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Inheritance of Three Major Genes Involved in the Synthesis of Aliphatic Glucosinolates in *Brassica oleracea*

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Abstract. **Inheritance of three major genes involved in synthesis of aliphatic glucosinolates (GSL) was followed in segregating populations of *Brassica oleracea* L. generated from three crosses: broccoli x cauliflower, collard x broccoli, and collard x cauliflower. Two of these genes, *GSL-PRO* and *GSL-ELONG,* regulate sidechain length. The action of the former results in three-carbon GSL, whereas action of the latter produces four-carbon GSL. We determined that these two genes act and segregate independently from each other in *B. oleracea*. The double recessive genotype produces only trace amounts of aliphatic GSL. The third gene, *GSL-ALK* controls sidechain desaturation and, as it has been observed in *Arabidopsis thaliana* (L.) Heynh., we found that this gene cosegregates with a fourth gene, *GSL-OH*, that is responsible for sidechain hydroxylation. Elucidation of the inheritance of major genes controlling biosynthesis of GSL will allow for manipulation of these genes and facilitate development of lines with specific GSL profiles. This capability will be important for improvement of *Brassica* breeding lines with high content of desirable GSL, like glucoraphanin, a demonstrated precursor of anticarcinogenic compounds. Additionally, this work is the first step towards cloning the major genes of the aliphatic GSL pathway, and to use these clones in transformation strategies for further crop enhancement.**

A number of studies suggest that consumption of vegetables, in particular crops such as broccoli [*Brassica oleracea (Italica* Group)] and other crucifers, reduces the incidence of cancer in humans and other mammals (Block et al., 1992; Fahey and Talalay 1995, Prochaska et al., 1992). This seems to be due to the presence of inducers of phase II enzymes, that detoxify carcinogens and mutagens in various mammalian organs (Prestera et al., 1996; Prochaska et al., 1992; Talalay et al., 1995). In broccoli, the isothiocyanate sulfuraphane, derived from the GSL glucoraphanin by the action of the enzyme myrosinase, was identified as a potent inducer of these enzymes, conferring protection against mammary tumor growth in rats after treatment with dimethyl benzanthracene, a carcinogenic agent (Zhang et al., 1992, 1994). Glucoraphanin is one of the major GSL present in some crops of *B. oleracea* such as broccoli (Farnham et al., 2000), cauliflower [*B. oleracea (Botrytis* Group)], cabbage [*B. oleracea (Capitata* Group)] and brussels sprouts [*B. oleracea (Gemmifera* Group)] (Rosa et al., 1997). Although certain GSL derivatives have a protective effect against cancer (Rosa et al., 1997), there are some that may have detrimental effects such those derived from alkenyl GSL in rapeseed seed meal (*Brassica napus* L.). These act as antinutrients affecting not only animal growth and development, but also lowering food intake. Additionally, modified isothiocyanates from the aliphatic GSL progoitrin may have goitrogenic effects in animals (Rosa et al., 1997). Therefore, a common breeding objective for *Brassica* L. crops grown for oilseed or forage is to lower the amount of these antinutritional GSL (Bell, 1993).

GSL are a diverse class of thioglucosides that are synthesized by many species of the order Capparales, including *Brassica* and *Arabidopsis* Heynh. The GSL molecule consists of two parts; a common glycone moiety and a variable aglycone side chain (Fenwick et al., 1983; Rosa et al. 1997). The aglycone part may contain aliphatic, indolyl, or aromatic side chains and is derived from a corresponding α-amino acid. The general GSL biosynthetic pathway proposed by Underhill (1980), Larsen (1981) and (Haughn et al., 1991) considers that aliphatic GSL are derived from methionine. Genetic studies in *Arabidopsis thaliana* (Mithen et al., 1995; Mithen and Campos 1996) and *Brassica* sp. (Magrath et al. 1993; 1994) support the biochemical pathway proposed for biosynthesis of aliphatic GSL. The synthesis of these compounds is determined by a simple genetic system containing two distinct sets of genes, one determining sidechain elongation and the second one chemical modification of the sidechains. Aliphatic GSL profiles vary considerably in *A. thaliana* ecotypes and *Brassica* sp. These GSL are synthesized in the following sequence: methylsulfinylalkyl, alkenyl and hydroxy types, which can be divided into three-carbon (3C), four-carbon (4C), and five-carbon (5C) groups based on their sidechain length.

