A Novel 2-Oxoacid-Dependent Dioxygenase Involved in the Formation of the Goiterogenic 2-Hydroxybut-3-enyl Glucosinolate and Generalist Insect Resistance in Arabidopsis

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Plants utilize a suite of defensive compounds to provide resistance to the attacks of other organisms. These secondary metabolites show high levels of variation in terms of both accumulation and structure (Rodman et al., 1981; Zangerl and Berenbaum, 1990; Berenbaum and Zangerl, 1998). This variation is believed to result from an arms race between the plant and its biotic attackers such that each evolutionary innovation in plant defense elicits the evolution of a counteradaptation in the biotic attacker. The repetition of this process may lead to the high level of diversity observed in chemical structures and their amounts (Ehrlich and Raven, 1964) and variation in bioactivity of the different structures (Bowers and Puttick, 1988). An alternative option is that the variation may represent a stable cycling of phenotypic classes (Berenbaum and Zangerl, 1998; Zangerl et al., 2008). The concomitant and often correlated variation in both chemical structure and the total amount of a given class of chemical defense complicates the ability to separate the contribution of these two factors to plant protection (Puttick and Bowers, 1988; Mithen et al., 1995; Giamoustaris and Mithen, 1996). The development of precise tools to tease apart chemical structure and content would make an important contribution to the study of plant chemical defense.

An important model system for studying the role of chemical defenses in plants is the glucosinolates, a class of naturally occurring thioglycosides that are found throughout the order Brassicales. These sulfur-rich plant secondary metabolites are synthesized from a variety of protein amino acids (Met, Leu, Ile, Val, Trp, and Phe; Halkier and Du, 1997; Halkier and Gershenzon, 2006). The glucosinolates are part of a two-component defensive system whereby the intact glucosinolates are stored separate from their activating enzyme myrosinase (Bones et al., 1991; Bones and Rossiter, 1996; Kelly et al., 1998; Koroleva et al., 2000). Upon tissue disruption...
tion, the glucosinolate and myrosinase combine and generate a suite of biologically active chemicals that provide resistance against lepidopteran herbivores, aphids, and pathogens (Mithen et al., 1987; Mithen, 1992; Tierens et al., 2001; Barth and Jander, 2006; Kim and Jander, 2007; de Vos et al., 2008). This bipartite system also contains a set of myrosinase-associated proteins that determine the final chemical structure and their resulting biological activity (Burrow et al., 2006; Zhang et al., 2006).

Another important determinant of the final biological activity is the structure of the glucosinolate side chain prior to activation. One example of this structural variation from Arabidopsis (Arabidopsis thaliana) is the dihomomethionine-derived methylthioalkyl side chain, which can be modified to form methylthioalkyl, methylsulfanylalkyl, alkenyl, and hydroxylalkenyl glucosinolates (Fig. 1; Kliebenstein et al., 2001c; Lambrix et al., 2001; Wentzell et al., 2007). Frequently, the chemical structure of the side chain is highly variable within a crucifer species and is often controlled by a set of four genetic loci (Parkin et al., 1994; Giamoustaris and Mithen, 1996; Kliebenstein et al., 2001c). In Arabidopsis and Brassica species, these loci are GSL-Elong, GSL-OX, GSL-ALK, and GSL-OH. The GSL-Elong locus encodes a set of methylthioalkylmalate synthases that control the carbon chain length of the final glucosinolate structure (Benderoth et al., 2006). GSL-OX is controlled by differential expression of a family of flavin monoxygenases that oxidize methylthioalkyl glucosinolates to their corresponding methylsulfanylalkyl derivatives (Hansen et al., 2007). The conversion of methylsulfanylalkyl glucosinolates to alkenyl and hydroxylalkyl glucosinolates in Arabidopsis is mediated by the GSL-AOP locus, which encodes two 2-oxoacid-dependent dioxygenases (2-ODDs) that have no close relationship with any other members of this large gene family (Fig. 1; Kliebenstein et al., 2001a).

