as the Marathon extract alone has no significant effect on induction activity. Therefore, it is possible in Marathon (and also other cultivars) that only a proportion of the glucosinolate had been converted to the isothiocyanate.

Extracts of *B. villosa* and *B. drepanensis* were potent inducers of QR activity. In contrast, extracts of *B. atlantica* did not induce QR activity, despite the high glucosinolate content (Fig. 3). In figure 3, the effects of the extracts of: *B. drepanensis* (■); *B. villosa* (◆); and *B. atlantica* (□)

on the induction of QR in murine hepatoma Hepa 1c1c7 cells is shown. If it is assumed that 100% of the glucosinolate in these taxa had been converted to the isothiocyanate and not to other possible hydrolytic breakdown products (thiocyanate and nitrile derivatives), the apparent CD values for 3-methylthiopropyl isothiocyanate and 3-methylsulfinylpropyl isothiocyanate are 1.6 mM and 0.5 mM respectively. These values are both lower than those reported for synthetic isothiocyanates of 3.5 mM and 2.4 mM, as illustrated in Table 2. Indeed, if less of the glucosinolates had been converted to isothiocyanates the apparent CD values would be even lower. Table 2 below illustrates the induction of QR activity (phase II enzymes) in Hepa 1c1c7 cells by vegetable extracts.

TABLE 2

Potency of induction of QR in murine Hepa 1c1c7 cells by vegetable extracts.

	Predominant Isothiocyanate	Apparent CD value* (μM)	CD value for synthetic isothiocyanate (μM) (See Zhang (2))
B. drepanensis	3-methylthiopropyl	1.6†	3.5
B. villosa	3-methylsulfinylpropyl	0.5†	2.4
GD‡ x *B. drepanensis*	4-methylsulfinylbutyl	0.3†	0.4-0.8
GD x *B. villosa*	4-methylsulfinylbutyl	0.3†	0.4-0.8
Synthetic sulforaphane	4-methylsulfinylbutyl	0.3	0.4-0.8§

*CD value: Concentration of parent glucosinolate required to double the induction activity of QR. †Values quoted were calculated assuming 100% conversion of the parent glucosinolates to the corresponding isothiocyanate. ‡GD: GD DH. §Other studies have quoted a CD value of 0.2 μM. See Prochaska (10).

The difference in potency could be due to either chemical differences between natural and synthetic isothiocyanates (e.g. nature of stereoisomers) or to other factors in the plant extracts which may include synergistic effects of low levels of other isothiocyanates. The lack of activity of extracts from *B. atlantica* emphasizes the importance of the structure of the glucosinolate side chain.

Extracts from both [GD DH x *B. villosa*] and [GD DH x *B. drepanensis*] were potent inducers of QR activity (Fig. 4). In Figure 4, the effect of extracts of the cultivar GD DH (●); and the hybrid crosses [GD DH x *B. drepanensis*] (■); [GD DH x *B. villosa*] (◇); and [GD DH x *B. atlantica*] (△); on the induction of QR in murine hepatoma Hepa 1c1c7 cells is shown. Based on 100% conversion of the glucosinolates to isothiocyanates, the apparent CD values of both extracts were 0.3 mM (4-methylsulfinylbutyl glucosinolate equivalent), which is similar to that of pure sulforaphane (above) and to that reported in previous studies. Thus, while there was an approximate ten-fold increase in the levels of 4-methylsulfinylbutyl glucosinolate in [GD DH x *B. villosa*] and [GD DH x *B. drepanensis*] compared to Marathon and GD DH, there was more than a 100-fold difference in ability to induce QR activity (i.e. it requires at least one hundred times the amount of Marathon tissue to induce equivalent QR activity compared to the GD DH hybrids).