In *A. thaliana,* several genes involved in the GSL pathway have been identified by genetic analysis, including *GSL-ELONG, GSL-ALK, GSL-OHP,* and *GSL-OH* (Campos de Quiros et al., 2000; Magrath et al., 1994; Mithen et al., 1995; Mithen and Campos 1996). The *GSL-ELONG* locus regulates sidechain length, whereas *GSL-ALK* controls sidechain desaturation. *GSL-OHP* and *GSL-OH* are responsible for sidechain hydroxylation (Magrath et al., 1993; 1994). In *B. oleracea,* presence of homologs to *GSL-PRO, GSL-OXID*, *GSL-ELONG, GSL-ALK* and *GSL-OH* loci (Giamoustaris and Mithen,1996; Mithen et al., 1995) has been inferred from inspection of GSL profiles. In rapeseed, (Magrath et al., 1993; Parkin et al., 1994) a similar set of genes has also been proposed. Therefore, the genetic evidence suggests that biosynthesis of GSL is highly conserved in *Brassica* and *Arabidopsis*. However, many steps in sidechain elongation, glycone formation, and aglycone modification remain to be characterized biochemically and genetically.

According to the present model, the 3C, 4C, and 5C GSL are closely related biosynthetically because all of them originate from the same precursor, methionine (Fig. 1). In this model, it is expected that the presence of the dominant allele for the *GSL-ELONG* gene will result in 4C GSL, whereas presence of the dominant allele for *GSL-PRO* will result in 3C GSL. Although it is clear that plants carrying both dominant alleles will produce both 3C and 4C GSL,

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Table 1. Composition of the GSL phenotypes reported in this paper for major three loci involved in the biosynthesis of aliphatic GSL.

Phenotype	GSL composition
$PRO^+/ELONG^+/ALK^+$	Glucoiberin, progoitrin,[z] glucoraphanin, sinigrin, gluconapin
$PRO^-/ELONG^+/ALK^+$	Glucoraphanin and progoitrin[z]
$PRO^+/ELONG^+/ALK^-$	Glucoiberin , glucoraphanin
$PRO^+/ELONG^-/ALK^+$	Sinigrin, glucoiberin
$PRO^-/ELONG^+/ALK^-$	Glucoraphanin
$PRO^+/ELONG^-/ALK^-$	Glucoiberin
$PRO^-/ELONG^-/ALK^-$	Trace amounts of GSL

[z]Results from the action of a fourth locus, GSL-OH, where allele GSL-OH hydeoxylates gluconapin into progoitrin (Fig. 1).

it is not clear what the phenotype of the plant is when both alleles are recessive. Another question is whether it is possible to manipulate independently the 3C and 4C GSL determining genes. In this paper, we address these questions by following segregation of the major GSL chain elongation and modification genes. These results lay the foundation for isolation of important genes involved in the GSL pathway, which will ultimately make it possible to genetically engineer *Brassica* crops with optimal contents of specific GSL.

Materials and Methods

Plant material. The following *B. oleracea* populations were used for the inheritance studies: 1) 52 recombinant inbred (RI, F_5) and 88 F_2 plants of collard x broccoli. 2) 89 RI (F_6) lines of collard x cauliflower, and 3) 195 F_2 plants of a doubled haploid (DH) cauliflower x DH broccoli cross.

