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Biochemical genetics of glucosinolate modification in *Arabidopsis* and *Brassica*

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Abstract Fine mapping of the glucosinolate biosynthesis gene *OHP*, which regulates the conversion of 3-methylsulphinylpropyl to 3-hydroxypropyl glucosinolate, in an *Arabidopsis thaliana* Columbia × Landsberg *erecta* RI line population positioned the gene within 54 kb of DNA on chromosome IV. Sequence data identified a family of genes encoding 2-oxoglutarate-dependent dioxygenases in this region. A probe based on these genes co-segregated with *ALK* in *Brassica oleracea*, a gene regulating the synthesis of alkenyl glucosinolates. The reactions catalysed by the *OHP* and *ALK* enzymes utilise similar substrates and may have a common mechanism. Thus, these dioxygenases are prime candidates for controlling the side chain modification of glucosinolates.

Keywords *Arabidopsis thaliana* · *Brassica oleracea* · Glucosinolates · 2-Oxoglutarate dependent dioxygenases

Introduction

Glucosinolates are sulphur-containing glycosides found in the Brassicales. Following tissue damage, glucosinolates are hydrolysed by myrosinase to an array of products, of which the isothiocyanates are of particular importance. Glucosinolates and their breakdown products are an important class of phytochemicals involved in the mediation of plant-herbivore interactions. Isothiocyanates have also been shown to be potentially important anticarcinogenic compounds in *Brassica* vegetables and salad crops (Faulkner et al. 1998). The glucosinolate molecule consists of a glycone moiety and a variable aglycone side chain derived from one of seven amino acids. More than 100 side chain variants have been described. Approximately half of these are derived from methionine via two processes: (1) elongated homologues of methionine are formed by repeated condensation reactions with acetyl CoA (Campos et al. 2000); (2) subsequent modifications of the methylthioalkyl side chain structure. These modifications may involve oxidation, hydroxylation and desaturation reactions.

Genetic studies within *Brassica* and *Arabidopsis* have demonstrated that alleles at a series of Mendelian loci regulate these side chain modifications (Giamoustaris and Mithen 1996; Mithen et al. 1995). One of the most important modifications is the conversion of the methylsulphinylalkyl side chain to an alkenyl side chain; this step is controlled by the *ALK* gene and involves the removal of the terminal methylsulphinyl group and the insertion of a double bond. This modification has considerable effects on the physico-chemical and biological properties of the isothiocyanates (ITCs), which are formed after hydrolysis. For example, alkenyl ITCs are more volatile than the corresponding methylsulphinylalkyl ITCs, which affects the attraction of insect herbivores and their parasities (Bradburne and Mithen 2000) and, while methylsulphinylalkyl isothiocyanates are potent inducers of phase II detoxification enzymes in mammals alkenyl isothiocyanates have little or no inducer activity (Faulkner et al. 1998).

Arabidopsis thaliana ecotypes have a similar range of methionine-derived glucosinolates to *Brassica* with two exceptions. Firstly, all *Arabidopsis* ecotypes make methionine-derived glucosinolates with 6C, 7C and 8C side chains. These are not found in *Brassica* but do occur in other genera such as *Rorippa*. Secondly, certain *Arabidopsis* ecotypes synthesise hydroxyalkyl and benzoxoylalkyl glucosinolates, which are also not found in *Brassica*. Analysis of the Lister and Dean Landsberg *erecta* × Columbia RI lines mapped a single Mendelian gene (*OHP*) that converts 3-methylsulphinylpropyl glucosinolate to 3-hydroxypropyl glucosinolate to the short arm of chromosome IV (Mithen et al. 1995). This gene mapped to the

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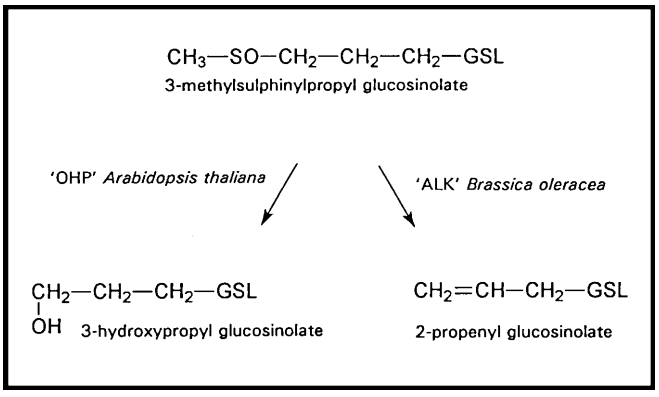