The final member of this set of modular genetic loci is the uncloned GSL-OH locus, which controls the production of 2-hydroxybut-3-enyl glucosinolate (Fig. 1; Parkin et al., 1994; van Doorn et al., 1999; Kliebenstein et al., 2001c). This glucosinolate arises by oxidation of 3-butenyl glucosinolate to produce either the 2R- or 2S-hydroxylated derivative (Rossiter et al., 1990; Daubos et al., 1998). Arabidopsis contains both the 2R and 2S enantiomers of 2-hydroxybut-3-enyl glucosinolate, whereas Brassica napus makes only the 2R enantiomer and Crambe abyssinica makes only the 2S enantiomer (Daubos et al., 1998; van Doorn et al., 1998). This suggests that there may be enzymatic differences in the production of 2-hydroxybut-3-enyl glucosinolate among different plant species.

The production of 2-hydroxybut-3-enyl glucosinolate has diverse biological consequences. It is toxic to insects, bacteria, and nematodes, inhibits the germination of various plant species, and is a major source of bitter flavor in Brassica vegetables (Donkin et al., 1995; Angelini et al., 1998; van Doorn et al., 1998; Manici et al., 2000; Peterson et al., 2000). In addition, upon tissue disruption, 2-hydroxybut-3-enyl glucosinolate can be hydrolyzed to form an oxazolidine-2-thione derivative (goitrin), a compound that causes goiter disease in mammals and is a major impediment to the use of Brassica crops as cattle feed (Greer, 1956; Greer and Deeney, 1959; Pearson et al., 1983). Interestingly, if 2-hydroxybut-3-enyl glucosinolate is converted to the simple nitrile instead, as can occur in several Brassica and Arabidopsis genotypes, the resulting compound can stimulate antioxidant pathways in humans (Nho and Jeffery, 2004). Thus, identification of the gene(s) responsible for the GSL-OH locus could aid investigative inquiries into numerous biological phenomena.

To identify the genes required to produce 2-hydroxybut-3-enyl glucosinolate and its resulting biological activity, we have undertaken a genetic, biochemical, and molecular analysis of the production of 2-hydroxybut-3-enyl glucosinolate. We utilized natural variation in gene expression as well as T-DNA mutations within Arabidopsis to show that a novel 2-ODD is required to form 2-hydroxybut-3-enyl glucosinolate. The genetic analysis also suggested that this enzyme determines the total level of aliphatic glucosinolates accumulated and resistance to a generalist insect herbivore. Interestingly, this insect resistance appears to be dependent upon the specific structure of the glucosinolate rather than the total accumulation of glucosinolates, as had

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**Figure 1.** Dihomomethionine-derived glucosinolate side chain modification. Shown are the major foliar dihomomethionine glucosinolates that accumulate in Arabidopsis accessions. The names of the glucosinolates are below the corresponding structures. The QTL controlling each reaction is listed to the right of the reaction’s arrow. In parentheses is included the gene or enzyme underlying the associated QTL for each given reaction.
been found previously (Kliebenstein et al., 2002; Lankau, 2007; Beekwilder et al., 2008).

RESULTS
Natural Variation of 2-Hydroxybut-3-enyl Glucosinolate in Arabidopsis

In Brassinia, the occurrence of 2-hydroxybut-3-enyl glucosinolate is controlled by the allelic status at the GSL-OH locus and the presence of its precursor, but-3-enyl glucosinolate, whose production is in turn regulated by the GSL-Elong and GSL-AOP loci (Magrath et al., 1994; Mithen and Toroser, 1995; Fig. 1). A previous analysis suggested that there is a GSL-OH locus in Arabidopsis but that the segregation of naturally occurring knockout alleles at GSL-Elong and GSL-AOP limits the accessions that accumulate 3-butenyl glucosinolate (Kliebenstein et al., 2001a, 2001b, 2001c). Analysis of 38 accessions identified 27 that accumulated but-3-enyl glucosinolate in the leaves and seeds. The presence or absence of 2-hydroxybut-3-enyl glucosinolate in leaf and seed tissues suggested three different phenotypic GSL-OH variants (Supplemental Table S1). Most accessions, such as Aberdeen, have a full-functional variant that contain high levels of 2-hydroxybut-3-enyl glucosinolate in both leaves and seeds (Supplemental Table S1). In contrast, Sorbo, Shakdara, and Kas-1 (and others) contain 2-hydroxybut-3-enyl glucosinolate only in the seed (seed-functional variant), while Cape Verde Island (Cvi) contains no 2-hydroxybut-3-enyl glucosinolate in either tissue (null variant; Supplemental Table S1). It remains to be tested if these variants are alleles of the same locus. All accessions with 2-hydroxybut-3-enyl glucosinolate contained both the 2R and 2S enantiomers in the identical ratios (2R:2S) of approximately 1:3, as found previously, and as such the two forms are summed and presented as the total 2-hydroxybut-3-enyl glucosinolate (Kliebenstein et al., 2001c).