GSL analysis. Analysis of GSL was performed with the protocol based on Kraling et al. (1990), which was modified for leaf extraction (instead of seeds). For this purpose ≈2 g of fresh leaves collected from 6-week-old seedlings were ground in liquid nitrogen. The tissue was extracted twice with 70% methanol at 80 °C for 10 min. After applying the supernatant to a DEAE-Sephadex A-25 column (Sigma, St. Louis, Mo.), the GSL were converted into desulfo-GSL with sulfatase (0.5% enzyme in water for 12 h at 24 °C, Sigma H-1 type). The desulfo-GSL were then eluted by adding 1.5 mL distilled water. The resulting mixture was separated by high-performance liquid chromatography (HPLC) (model SCL-10AVP; Shimadzu, Columbia, Md.) fitted with an ultraviolet detector set at 230 nm and a Lichrosorb 100 RP-18 column (Alltech Assoc. Inc., Deerfield, Ill.) using a linear solvent gradient from 1% to 19% acetonitrile in water over 20 min. The flow rate was 1.5 mL·min^{-1} at 32 °C. The HPLC chromatogram was compared to the desulfo-GSL profile of 'Linetta' rapeseed, a cultivar used widely as a standard for GSL identification to compare the peaks with the corresponding GSL. The presence of desulfosinigrin and desulfoglucoraphanin peaks was confirmed by using pure authentic sinigrin (Sigma) as an internal standard. Qualitative assessment of GSL was done visually by the presence or absence of the specific peaks. On the basis of this assessment, we assigned the phenotypes of each plant in the segregating populations as described in Table 1. GSL content was quantified with glucotropaeolin (E.M. Science, Gibbstown, N.J.) as an internal standard.

Results and Discussion

Broccoli x cauliflower population. This population was useful to follow segregation of two genes involved in the GSL pathway, *GSL-PRO* determining synthesis of 3C aliphatic GSL and *GSL-ELONG* determining synthesis of 4C GSL (Fig. 1). The parental cauliflower DH line for this cross contained only the 3C side chain GSL, sinigrin, and glucoiberin (Fig. 2A). Therefore, the phenotype of this line is GSL-PRO^+/GSL-$ELONG^-$/GSL-ALK^+. The expression level of *GSL-ALK* seems to be very low in this parent, because sinigrin content was lower than glucoiberin content. The DH broccoli parent contained only glucoraphanin (Fig. 2B), so its phenotype was GSL-PRO^-/GSL-$ELONG^+$/GSL-ALK^-. The GSL profile of the resulting F_1 plants contained four major aliphatic GSL, namely glucoiberin, progoitrin, glucoraphanin, and sinigrin (Fig. 2C). As expected from the genetic constitution of the parental lines, the F_1 was heterozygous at the *GSL-PRO, GSL-ELONG,* and *GSL-ALK* loci. The presence of progoitrin also indicated activity of *GSL-OH,* which regulates sidechain hydroxylation. *GSL-OH* hydroxylates 4C but not 3C sidechains, and it requires a functional *GSL-ALK* allele to perform the modification reaction. Therefore, it is difficult to know in this population which parental line had a functional allele for the *GSL-OH* locus, because there were no 4C side chain GSL in the cauliflower parent, and *GSL-ALK* was nonfunctional in the broccoli parent. In any case, the low level of both progoitrin and

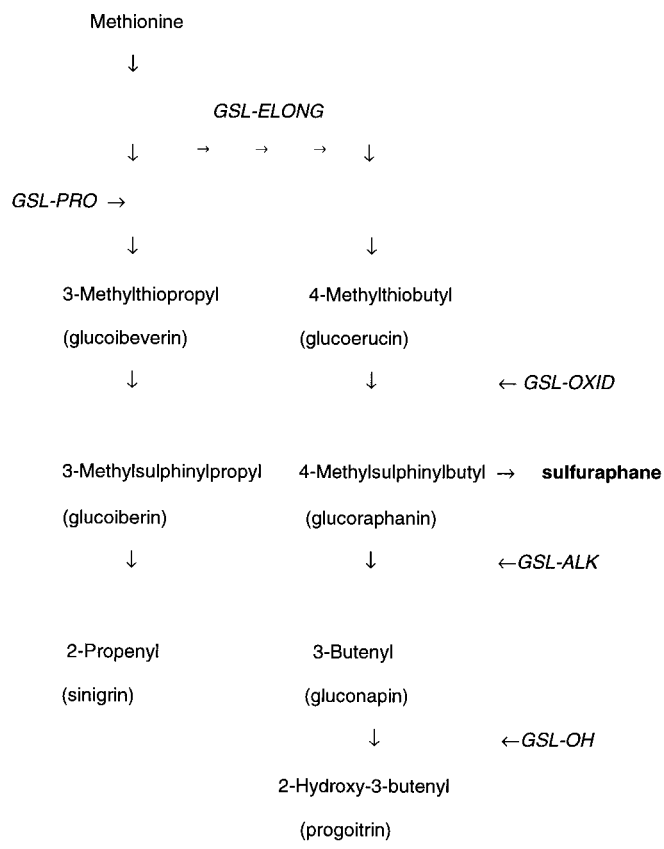

Fig. 1. Model for aliphatic GSL biosynthesis in *B. oleracea* , including the inferred major genes controlling this process (adapted from Mithen et al., 1995, 1996).