In order to examine the composition and nature of the hydrolytic products, extracts were analysed by GC-MS (Fig. 5). In Figure 5, gas chromatograph profile of an extract from [GD DH x *B. Drepanensis*] is shown. Mass spectrometry confirmed the peaks as (1), 3-methylsulfinylpropyl nitrile; (2), 4-methylsulfinylbutyl nitrile; (3), 3-methylsulfinylpropyl isothiocyanate; (4), 4-methylsulfinylbutyl isothiocyanate. Hydration of freeze-dried leaves of *B. drepanensis* and *B. atlantica* led to the production of large amounts of volatile 3-methylthiopropyl and 2-propenyl isothiocyanates. 3-Methyl-sulfinylpropyl isothiocyanate was detected in methylene chloride extracts from *B. villosa*, which was consistent with

the glucosinolate profiles. In extracts of [GD DH x *B. villosa*] and [GD DH x *B. drepanensis*], the dominant isothiocyanate was 4-methylsulfinylbutyl isothiocyanate as expected. Relatively low levels of 3-methylsulfinylpropyl isothiocyanate and nitrile derivatives were also detected. In contrast, only trace amounts of 4-methylsulfinylbutyl isothiocyanate were detected in the cultivars. This indicated that the 100-fold difference in ability to induce QR activity between the two hybrids and the cultivars is due to both the increase in 4-methylsulfinylbutyl glucosinolate and to a greater conversion to the isothiocyanate.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 18 & 19, line 36-37 & 1-3

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
AB2 1RY
United Kingdom

Date of deposit	Accession Number
11 February 1999	NCIMB 41008

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS:

1. A method for the production of *Brassica oleracea* with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, which comprises:

(a) crossing wild *Brassica oleracea* species with *Brassica oleracea* breeding lines; and

(b) selecting hybrids with levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, elevated above that initially found in *Brassica oleracea* breeding lines.

2. A method for the production of *Brassica oleracea* with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, which comprises:

(a) crossing wild *Brassica oleracea* species with broccoli breeding lines;

(b) selecting hybrids with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both;

(c) backcrossing to broccoli breeding lines; and

(d) selecting plants with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both.

3. The method according to claim 2 which additionally comprises:

(e) selecting a broccoli line with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, capable of causing a strong induction of phase II enzymes.

4. A method for the production of *Brassica oleracea* with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, and specific SI alleles which comprises:

(a) crossing wild species with broccoli double haploid

breeding lines containing specific SI alleles;

(b) selecting hybrids with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both; and

(c) screening for the specific SI alleles with RFLP markers.

5. The method according to claim 4 which additionally comprises:

(b1) backcrossing and selecting plants with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both; and

(d) selecting a broccoli line with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, and proper SI alleles which is capable of causing a strong induction of phase II enzymes.

6. A method for the production of *Brassica oleracea* with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, which comprises:

(a) crossing wild species with broccoli double haploid breeding lines;

(b) using DNA probes to select hybrids with a genetic combination encoding expression of elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both;

(c) backcrossing and selecting plants with the genetic combination encoding the expression of elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both; and

(d) selecting a broccoli line with elevated levels of 4-methylsulfinylbutyl glucosinolates, or 3-methylsulfinylpropyl glucosinolates, or both, capable of causing a strong induction of phase II enzymes.

7. The method according to any one of claims 1 to 6, wherein only 4-methylsulfinylbutyl glucosinolate is elevated.

8. The method according to any one of claims 1 to 6, wherein only 3-methylsulfinylpropyl glucosinolate is elevated.

9. An edible *Brassica* plant produced according to the method of any one of claims 1 to 6.

10. An edible portion of a broccoli plant produced according to the method of any one of claims 1 to 6.

11. Seed of a broccoli plant produced according to the method of any one of claims 1 to 6.

12. The method according to claim 6 where the DNA probes used are selected from the group comprising: pW176, pW141, pW207, pW224, pW114, pW145, pW123, pW138, pW197, pW228 and pW106.

13. A broccoli plant having elevated levels of 3-methylsulfinylpropyl glucosinolates, or 4-methylsulfinylbutyl glucosinolates, or both.

14. A broccoli plant according to claim 13, wherein the concentration of 3-methylsulfinylpropyl glucosinolates, or 4-methylsulfinylbutyl glucosinolates, or both, is between 10 and 100 μmoles per gram of dry weight.

15. A broccoli inflorescence having elevated levels of 3-methylsulfinylpropyl glucosinolates, or 4-methylsulfinylbutyl glucosinolates, or both.