Genetics of 2-Hydroxybut-3-enyl Glucosinolate in a Ler × Cvi Population

Neither Landsberg erecta (Ler) nor Cvi accumulates 2-hydroxybut-3-enyl glucosinolate (Kliebenstein et al., 2001b, 2001c). However, analysis of the Ler × Cvi recombinant inbred line (RIL) population showed that 37 of the 162 RILs accumulated 2-hydroxybut-3-enyl glucosinolate in the seeds (Kliebenstein, et al., 2001b). Utilizing these data, we mapped quantitative trait loci (QTL) for the production of 2-hydroxybut-3-enyl glucosinolate. Two of the loci responsible for this conversion mapped to the cloned GSL-Elong and GSL-AOP loci, which are required for production of the precursor but-3-enyl glucosinolate (Fig. 2; Kliebenstein et al., 2001a, 2001c). The third locus, GSL-OH, mapped to chromosome II between the markers FD150.C and Erecta (Parkin et al., 1994; Alonso-Blanco et al., 1998; Fig. 2). The Ler allele at this locus allowed the production of 2-hydroxybut-3-enyl glucosinolate in the seed but not the leaf. This suggests that the Ler parent contains the seed-functional GSL-OH variant while Cvi contains the null GSL-OH variant and that they are alleles of the same locus. Moreover, Ler × Cvi F1 individuals displayed the seed-functional phenotype showing that the seed-functional GSL-OH comple-
ments the null functional variant, further supporting the hypothesis that the two variants are alleles of the same locus.

Further mapping showed that GSL-OH mapped between microsatellite markers: 2 centimorgan (cM) north of T1D16 and 0.3 cM south of F13D4 (Fig. 3; Supplemental Table S2). This genomic region contains seven bacterial artificial chromosomes (BACs) and two potential genes that encode enzymes capable of hydroxylating but-3-enyl glucosinolate to its 2-hydroxyl derivative, a cytochrome P450 monoxygenase and a 2-ODD (Fig. 3).

**Fine-Scale Mapping of GSL-OH in Cvi × Tacoma**

To fine-scale map GSL-OH, we generated F2 progeny from a cross between Cvi (nonfunctional GSL-OH) and Tacoma (Tac; fully functional GSL-OH; Supplemental Table S1). These two parents were chosen because they have the same GSL-Elong and GSL-AOP alleles and, as such, only GSL-OH segregates. A total of 192 F2 progeny were analyzed by HPLC for the presence of 2-hydroxybut-3-enyl glucosinolate and genotyped at T1D16 and F13D4. GSL-OH again mapped to a single locus 2.6 cM north of T1D16 and 1.5 cM south of F13D4 (Fig. 3). Additionally, in Cvi × Tac F1 individuals, the full-functional GSL-OH phenotype complemented the seed-functional GSL-OH allele (data not shown). Thus, the full-functional, seed-functional, and null GSL-OH phenotypes are caused by different alleles at a single GSL-OH locus. To further fine-scale map GSL-OH, we generated microsatellite markers on T19L18, F17H15, F13B15, and T22F11. This showed that there was no recombination between GSL-OH and microsatellite F13B15 in a total of 580 F2 progeny (Fig. 3). The F13B15 BAC contains only the 2-ODD candidate gene, At2g25450, suggesting that the enzyme encoded by this gene may have the capacity to catalyze the hydroxylation of but-3-enyl glucosinolate to form 2-hydroxybut-3-enyl glucosinolate.