sinigrin in the F_1 progeny, which was <10% of all the aliphatic GSL, indicates the expression level of *GSL-ALK* and *GSL-OH* was very low. For this reason, the inheritance of these two genes was not followed in this F_2 population because their low expression makes it difficult to accurately classify the phenotype of the plants based on their GSL profiles. The F_2 plants segregated as expected for independent assortment for the *GSL-PRO* and *GSL-ELONG* genes (9:3:3:1 ratio, $\chi^2 = 0.44$, $P = 0.93$, 3 df) (Table 2). The phenotypes of these four segregating groups are GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^-$, (containing glucoiberin and glucoraphanin); GSL-PRO$^+$/GSL-ELONG$^-$/GSL-ALK$^-$ (containing glucoiberin, but no glucoraphanin), GSL-PRO$^-$/GSL-ELONG$^+$/GSL-ALK$^-$ (containing glucoraphanin, but no glucoiberin), and GSL-PRO$^-$/GSL-ELONG$^-$/GSL-ALK$^-$ (plants containing either traces, (<1% of total GSL) or nondetectable amounts of aliphatic GSL (Fig. 2D).

Two aspects of our results have important implications for crop improvement. First, the plants without detectable aliphatic GSL confirm that the 3C and 4C GSL are independently regulated by the *GSL-PRO* and *GSL-ELONG* genes. Therefore it is possible to manipulate 3C and 4C GSL content independently, making it possible to channel the