Fig.1 Comparison of the modification reactions regulated by alleles at the *Arabidopsis OHP* locus and *Brassica oleracea ALK* locus

same interval as a second Mendelian gene (*ALK*) that regulates the conversion of methylsulphinylalkyl glucosinolates to alkenyl glucosinolates. The *ALK* gene was located by analysis of progeny from a cross between the ecotypes Limburg 5 and H51, which have methylsulphinylalkyl and alkenyl glucosinolates, respectively. Thus, in *Arabidopsis*, the *ALK* and *OHP* loci may either represent two closely linked genes or different alleles of the same gene (Fig. 1).

In this paper we describe the fine mapping of the *OHP* locus in *Arabidopsis thaliana* and identify a family of dioxygenase genes as prime candidates for both *OHP* and *ALK*. We further show that probes based upon these genes co-segregate with the *ALK* locus in *Brassica oleracea (sensu lato)*.

Materials and methods

Plant material

Previous mapping data (Mithen et al. 1995) was used to identify 17 recombinant inbred (RI) lines from a Landsberg *erecta* × Columbia cross (Lister and Dean 1993) with recombination events in the region of *OHP*. Thirty plants of each of 17 selected RI lines were grown in 8.5 × 8.5-cm pots under standard glasshouse conditions, and after 6 weeks of growth, leaf tissue was harvested from the plants, frozen in liquid nitrogen and freeze-dried. Glucosinolates were analysed as described previously (Magrath et al. 1994). Thirty plants from a *B. atlantica* × *B. drepanensis* backcross population described by Giamoustaris and Mithen (1996) were grown under standard glasshouse conditions for 8 weeks before harvesting and analysis of leaf tissue as described above.

Isolation and analysis of plant DNA

Arabidopsis thaliana and *Brassica oleracea* plant DNA was isolated and quantified as described previously (Magrath et al. 1994). Restriction digestion was performed on 20 µg of genomic DNA following the supplier's (Gibco) recommendations. The resultant fragments were separated by gel electrophoresis, capillary-blotted onto nylon membrane (Hybond N+, Amersham Pharmacia) and subsequently probed (Magrath et al. 1994).

Isolation of large insert clones and library screening

Isolation of yeast artificial chromosome (YAC) DNA was as described by Bancroft (2000). Bacterial artificial chromosome (BAC) DNA was prepared from 50-ml overnight cultures by alkaline lysis (Birnboim and Doly 1979) followed by propan-2-*ol* precipitation at 4°C. Nucleic acids were recovered by centrifugation (2000 *g* for 20 min at 4°C) and the dried pellet resuspended in 2 ml TE (10 m*M* TRIS-HCl pH 7.4, 1 m*M* EDTA). Subsequently, 2 ml 4 *M* lithium acetate was added prior to a 20-min incubation at 4°C and centrifugation as before. The supernatant was retrieved and DNA precipitated with 0.2 ml 3 *M* sodium acetate pH 5.5, and 9 ml absolute ethanol at 4°C for 1 h. The dried pellet was resuspended in 4 ml TE, then extracted once with phenol/chloroform, ethanol-precipitated and resuspended in 50 µl TE.

Assembly of BAC contig

From the above preparation, 4 µl of BAC DNA was digested with *Eco*RI and *Sal*I, separated by gel electrophoresis and capillary-blotted. Filters were probed with 0.4 µl *Hind*III-digested BAC DNA.