**Association of At2g25450 Sequence Variation and the GSL-OH Allele**

We sequenced the predicted full-length At2g25450 open reading frame (ORF) from accessions that contained the three GSL-OH variants to determine whether the candidate gene sequence covaried with changes in the accumulation pattern of 2-hydroxybut-3-enyl glucosinolate. The At2g25450 ORF is 1,077 nucleotides long and encodes a protein of 359 amino acids. The cDNAs from accessions with full-functional or seed-functional GSL-OH alleles are nearly identical, with only one to three nucleotide differences among the various copies (Fig. 3). In contrast, the cDNA sequences from accessions with null GSL-OH alleles clearly separated into two groups. Analysis of the At2g25450 cDNA sequences from the GSL-OH null phenotype confirmed the 1-mL reaction results and repeated three times.
accessions Kondara and Hodja showed that they share a C-to-T substitution, which generates a TAG stop codon. This truncates the protein to 131 amino acids and is probably the reason for the lack of GSL-OH activity in these two accessions. In contrast, the Cvi At2g25450 cDNA encoded an intact ORF that has six nucleotide polymorphisms in comparison with the other sequences, with five of the six polymorphisms being nonsynonymous substitutions (R288-I, G254-E, P218-H, L184-F, and G105-S, with the first amino acid being the consensus and the second being Cvi). Additionally, all five amino acid changes are nonconservative substitutions that could significantly alter the structure of the encoded enzymes. Any of these changes may be sufficient to inactivate At2g25450 and cause the null phenotype in Cvi.

At2g25450 Expression Correlates with GSL-OH Allelic Status

To further corroborate the relationship between At2g25450 and GSL-OH, the expression of At2g25450 in leaves of Arabidopsis accessions was compared with the GSL-OH allele variant present in each accession based on its glucosinolate composition. In all accessions tested, the fully functional GSL-OH allele was associated with high At2g25450 transcript accumulation in the leaf (Fig. 3). In contrast, all accessions with the seed-functional allele had low to nondetectable GSL-OH expression in the leaf (Fig. 3).

HPLC analysis of leaves from accessions with the fully functional allele identified heritable differences in the ratio of but-3-enyl glucosinolate to 2-hydroxybut-3-enyl glucosinolate (Kliebenstein et al., 2001c). Interestingly, there are also differences in At2g25450 expression among these functional GSL-OH accessions (Fig. 3, compare Tac with Mt-0). To test if differences in At2g25450 gene expression could explain the different 2-hydroxyl-but-3-enyl glucosinolate production, we compared GSL-OH expression with the accumulation of 2-hydroxyl-but-3-enyl glucosinolate in 11 accessions containing but-3-enyl glucosinolate. This showed a strong positive correlation between At2g25450 expression and the amount of 2-hydroxybut-3-enyl glucosinolate (Fig. 4; n = 11, r = 0.817, P = 0.001). The combination of fine-scale mapping and association in the accessions suggests that the enzyme encoded by At2g25450 could be responsible for the 2-hydroxylation of 3-butenyl glucosinolate.

At2g25450 Expression and GSL-OH Enzyme Activity

At2g25450 is expressed in some accessions that do not contain but-3-enyl glucosinolate, the substrate for the encoded enzyme (Fig. 3). For example, the Ei-2 accession does not accumulate any but-3-enyl or 2-hydroxybut-3-enyl glucosinolate (Fig. 5A). However, after incubating Ei-2 leaves with extract containing but-3-enyl glucosinolate (Fig. 5B), the leaves took up the but-3-enyl glucosinolate precursor and converted it into both enantiomeric forms of 2-hydroxybut-3-enyl glucosinolate in the same ratio as observed previously for accessions producing 2-hydroxybut-3-enyl glucosinolate (Fig. 5C). This shows that At2g25450 expression and but-3-enyl glucosinolate-2-hydroxylase activity are found in accessions that do not contain the precursor glucosinolate. Utilizing this substrate-feeding protocol, we tested the correlation between At2g25450 expression and in vivo but-3-enyl glucosinolate-2-hydroxylase activity in 12 accessions that do not naturally contain the substrate. The absence of the precursor in these plants allowed for a simultaneous direct measurement of but-3-enyl glucosinolate uptake and its subsequent conversion to 2-hydroxybut-3-enyl glucosinolate. We observed a positive correlation between the accumulation of At2g25450 mRNA and but-3-enyl glucosinolate-2-hydroxylase activity (Fig. 5D; n = 11, r = 0.913, P < 0.0001).