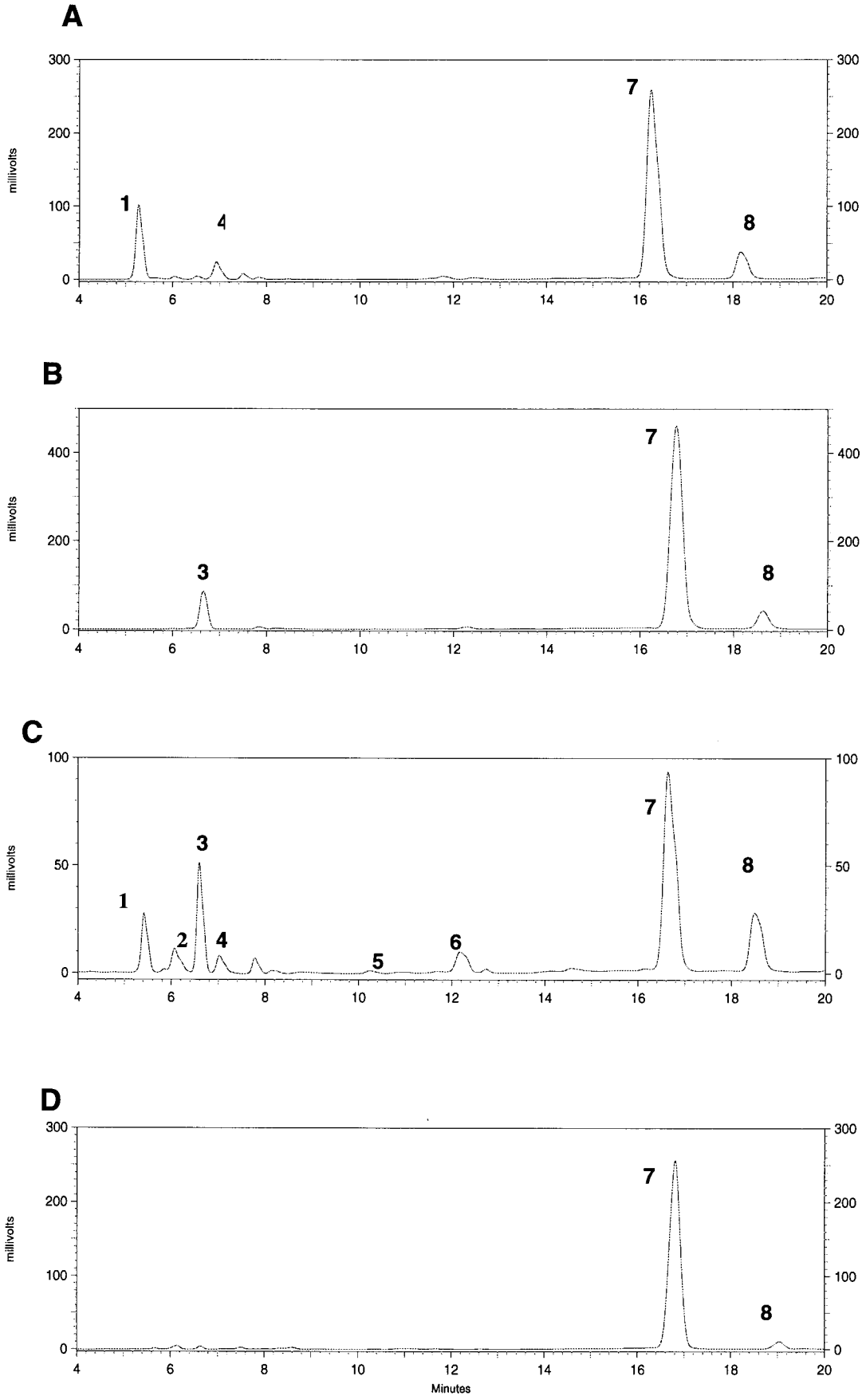

Fig. 2. GSL profiles for parental lines and derived progeny from the cross of doubled haploid lines of broccoli with cauliflower. (**A**) Cauliflower parent of phenotype GSL-PRO$^+$/GSL-ELONG$^-$/GSL-ALK$^+$, (**B**) broccoli parent of phenotype GSL-PRO$^-$/GSL-ELONG$^+$/GSL-ALK$^-$, (**C**) F_1 hybrid of phenotype GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^+$, and (**D**) recombinant double recessive individual of phenotype GSL-PRO$^-$/GSL-ELONG$^-$/GSL-ALK$^-$. Peak identities: 1 = glucoiberin, 2 = progoitrin, 3 = glucoraphanin, 4 = sinigrin, 5 = gluconapin, 6 = 4-hydroxyglucobrassicin, 7 = glucobrassicin, 8 = 4methoxy-glucobrassicin (6, 7 and 8 are indolyl GSL).

Table 2. Segregation ratios (expected values in parenthesis) for four progenies segregating for three major aliphatic GSL genes.

Phenotype for GSL	Population[z] c x b (RI) (1:1:1:1)[y]	c x b (F_2) (9:3:3:1)[y]	c x cw (RI) (1:1)[y]	b x cw (F_2) (9:3:3:1)[y]
$PRO^-/ELONG^+/ALK^-$	14 (13)	5 (5.5)		37 (36.6)
$PRO^-/ELONG^+/ALK^+$	8 (13)	18 (16.5)		
$PRO^+/ELONG^+/ALK^-$	13 (13)	17 (16.5)	36 (44.5)	112 (109.7)
$PRO^+/ELONG^+/ALK^+$	17 (13)	48 (49.5)	53 (44.5)	
$PRO^-/ELONG^-/ALK^-$				9 (12.2)
$PRO^+/ELONG^-/ALK^-$				37 (36.6)
χ^2 values	1.76	0.14	1.62	0.44

[z]c = collard, b = broccoli, cw = cauliflower, RI = recombinant inbreds.
[y]Expected ratios.

GSL pathway toward the synthesis of glucoraphanin by increasing expression level of *GSL-ELONG* and reducing expression of *GSL-PRO*. This may lead to development of *B. oleracea* crops with high content of the putative anticarcinogen, sulfuraphane. Secondly, the double recessive genotype may be valuable in rapeseed breeding to further reduce GSL content to improve the quality of seed meal used as livestock feed.