Inverse (i) polymerase chain reaction (PCR)

Restriction fragment length polymorphism (RFLP) probes were prepared from BAC ends by restriction digestion of 5 µl BAC DNA. Digested DNA was extracted with phenol/chloroform and dialysed with Sepharose CL 6B spin columns (Pharmacia) into 20 µl TE. Self-ligation was carried out in a 100-µl reaction volume with 1 U T4 DNA ligase (Gibco) and supplied buffer at 12°C overnight. An iPCR reaction (Ochman et al. 1988) was conducted using 3 µl of the ligation product with 0.25 m*M* dNTPs, 10 µ*M* of each primer, 1.5 U Amplitaq (Perkin Elmer) and 1× supplied buffer in a total volume of 50 µl. The primers used were 5'-CGACCTGCAGGCATGCAAGCTT-3' and 5'-ACTCTAGAGGATCCCCGGGTAC-3' for the right side, and 5'-CCTAAATAGCTTGGCGTAATCATG-3' and 5'-TGACACTATAGAATACTCAAGCTT-3' for the left side. The cycling conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 71°C for 2 min and finally by an extension at 72°C for 10 min. The products were resolved by electrophoresis and purified using a QIAquick (Qiagen) gel band purification kit.

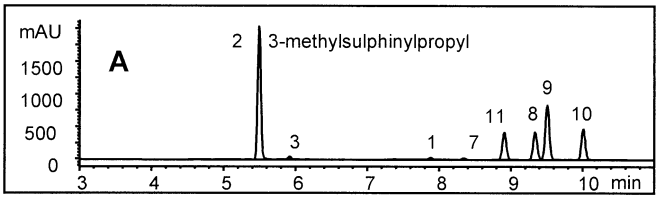
Isolation of sequence polymorphic PCR products

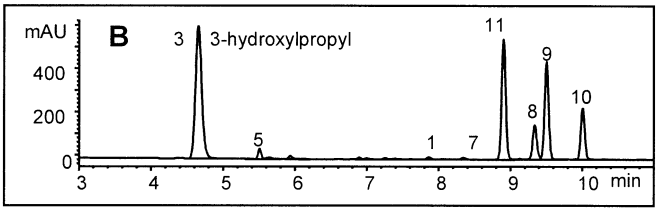
PCR reactions were performed using 50 ng of genomic DNA with 0.125 m*M* dNTPs, 0.5 µg of each primer and 2.5 U of *Taq* polymerase (Promega) with supplied buffer in a 100 µl reaction volume. The cycling conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 51°–56°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. Primers designed to BAC T4I9 were: pT4–47, 5'-AAGGAGAAGGAGAAGGAGG-3' and 5'-TTGATGATTGAACGGTGG-3'; pGLU30–31, 5'-AAGCTTGGAGAGTAGATCGAGC-3' and 5'-TGATGTTGTTGTTGTGGCAGGC-3'; pDIOX2, 5'-TCTACGCGTCAAGAAATGGATTCAGACT-3' and 5'-TACTCTTGTTCCCATGATCAGGCC-3'; pDIOX3, 5'-GGCACGCGTGAAAGAAGAATGGGTTCATGC-3' and 5'-TGATACTCTTGTTACCCTCAGGCC-3'. Primers designed to BAC F4C21 were: pF4–23, 5'-GAAGAAGCTGAAGGAGACTG-3' and 5'-CACGCATCAAATCTAGCC-3'. The products were gel band purified (Qiagen) and digested or sequenced. Sequence analysis of the PCR products was conducted using an ABI 377 Sequencer and BigDye Terminator kit (PE Biosystems).

Analysis of the Columbia DNA sequence

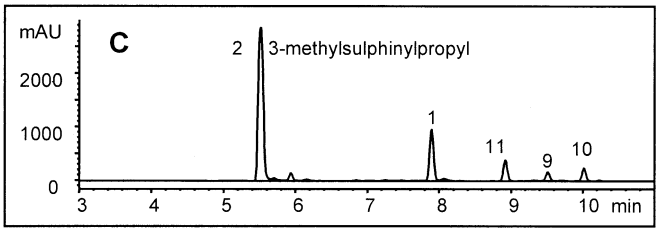
Analysis was carried out using the annotated sequence of BACs T4I9 and F4C21 available at the Munich Information Centre for Protein Sequences (MIPs) website (http://www.mips.biochem.mpg.de/proj/thal/).