T-DNA Mutation in At2g25450 Abolishes Glucosinolate-2-hydroxylase Activity

To further confirm the causal relationship between At2g25450 and 2-hydroxylase activity, we obtained a homozygous T-DNA insertion mutant in the Columbia (Col-0) background (SALK_089807). The T-DNA insertion is located in the second exon of At2g25450 and abolishes At2g25450 expression (Supplemental Fig. S1). While the standard Arabidopsis reference accession for T-DNA knockouts, Col-0, does not contain 2-hydroxybut-3-enyl glucosinolate due to a lack of
its precursor but-3-enyl glucosinolate, we relied on the fact that introduction of a functional AOP2 into Col-0 causes the accumulation of both but-3-enyl glucosinolate and 2-hydroxybut-3-enyl glucosinolate (Li and Quiros, 2003; Wentzell et al., 2007). Thus, Col-0 has a functional At2g25450. The At2g25450 T-DNA knockout was then crossed to Col-0 expressing AOP2, and homozygotes were obtained for all four genotypic combinations. Lines containing AOP2 and a functional At2g25450 accumulated both but-3-enyl glucosinolate and 2-hydroxybut-3-enyl glucosinolate (Fig. 6). In contrast, lines with AOP2 and the At2g25450 T-DNA knockout only accumulated the precursor but-3-enyl glucosinolate, with no detectable accumulation of 2-hydroxybut-3-enyl glucosinolate (Fig. 6).

As observed previously, introduction of a functional AOP2 into Col-0 results in an elevated total aliphatic glucosinolate level (Fig. 6; Wentzell et al., 2007). Interestingly, combining AOP2 expression in Col-0 with an At2g25450 T-DNA mutation led to a further increase in aliphatic glucosinolate accumulation. This suggests that At2g25450, in addition to producing 2-hydroxybut-3-enyl glucosinolate, also affects aliphatic glucosinolate accumulation. As this effect requires a functional AOP2 gene, it suggests that this repressive function is not an inherent property of the At2g25450 transcript or protein. Instead, it is possible that the 2-hydroxybut-3-enyl glucosinolate product may feedback regulate the biosynthetic pathway or that At2g25450 directly influences the inductive capacity of AOP2 (Fig. 6; Wentzell et al., 2007). These results confirm that At2g25450 is the GSL-OH locus and likely encodes the enzyme responsible for the conversion of but-3-enyl to 2-hydroxybut-3-enyl glucosinolate, which, interestingly, also affects the regulation of the aliphatic glucosinolate pathway.

GSL-OH and Trichoplusia ni Herbivory

Arabidopsis resistance against generalist lepidopteran herbivory has been consistently shown to be controlled by increasing glucosinolate content or activation (Kliebenstein et al., 2002; Barth and Jander, 2006; Gigolashvili et al., 2007; Beekwilder et al., 2008). This negative relationship between glucosinolate accumulation and T. ni herbivory would predict that the higher glucosinolate content in Col-0 with AOP2 and At2g25450 should increase resistance to T. ni herbivory (Fig. 6). Until now, most studies on Arabidopsis-insect interactions have focused on the Col-0 accession, due to the plethora of tools and information available for this accession. However, Col-0 accumulates only nonalkenyl glucosinolate structures; therefore, it has not been tested what effect the different