16. Broccoli inflorescence according to claim 15, wherein the concentration of 3-methylsulfinylpropyl glucosinolates, or 4-methylsulfinylbutyl glucosinolates, or both, is between 10 and 100 μmoles per gram of dry weight.

17. A *Brassica* plant cell having elevated levels of 3-methylsulfinylpropyl glucosinolates, or 4-methylsulfinylbutyl glucosinolates, or both.

18. A plant cell according to claim 17, wherein the cell is an inflorescence cell.

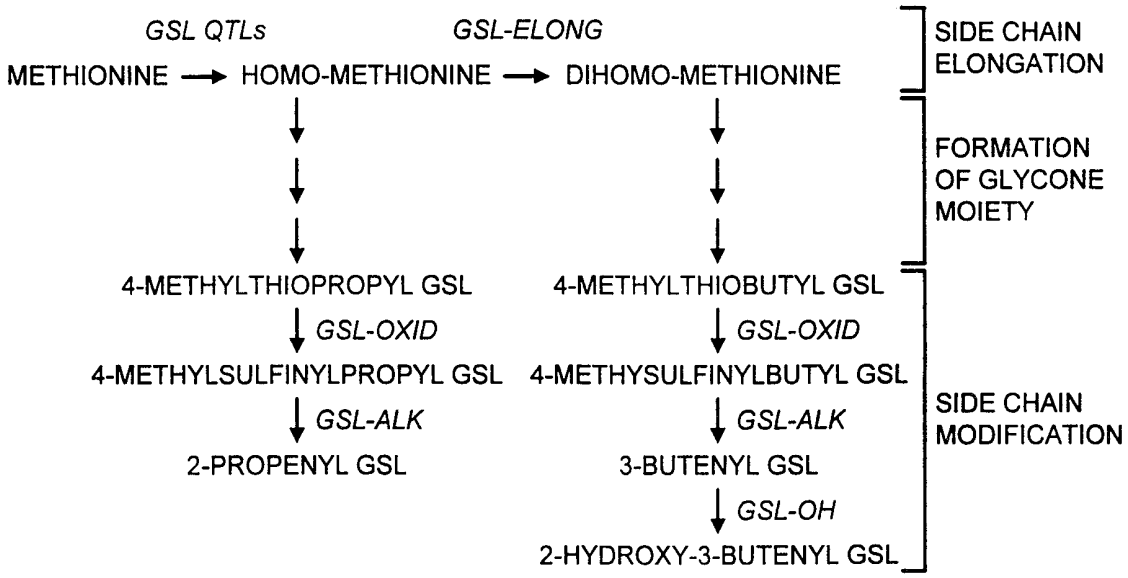
GSL QTLs
GSL-ELONG
METHIONINE
HOMO-METHIONINE
DIHOMO-METHIONINE
SIDE CHAIN ELONGATION
FORMATION OF GLYCONE MOIETY
4-METHYLTHIOPROPYL GSL
4-METHYLTHIOBUTYL GSL
GSL-OXID
GSL-OXID
4-METHYLSULFINYLPROPYL GSL
4-METHYSULFINYLBUTYL GSL
GSL-ALK
GSL-ALK
SIDE CHAIN MODIFICATION
2-PROPENYL GSL
3-BUTENYL GSL
GSL-OH
2-HYDROXY-3-BUTENYL GSL