Arabidopsis





Brassica



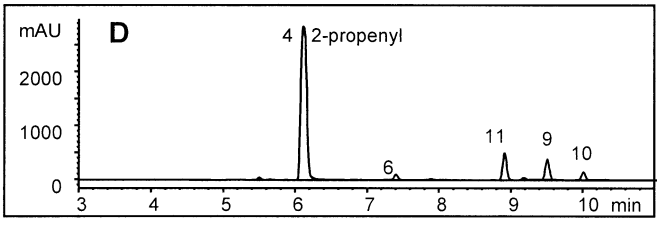


Fig. 2 Separation of desulphoglucosinolates occurring in the leaves of *Arabidopsis thaliana* and *Brassica oleracea*. 3-Hydroxylpropyl glucosinolate is the major glucosinolate in Landsberg *erecta* (**B**), while the RI line N1937 (**A**), which has a non-functional Col allele at *OHP*, has high levels of methylsulphinylpropyl glucosinolate. The major glucosinolate of *B. atlantica* (**D**) is 2-propenyl glucosinolate, while backcross plant DRP30 (**C**), which has a non-functional allele at *ALK*, has high levels of methylsulphinylpropyl glucosinolate. Desulphoglucosinolates were identified through liquid chromatography-mass spectrometry with the use of atmospheric pressure chemical ionisation. *Peaks: 1* 3-methylthiopropyl, *2* 3-methylsulphinylpropyl, *3* 3-hydroxypropyl, *4* 2-propenyl, *5* 4-methylsulphinylbutyl, *6* 3-butenyl, *7* 7-methylsulphinylheptyl, *8* 8-methylsulphinyloctyl, *9* 3-indolylmethyl, *10* 4-methoxy-1-indolylmethyl, *11* benzyl (internal standard)

B. oleracea RFLP mapping

Filters of *Eco*RI-digested DNA were probed with anonymous genomic probes selected with reference to the *Brassica* RFLP maps as described by Sebastian et al. (2000) and the linkage group described by Giamoustaris and Mithen (1996). The linkage group was assembled with MAPMAKER (version 3.0) software (Lincoln et al. 1992), and the Kosambi function used to convert recombination fractions into centiMorgans.

Results

Genetic mapping of *OHP* in *Arabidopsis*

The *OHP* locus was mapped previously (Mithen et al. 1995) to the short arm of chromosome IV between RFLP markers mi390 and mi233 using 300 RI lines from a Columbia (Col) × Landsberg *erecta* (Ler) cross (Lister and Dean 1993). Fine mapping of this locus was carried out using these lines. Four different glucosinolate profiles were found within the RI lines: Col type, 4-methylsulphinylbutyl and lower levels of 3-methylsulphinylpropyl glucosinolate; Ler type, 3-hydroxypropyl glucosinolate and two novel recombinant phenotypes, one containing 4-methylsulphinylbutyl and 3-hydroxypropyl glucosinolates and the other only 3-methylsulphinylpropyl glucosinolate (Fig. 2). The presence of a Ler allele at the *OHP* locus is required for the conversion of 3-methylsulphinylpropyl to 3-hydroxypropyl glucosinolate; Col has a non-functional allele at this locus. Glucosinolate and RFLP analysis of the 17 selected RI lines identified two recombinants (N1917, N1960) between GA1 and *OHP* and four recombinants (N1966, N4739, N4741, N4798) between PetC and *OHP*.

Physical mapping of *OHP* in *Arabidopsis*

We developed a physical map around the location of *OHP* in order to identify BAC clones in this region and to reduce the non-recombinant interval for *OHP*. YAC clones CIC11B4 and CIC4A7 that span the region between GA1 and PetC (http://nasc.nott.ac.uk/JIC-contigs/Chr4_YACcontigla.GIF) were used as probes to the TAMU BAC library (Choi et al. 1995). Thirty-three clones were selected, assembled into a contig and orientated through hybridisation to GA1 and PetC. Ends of several of these clones were isolated by iPCR and used as RFLP probes to genomic DNA of the 6 selected RI lines. Polymorphisms were obtained with the following BAC end probes/enzyme combinations: T4I9LE/*Eco*RV, T5J8RE/*Eco*RV, T4I9RE/*Eco*RI, T5J8LE/*Cfo*I, T2M8LE/*Xho*I and T9O18LE/*Pst*I. Recombination was observed between T2M8LE and *OHP* towards the centromere and T3J21LE towards the telomere, allowing *OHP* to be positioned on these two overlapping BACs covering 153 kb of DNA. The end sequences of BACs available through the TIGR database (http://www.tigr.org/) were used to integrate this physical map with BAC sequences available (http://nucleus.cshl.org/protarab/) as part of the international sequencing initiative. Two BACs covering this region, T4I9 (GB: AF069442) and F4C21 (GB: AC005275), were identified.