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**Note:** The diagram in Figure 5 illustrates the conversion of exogenous but-3-enyl to 2-hydroxybut-3-enyl glucosinolate in selected Arabidopsis accessions. The experimental conditions described above are important for understanding the role of chemical structure in biotic resistance.
chemical structures have on \( T. ni \). To test if the chemical structure or content of glucosinolates modulate insect herbivory, we conducted a no-choice herbivory assay with first instar \( T. ni \) larvae using the above lines that vary for the AOP2 and \( \text{At2g25450} \) loci. This showed a highly significant effect of glucosinolate chemical structure, rather than total amounts of glucosinolates upon \( T. ni \) herbivory (\( n = 88, F_{3/88} = 9.01, P < 0.001 \)). Plant genotypes accumulating alkenyl glucosinolates were less resistant to \( T. ni \) herbivory than the Col-0 parent, with or without a functional \( \text{At2g25450} \) (Fig. 7). This is not due to the methylsulfinylalkyl glucosinolates being differentially induced, as glucosinolate content after 96 h of herbivory showed the same relationship of genotype to glucosinolate content as was found previously in the absence of herbivory (Fig. 6). This suggests that the glucosinolate structure is an important determinant of Arabidopsis-\( T. ni \) interactions and that alkenyl glucosinolates are less effective defense metabolites than the 4-methylsulfinylbutyl glucosinolate predominant in wild-type Col-0. Interestingly, the presence of a functional \( \text{At2g25450} \) imparted increased resistance to \( T. ni \) herbivory in the presence of the functional AOP2 gene (Fig. 7). Thus, converting a methylsulfinylalkyl glucosinolate to the alkenyl decreases resistance, but further conversion to the hydroxyl-alkenyl begins to restore insect defense in an evolutionary sense.

**GSL-OH Promoter Variation and Expression**

To identify the polymorphisms responsible for differential gene expression, we sequenced 4.9 kb of the

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**Figure 6.** Analysis of GSL-OH activity in an \( \text{At2g25450} \) T-DNA knockout mutant in the presence and absence of AOP2. Glucosinolate (GLS) content was determined in a segregating population from a Col-0 plant heterozygous for both \( 35S:AOP2 \) and the \( \text{At2g25450} \) T-DNA knockout allele. Values shown are mean accumulation and SE of the major aliphatic glucosinolates in nanomoles per milligram fresh weight of leaf tissue from at least 10 independent replicates. Glucosinolates that were not detected are listed as nd; other letters show mean glucosinolate contents that differ significantly by Student’s \( t \) test within that graph. The presence of the \( 35S:AOP2 \) gene is shown as + or −, and the status of \( \text{At2g25450} \) is shown as WT (wild type) or KO (knockout) at bottom. [See online article for color version of this figure.]

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**Figure 7.** Chemical structure rather than total glucosinolate content affects \( T. ni \) herbivory. Four genotypes generated with the \( \text{At2g25450} \) T-DNA knockout mutant and the AOP2 expression construct in a Col-0 background were assayed for resistance to \( T. ni \) herbivory. Two first instar larvae per plant were allowed to feed for 96 h on at least 20 plants per genotypic class. Each plant was physically separated to prevent the larvae from moving between plants. The presence of the \( 35S:AOP2 \) gene is shown as + or −, and the status of \( \text{At2g25450} \) is shown as WT (wild type) or KO (knockout) at bottom. The amount of leaf area removed by \( T. ni \) at 48, 72, and 96 h on each plant per genotype was measured in two independent experiments and analyzed via ANOVA. There was a significant difference between the genotypes at all three time points; here, 96-h herbivory data ± se are presented with significant differences as determined by \( t \) tests shown by letters. [See online article for color version of this figure.]
At2g25450 genomic region from the 12 accessions that differ in At2g25450 expression levels (Fig. 5). The sequenced region extended from the stop codon of the upstream gene to the start codon of the downstream gene and is expected to include all regulatory elements. Sequence comparison identified a unique 120-bp motif that is repeated five times in accessions with low leaf GSL-OH alleles and four times in full-functional alleles (Fig. 8; Supplemental Table S3). Two motifs overlap with the putative transcription start and stop sites, while the others are in the promoter region (Fig. 8). BLAST analysis against the complete Arabidopsis genomic sequence showed that this motif is not found elsewhere. The difference in motif number between the expression classes is caused by a deletion removing part of two intact motifs and the intervening DNA to produce one intact motif in the high-expression accessions (Fig. 8). The one accession with undetectable leaf expression, Ler, has two separate deletions that remove two-thirds of the two motifs flanking the ORF. Because the sequence extended from the upstream gene to the downstream gene in all accessions, this eliminates the possibility of a tandemly duplicated At2g25450. Thus, the three different variant classes are probably due to differential expression of At2g25450 caused by polymorphisms altering these unique motifs.