FIG. 1

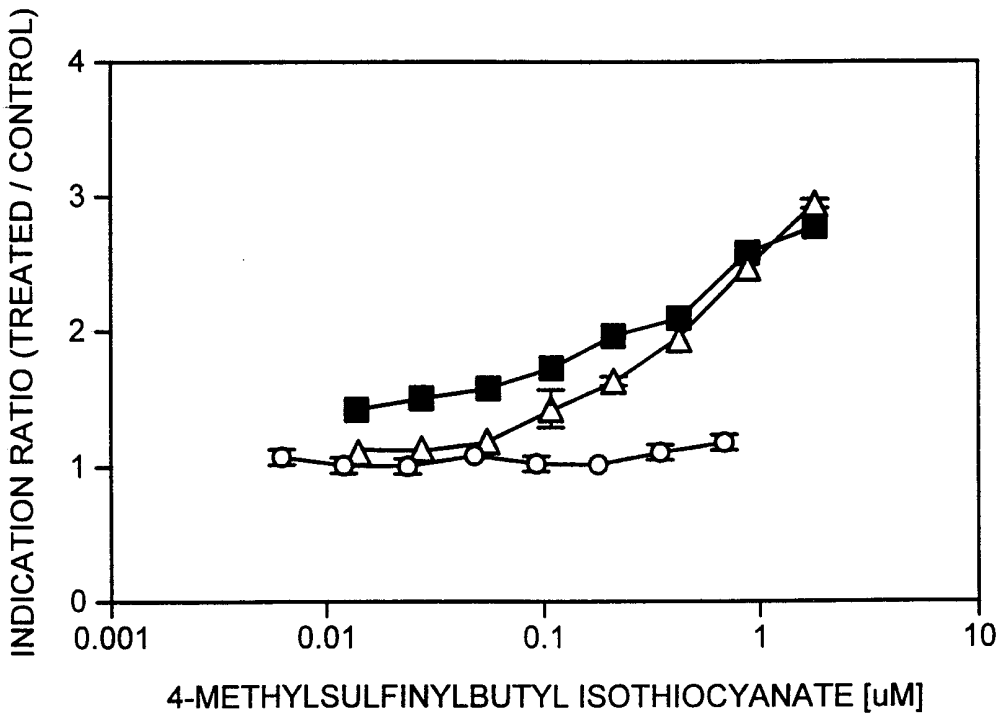
INDICATION RATIO (TREATED / CONTROL)
0
1
2
3
4
0.001
0.01
0.1
1
10
4-METHYLSULFINYLBUTYL ISOTHIOCYANATE [uM]

FIG. 2

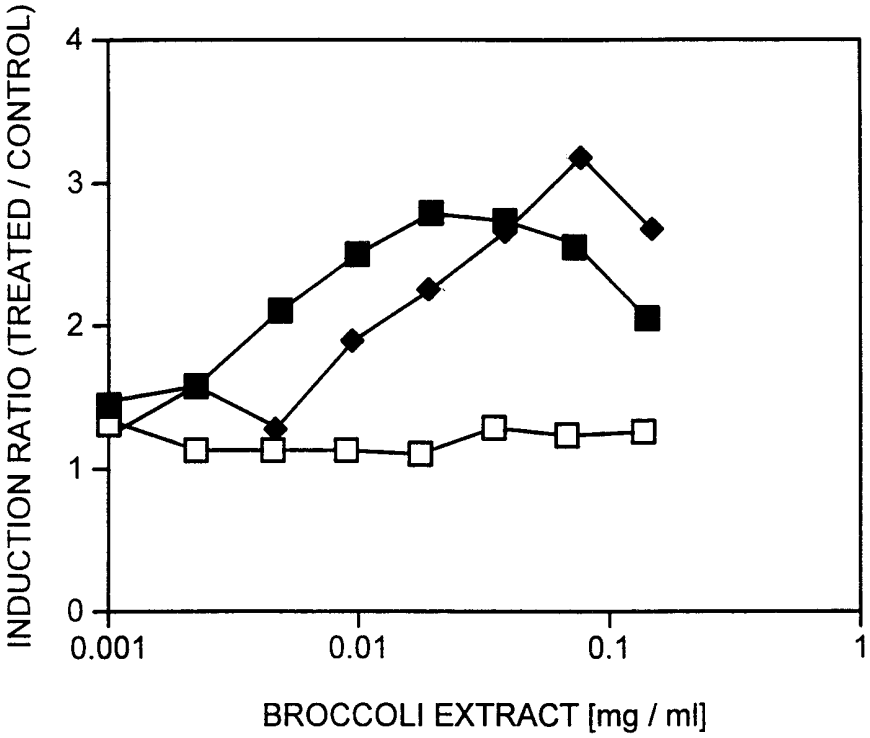
INDUCTION RATIO (TREATED / CONTROL)
4
3
2
1
0
0.001
0.01
0.1
1
BROCCOLI EXTRACT [mg / ml]