COLLARD X BROCCOLI POPULATIONS. These populations allowed us to follow segregation of two genes in the aliphatic GSL pathway, *GSL-PRO* and *GSL-ALK* (Fig. 1). The GSL profile of the collard parent indicated that over 90% of its aliphatic GSL were represented by sinigrin and progoitrin (42% and 55%, respectively). Thus, its phenotype is GSL-PRO^+/GSL-$ELONG^+$/GSL-ALK^+/GSL-OH^+. The *GSL-ALK* and *GSL-OH* genes are expressed at high levels because glucoiberin and glucoraphanin were nearly absent and completely converted into sinigrin and progoitrin, respectively (Fig. 3A). However, the independent phenotypes for *GSL-ALK* and *GSL-OH* could not be resolved in this population because they clearly cosegregated. This situation has been also observed in *A. thaliana* (Mithen et al., 1995). Therefore, *GSL-ALK* and *GSL-OH* loci either cor-

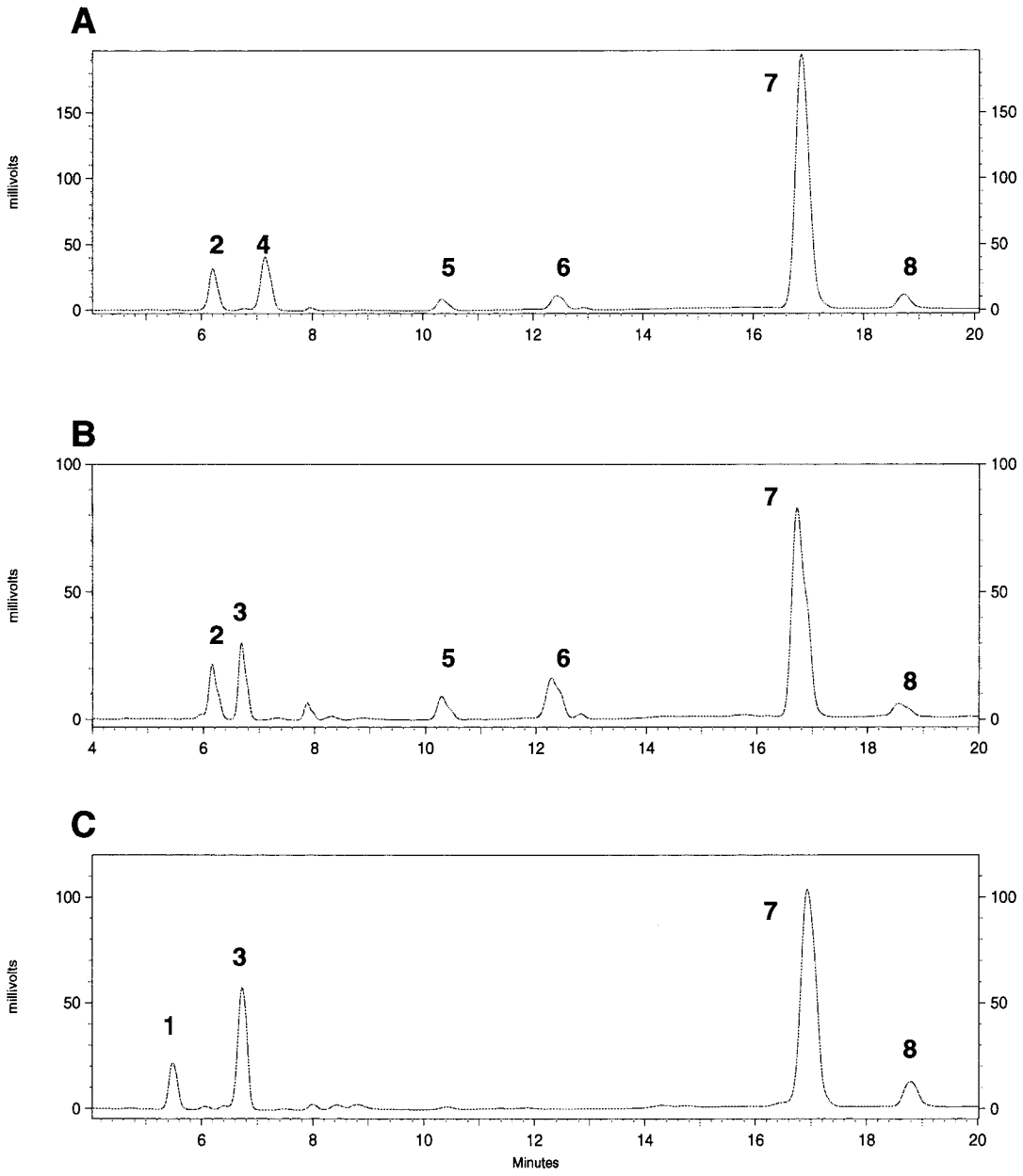

Fig. 3. GSL profiles of the collard parent used to generate collard x broccoli segregating progenies and of two F_2 individuals. (**A**) Collard parental line of phenotype GSL-PRO^+/GSL-$ELONG^+$/GSL-ALK^+/GSL-OH^+, (**B**) recombinant individual of phenotype GSL-PRO^-/GSL-$ELONG^+$/GSL-ALK^-, and (**C**) recombinant individual of phenotype GSL-PRO^+/GSL-$ELONG^+$/GSL-ALK^-. For peak identities, see caption of Fig. 2.

respond to a single gene encoding a bifunctional enzyme catalyzing desaturation and hydroxylation, or they represent two tightly linked loci.

The broccoli parent had a similar profile as that of the DH broccoli line used in the previous cross. The phenotype of this line is GSL-PRO$^-$/GSL-ELONG$^+$/GSL-ALK$^-$/GSL-OH$^-$. For 88 F_2 plants of this progeny, four phenotypic groups were observed segregating in the expected 9:3:3:1 ratio for two independent genes, *GSL-PRO* and *GS-ALK* ($\chi^2 = 0.14$, $P = 0.99$, 3 df) (Table 2). One phenotypic group, GSL-PRO$^-$/GSL-ELONG$^+$/GSL-ALK$^-$ had the same GSL profile as the broccoli parent, which contained only glucoraphanin. Another phenotypic group, GSL-PRO$^-$/GSL-ELONG$^+$/GSL-ALK$^+$, contained progoitrin and glucoraphanin (Fig. 3B) and a third group, GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^-$, contained glucoiberin and glucoraphanin (Fig. 3C). These represent the expected recombinant phenotypes. The fourth and largest group had the same phenotype as the collard parent, GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^+$, containing all four major aliphatic GSL. Fifty-three RI lines of the population from the same cross, collard x broccoli, could also be classified into the same four groups as observed for the F_2 plants segregating in the expected 1:1:1:1 ratio ($\chi^2 = 1.76$, $P = 0.62$, 3 df) (Table 2).