**DISCUSSION**

Several lines of evidence strongly support the identification of At2g25450 as the causal gene for the GSL-OH QTL. First, fine-scale mapping of 2-hydroxybut-3-enyl glucosinolate production in several Arabidopsis populations identified At2g25450 as the best candidate gene with the appropriate enzymatic activity to convert but-3-enyl to 2-hydroxybut-3-enyl glucosinolate (Figs. 2 and 3). Second, association experiments using Arabidopsis accessions showed a nearly total congruence between At2g25450 expression and the production of 2-hydroxybut-3-enyl glucosinolate from both endogenous and exogenous precursor (Figs. 4 and 5). Furthermore, examination of existing genomic sequence polymorphism data indicates that At2g25450 does not have any linkage disequilibrium with its neighboring genes and hence varies independently of these genes in the accessions. This shows that this association between GSL-OH and sequence and expression variation in the accessions is centered on the At2g25450 gene itself and not on any proximal loci (Borevitz et al., 2007; Clark et al., 2007). Furthermore, three accessions with nonfunctional GSL-OH alleles have loss-of-function mutations in At2g25450. Finally, an independent T-DNA knockout in At2g25450 abolishes all measurable GSL-OH activity. In combination, these multiple and independent genetic lines of evidence show that At2g25450 is the GSL-OH QTL.

At2g25450 (GSL-OH) belongs to the 2-ODD gene superfamily, a group that has at least 100 members in Arabidopsis, but none of the closely related Arabidopsis 2-ODDs have any defined biochemical functions (Supplemental Fig. S2). 2-ODDs are nonmembranous dioxygenases with activities similar to those of the membrane-bound cytochrome P450 monooxygenases, but usually requiring Fe^{2+} ions and a 2-oxoacid such as ascorbate or oxoglutarate (De Carolis and De Luca, 1993; Prescott, 1993). 2-ODDs catalyze a range of hydroxylations, desaturations, and epoxidations, including the oxidation of 1-aminocyclopropane-1-carboxylic acid in ethylene biosynthesis and several different position-specific hydroxylations of gibberellins. As such, the allylic hydroxylation of a glucosinolate side chain is a likely reaction for a 2-ODD to catalyze. This is supported by the observation that other 2-ODDs closely related to the At2g25450 protein are known to catalyze hydroxylation reactions of sec-

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**Figure 8.** Variable regulatory motifs in the At2g25450 promoter. The long black rectangle represents the complete At2g25450 gene with the locations of the mRNA sequence and start/stop codon positions. The boxes below the genomic fragment show the locations of the identified repeat motifs. Black boxes are the positions of the repeated motif in accessions with low At2g25450 leaf expression, and light gray boxes are the motif positions in accessions with high At2g25450 leaf expression. The graph at right represents the average transcript accumulation and se of At2g25450 in the two allelic classes using the measured expression levels of the 12 accessions in Figure 5.
Glucosinolate Structure versus Content in Insect Herbivory Resistance

Total aliphatic glucosinolate content is often used as a variable to explain insect resistance without including information about the specific structures. Our data show that the different aliphatic glucosinolate structures are not functionally equivalent as herbivory deterrents in the Col-0 background (Fig. 7). The methylsulfinylalkyl glucosinolates were the most effective deterrents, with the hydroxyalkenyl glucosinolates being more effective than their alkenyl precursors. This is in spite of lines with methylsulfinylalkyl glucosinolates having the lowest levels of total aliphatic glucosinolates (Fig. 6). Thus, At2g25450, in addition to controlling hydroxyalkenyl glucosinolate production, also affects resistance to insect herbivory. The increased generalist lepidopteran resistance of hydroxyalkenyl glucosinolates over simple alkenyl glucosinolates may explain why most Arabidopsis accessions with alkenyl glucosinolates produce them as the hydroxyalkenyl form (Kliebenstein et al., 2001c; Lambrix et al., 2001).