FIG. 3

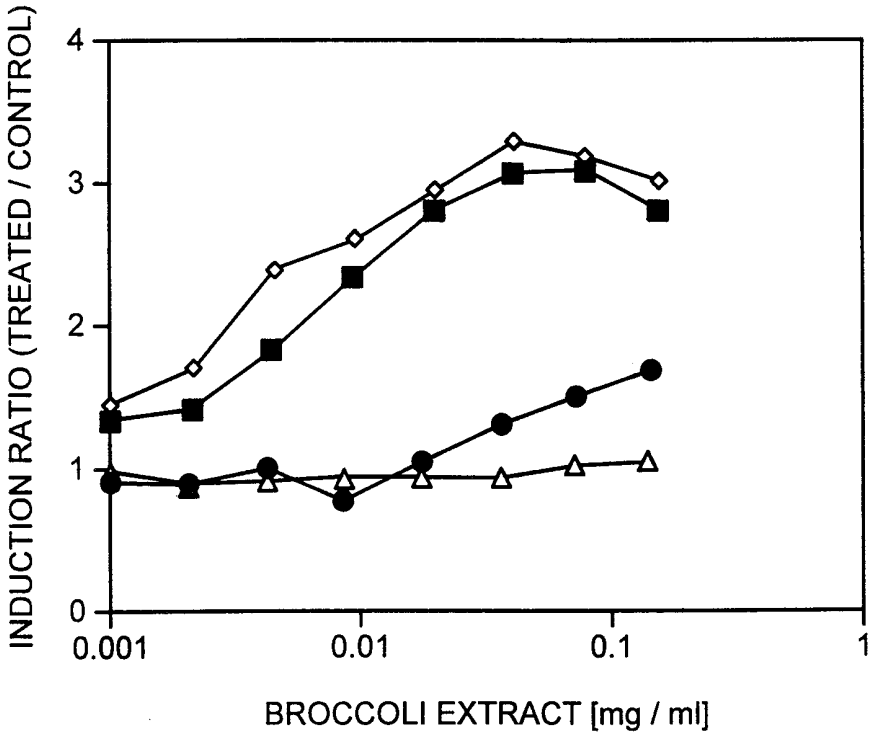
INDUCTION RATIO (TREATED / CONTROL)
4
3
2
1
0
0.001
0.01
0.1
1
BROCCOLI EXTRACT [mg / ml]