PCR primers designed to the Col sequence were used to amplify 1-kb products from Col, Ler and the 6 RI lines. The PCR products were sequenced to detect single nucleotide polymorphisms (SNIPs). The PCR products, pT4–47 (46845–47357) designed to the T4I9 sequence,

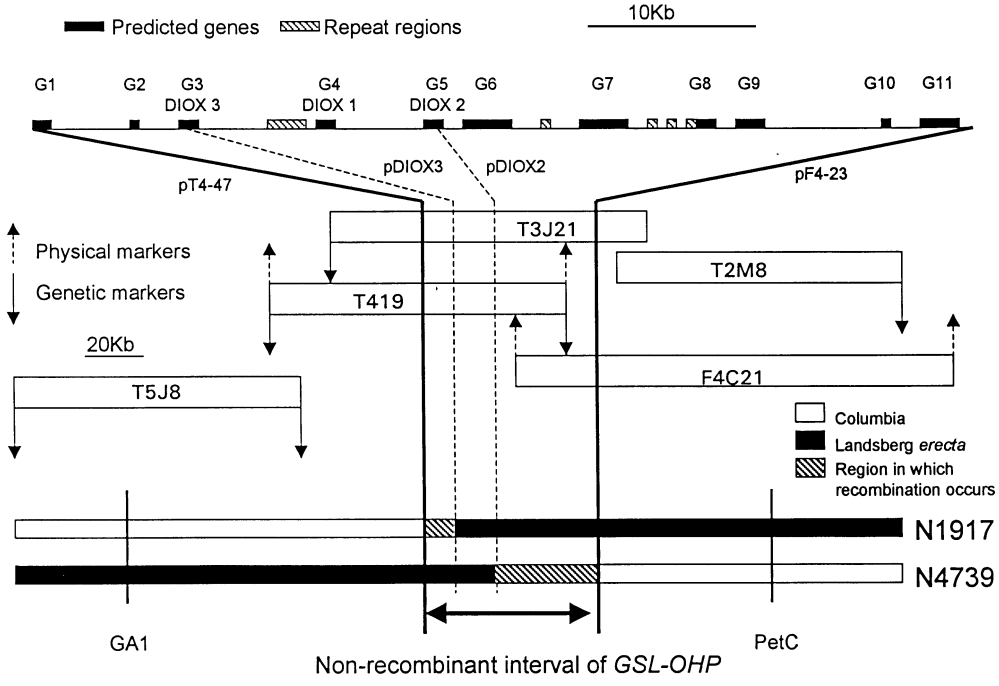


Fig. 3 Genetic and physical map in the region of *OHP*. Details of map construction are provided in the text. T5J8, T3J21, T2M8 (sequenced) and T4I9 (sequenced) BAC clones from the TAMU library, *F4C21* a sequenced BAC clone from the IGF library, *pT4–47, pF4–23, pDIOX2* and *pDIOX3* PCR products. Genetic anchors are provided by the use of BAC end sequences as RFLP probes and PCR products as CAPS (Cleaved Amplified Polymorphic Sequences) and SNPs (Single Nucleotide Polymorphisms). Physical anchors are provided by integrating BAC end and PCR product sequences to sequenced BACs. *G1–G11* Predicted genes within the nonrecombinant interval of *OHP*

Table 1 Analysis of the 11 putative genes/open reading frames predicted from the sequence within the non-recombinant interval of *OHP*. The genes have been assigned possible functions based on their homology to known proteins and signature patterns found within the sequence. Further details can be found at the Munich Information Center for Protein Sequences (MIPs) website