Collard x cauliflower population. Only the segregation of *GSL-ALK* and *GSL-OH* was followed in this population. The cauliflower parent of this cross contained only glucoiberin and glucoraphanin, therefore, its phenotype was GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^-$/GSL-OH$^-$. The alleles for *GSL-ALK* and *GSL-OH* were recessive in this parent because no desaturation and hydroxylation products were detected. As indicated in the previous cross, the phenotype of the collard parent was GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^+$/GSL-OH$^+$. Again *GSL-ALK* and *GSL-OH* cosegregated in this cross. If recombination between these two genes had occurred, one would expect to find plants containing gluconapin instead of progoitrin. None of the segregating plants from either the collard x broccoli or the collard x cauliflower populations had this GSL profile. Only two GSL profiles were detected in the RI segregating lines. One had the same phenotype as the cauliflower parent, GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^-$, containing glucoiberin and glucoraphanin, and the other had the same phenotype as the collard parent, GSL-PRO$^+$/GSL-ELONG$^+$/GSL-ALK$^+$, with all four GSL present. The segregation of this population fits the expected 1:1 ratio ($\chi^2 = 1.62$, $P = 0.21$, 1df) (Table 2) for a single segregating gene.

Conclusion

The populations generated in the present study will be essential to clone the major genes involved in the synthesis of aliphatic GSL in *B. oleracea* crops using the respective *A. thaliana* homologs. For example, one of the genes (*GSL-ELONG*) has been cloned from *A. thaliana* (Campos de Quiros, et al., 2000). Based on this sequence, we have identified several clones from a *B. oleracea* bacterial artificial chromosome library containing the homologous gene. This library was developed from the DH broccoli parent used to generate the first cross described in this paper (Li et al., 2000). A marker obtained from one of these clones cosegregates with 4C GSL. We are now in the process of sequencing the *B. oleracea* GSL-ELONG homolog. Additionally, using these populations, we found several molecular markers closely linked to *GSL-ALK* and *GSL-PRO* genes. Our future plans are to do complementary transformation to confirm cloning of the major GSL genes in *Brassica*.

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