FIG. 4

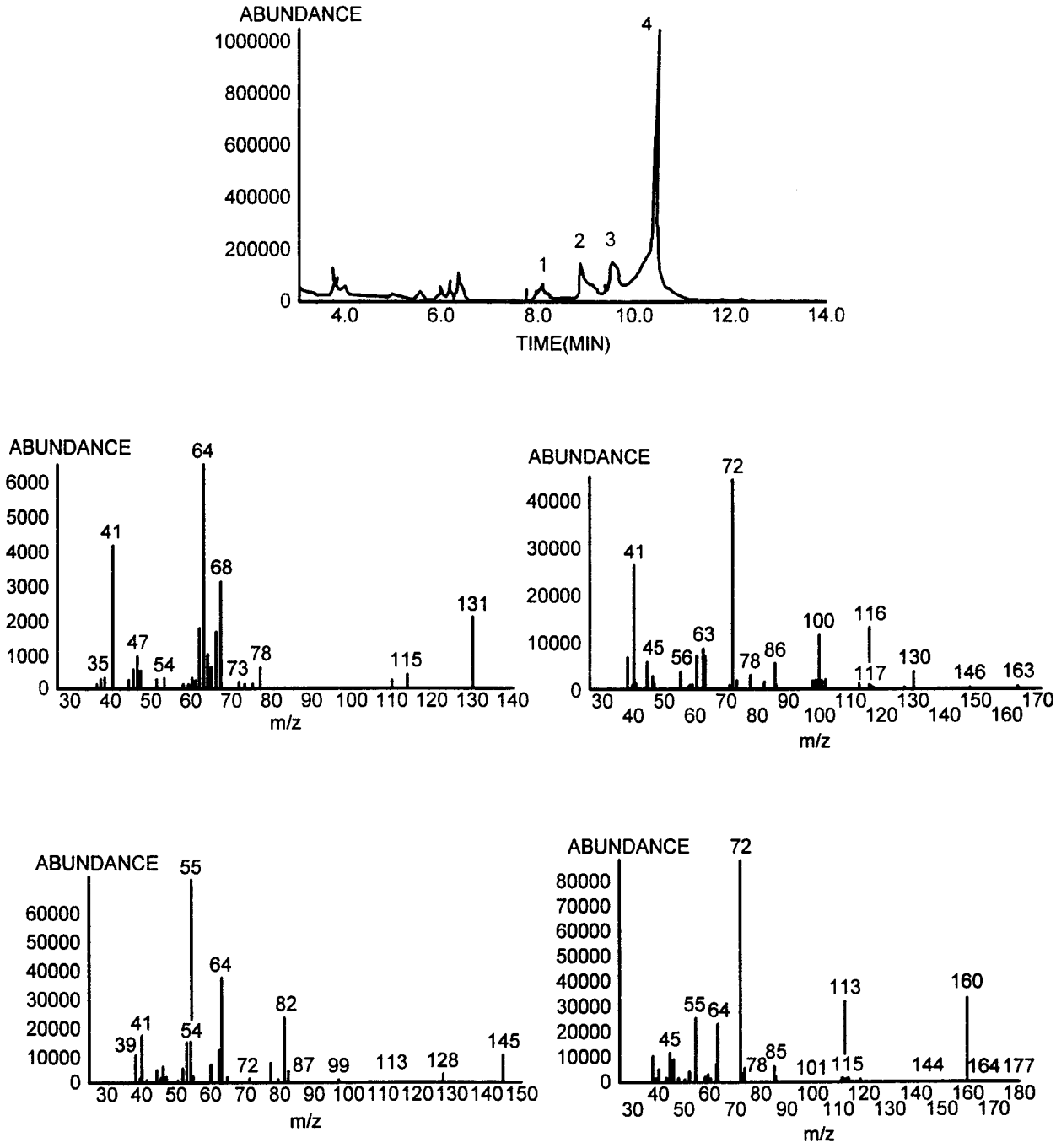
ABUNDANCE
1000000
800000
600000
400000
200000
0
4.0
6.0
8.0
10.0
12.0
14.0
TIME(MIN)
1
2
3
4
ABUNDANCE
m/z
64
41
68
131
47
35
54
73
78
115
ABUNDANCE
m/z
72
41
45
56
63
78
86
100
116
117
130
146
163
ABUNDANCE
m/z
55
64
41
39
54
82
72
87
99
113
128
145
ABUNDANCE
m/z
72
55
64
45
78
85
101
113
115
144
160
164
177

FIG. 5

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 99/01079

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01H5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MITHEN, R.F. ET AL: "Glucosinolates of wild and cultivated brassica species" PHYTOCHEMISTRY, vol. 26, no. 7, 1987, pages 1969-1973, XP002110359 cited in the application	13-15, 17,18
A	page 1969, right-hand column, paragraph 3; tables	1-12

	-/--	

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 July 1999	09/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fonts Cavestany, A

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/GB 99/01079

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	CARLSON, D.G. ET AL.: "Glucosinolates in Crucifer Vegetables: Broccoli, Brussels Sprouts, Cauliflower, Collards, Kale, Mustard Greens and Kohlrabi" JOURNAL OF THE AMERICAN SOCIETY OF HORTICULTURAL SCIENCE, vol. 112, no. 1, 1987, pages 173-178, XP002110360 cited in the application page 174, right-hand column, paragraph 4 - page 177, right-hand column, paragraph 1; tables	1-18
A	FAHEY J W ET AL: "Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 94, no. 19, 1997, pages 10367-10372, XP002086077 Correspondence (Reprint) address, P. Talalay, Dep. of Pharmacology & Molecular Sci., Johns Hopkins Sch. of page 10369, left-hand column, paragraph 3 - page 10372, paragraph 4; figures; tables	1,2,4,6, 9-11, 13-15, 17,18

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