Gene number	Protein type	MIPs code	Possible function
G1	OR23 protein	AT4g03030	Unknown function
G2	Hypothetical protein	AT4g03040	Unknown function
G3 DIOX 3	Oxidoreductase/ dioxygenase	AT4g03050	Secondary metabolism
G4 DIOX 1	Oxidoreductase/ dioxygenase	AT4g03060	Secondary metabolism
G5 DIOX 2	Oxidoreductase/ dioxygenase	AT4g03070	Secondary metabolism
G6	Phosphoserine/threonine phosphatase	AT4g03080	Phosphatase involved in cellular communication/signal transduction
G7	NDX1 homeobox protein	AT4g03090	Transcription/mRNA synthesis
G8	rac GTPase activating protein	AT4g03100	Cellular communication/signal transduction
G9	Ribonucleoprotein	AT4g03110	Protein synthesis/translational control
G10	C-type U1 small nuclear ribonucleoprotein	AT4g03120	Transcription/mRNA processing (splicing)
G11	Hypothetical protein	AT4g03130	Unknown function

and pF4–23 (23312–24214) designed to F4C21, had sequence polymorphisms between Col and Ler. Recombination was observed between *OHP* and pT4–47 in line N1917 and pF4–23 in lines N4739 and N4741 giving a non-recombinant interval of 54 kb DNA (Fig. 3).

Identification of a candidate gene family

Eleven putative open reading frames were identified within the non-recombinant interval of 54-kb DNA. The possible function(s) of putative spliced cDNAs were analysed to identify strong candidate genes for involvement in glucosinolate biosynthesis (Table 1). A family of three putative genes was identified as the most likely candidates for *OHP*. A pseudogene containing the terminal portion of a fourth member of the family was also located in this gene cluster. The three complete open reading frames were assigned the names DIOX1, DIOX2 and DIOX3; they showed high homology to each other and to genes encoding 2-oxoglutarate-dependent dioxygenases (2-ODDs). 2-ODDs carry out hydroxylation and desaturation reactions on a wide range of substrates involved in plant secondary metabolism. PCR analysis using CAPs designed to DIOX 2 (pDIOX2/*Cla*I) and DIOX 3 (pDIOX3/*Nco*I) confirmed co-segregation of both genes with *OHP* in the 3 RI lines N1917, N4741 and N4739.

Cosegregation of candidate genes with *ALK* in *Brassica*

The *B. oleracea ALK* phenotype was mapped previously as a single, dominant Mendelian gene in a *B. drepanensis* × *B. atlantica* backcross (Giamoustaris and Mithen 1996).

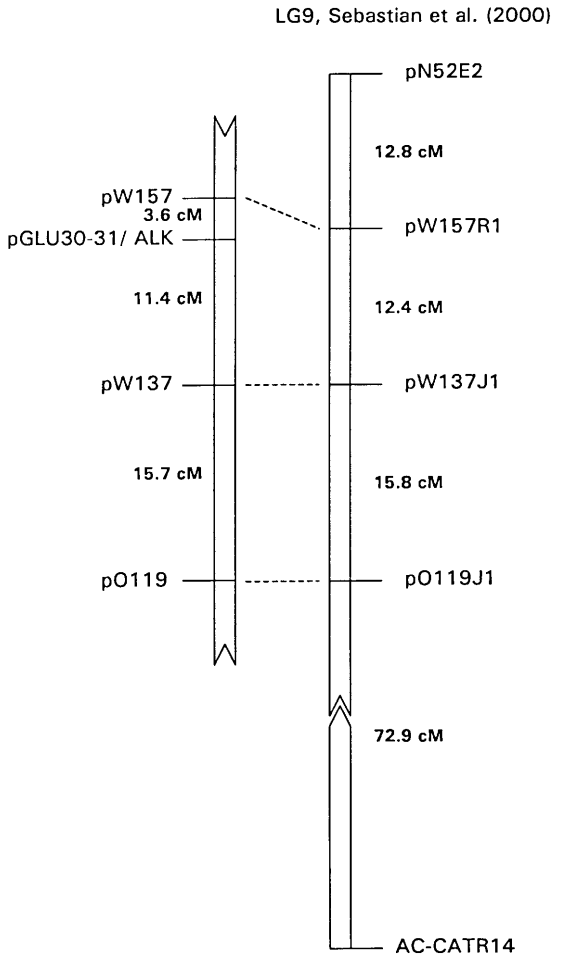


Fig. 4 Alignment of a linkage group from *B. atlantica* × *B. drepanensis* backcross on which *ALK* is positioned with part of *B.oleracea* linkage group LG9 (Sebastian et al. 2000). The marker pGLU30–31, designed with homology to the *Arabidopsis DIOX* gene family, co-segregates with *ALK*

Glucosinolate analysis (Fig. 1) and RFLP analysis on *Eco*RI-digested genomic DNA was undertaken with 30 plants from a second population of the same backcross. This confirmed the positioning of *ALK* between RFLP markers pW137 and pW157 on a linkage group that can be aligned to the *B. oleracea* linkage group, LG9 reported by Sebastian et al. (2000). The PCR product pGLU30–31 was designed to amplify a fragment of DIOX3, which has 75% and 79% sequence identity to DIOX2 and DIOX1, respectively. When used as an RFLP probe on the backcross population, pGLU30–31 hybridised to three *B. atlantica* bands of 17 kb, 11 kb and 6 kb, the smallest of which co-segregated with the *ALK* phenotype (Fig. 4).

Discussion

The results of this study have identified genes encoding 2-oxoglutarate-dependent dioxygenases (2-ODDs) as prime candidate genes for the conversion of methylsulphinylalkyl glucosinolates to both hydroxyalkyl and alkenyl glucosinolates.

In chemical terms, the reactions catalysed by *ALK* and *OHP* are similar, and both enzymes are proposed to utilise methylsulphinylpropyl glucosinolate (or possibly methylsulphinylpropyl desulphoglucosinolates) as a substrate. Hydroxylase and desaturase reactions may be catalysed by several groups of enzymes, including fatty acid modification enzymes, cytochrome P450 monooxygenases and 2-ODDS. Many of these enzymes are multifunctional, capable of catalysing either reaction depending on the substrate and/or reaction conditions (Halkier 1996; Prescott 2000; Broun et al. 1998a). Alternatively, minor modifications to the amino acid sequence can convert a fatty acid hydroxylase to a desaturase (Broun et al. 1998b), demonstrating the similarity in the two types of reaction. Analysis of the 11 genes located in the non-recombinant region of *OHP* identified three 2-ODDs as the only strong candidates for a hydroxylase and/or desaturase.

2-ODDs form a large gene family in *Arabidopsis* consisting of approximately 100 members (Prescott 2000). Most of these genes have not been assigned functions, but those of known activity are involved in the synthesis of plant growth regulators and other secondary metabolites (reviewed in Prescott 2000). Many 2-ODDs are multi-functional and catalyse a range of reaction types, the most common of which are hydroxylations and desaturations (Prescott and Lloyd 2000). Examples of multi-functional 2-ODDs characterised in *Arabidopsis* include the gibberellin (GA) C20 oxidases (Phillips et al. 1995), GA 3β-hydroxylases (Williams et al. 1998), GA 2β-oxidases (Thomas et al. 1999) and others involved in secondary metabolism (A. Prescott, unpublished data). The results of our study suggest that this broad class of enzymes may also function in glucosinolate biosynthesis. Confirmation of their involvement will be obtained by functional analysis.

The three 2-ODDs identified show a high degree of sequence similarity (74–76% nucleotide sequence identity), indicating that they are the result of gene duplication. Regions of repetitive DNA including a putative retrotransposon flank the three dioxygenase genes. DIOX1 has repetitive DNA within a putative intron and is in reverse orientation to the other two dioxygenase genes, suggesting a possible inversion. Clustered gene families have been shown to catalyse sequential (and similar) reactions in secondary metabolic pathways in other plant species; for example, the DIBOA pathway in maize (Frey et al. 1997). Sequence analysis did not give any indication of which of these genes may encode the *OHP* and/or *ALK* function. Thus, separate dioxygenases may be responsible for the hydroxylation and desaturation reactions, or they may be alleles of the same gene. Elucidation of the expression patterns of individual members of the family, biochemical analysis of enzyme function and transgenic studies will clarify which of the alternative hypotheses is correct.

Methionine-derived glucosinolates in *Arabidopsis* and *Brassica* exhibit several modifications to their structure, with associated biological consequences for plant-herbivore interactions. In this paper, we have identified a fam-

ily of closely related dioxygenase genes as prime candidates for controlling these chemical modifications. This study also illustrates the utility of the *Arabidopsis* sequencing programme for identifying gene candidates and molecular markers for traits of biological and agronomic importance in *Brassica*. Elucidating the nature of the *ALK* and *OHP* genes may provide both an insight into the evolution of biochemical diversity and facilitate the modification of glucosinolates in cruciferous crops by conventional and transgenic approaches.

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