This observation of alkenyl glucosinolates being a less effective generalist lepidopteran defense, specifically against T. ni, than methylsulfinylalkyl glucosinolates is in contradiction to the fact that half of the natural Arabidopsis accessions make alkenyl glucosinolates (Kliebenstein et al., 2001c; Lambrix et al., 2001). One explanation is that the Col-0 accession hydrolyzes glucosinolates to isothiocyanates, while nearly all wild Arabidopsis accessions with alkenyl glucosinolates form thionitrites on hydrolysis (Kliebenstein et al., 2001c; Lambrix et al., 2001). Thus, alkenyl glucosinolates may be a more effective defense in a nitrile-producing background. This was suggested by a previous analysis of the nitrile-producing Ler × Cvi RIL population, in which alkenyl glucosinolates showed an ability to prevent generalist insect herbivory and methylsulfinylalkyl glucosinolates showed no correlation with insect defense (Supplemental Fig. S4; Kliebenstein et al., 2002). Thus, genetic variation in glucosinolate hydrolysis may epistatically determine the relative effectiveness of the different glucosinolate structures in deterring generalist lepidopteran herbivory. Accordingly, adequate assessment of the role of glucosinolate content, structure, and hydrolysis in herbivore resistance will require the generation of a population of nearly isogenic lines that simultaneously vary with respect to glucosinolate content, glucosinolate structure, and hydrolysis structure. The recent identification of single gene mutants in all of these processes allows us to recreate this full phenotypic matrix in a single defined polygenic population (Haughn et al., 1991; Kroymann et al., 2001; Lambrick et al., 2001; Zhang et al., 2006; Gigorashvili et al., 2007; Hansen et al., 2007; Hirai et al., 2007; Sønderby et al., 2007; Wentzell et al., 2007).

The Expression of Hidden Enzymes

Numerous accessions lacking the but-3-enyl glucosinolate precursor nevertheless had detectable At2g25450 mRNA and measurable in vivo 2-hydroxylase activity (Fig. 8). If this enzyme has no natural substrate in these plants, one would expect the encoding gene to rapidly lose its function over the course of evolutionary time. Why then is GSL-OH activity found in lines lacking the necessary precursor? One possibility is that the levels of out-crossing and levels in Arabidopsis are sufficient to maintain a functional GSL-OH even in lines lacking precursor. An alternative is that the enzyme is multifunctional and required for another biosynthetic reaction. However, this second reaction, if it exists, must not be critical for basic growth and development, because the accessions Kondara and Hodja, which have premature stop codons, and the T-DNA insertion line are all viable (Fig. 9). Furthermore, this second reaction cannot affect insect herbivory or glucosinolate content in the absence of the but-3-enyl precursor (Figs. 6 and 7).

The presence of enzymes lacking precursors has interesting implications for the manipulation of secondary metabolic pathways. It has been proposed to utilize transgenic technologies to introduce new biosynthetic capacities into plants. However, the presence of hidden enzyme activities makes it difficult to predict the final compound that may accumulate from a given transgenic modification. As more and more genes of secondary metabolism are studied, it will be interesting to see what proportion of these encode enzyme activities that are hidden by the lack of an endogenous substrate.

Stereoselectivity of the 2-Hydroxylase

The stereoselectivity of but-3-enyl glucosinolate-2-hydroxylase varies among species within the Brassicaceae. In Arabidopsis, both the 2R and 2S enantiomers of 2-hydroxybut-3-enyl glucosinolate are produced in a constant ratio (2R:2S) of 1:3. While some enzyme reactions lead to a racemic mixture of products, it is unusual to find a reaction that yields a mixture of enantiomers in a fixed, nonequivalent ratio. In con-
Arabidopsis and Arabis alpina make only the 2R enantiomer of 2-hydroxybut-3-enzyme glucosinolate, while Crambe abyssinica makes only the 2S enantiomer (Daubos et al., 1998; van Doorn et al., 1998). The lack of stereospecificity in Arabidopsis could result from the fact that the enzyme has evolutionarily lost the ability to form a single enantiomer. Alternatively, the enzyme may not have been altered, but the species may have lost an associated protein that imparts stereospecificity to the enzyme. Another possibility is that the different species may use completely different enzymes for the production of 2-hydroxybut-3-enzyme glucosinolate. This final possibility is supported by the analysis of crucifer species thought to be more closely related to Arabidopsis than A. alpina is. Only one of these species contains the substrate, but-3-enzyme glucosinolate, and none of these species has 2-hydroxybut-3-enzyme glucosinolate (Daxenbichler et al., 1991; Fahey et al., 2001; Koch et al., 2001; Windsor et al., 2005). Because the split between Arabidopsis and A. alpina was at least 20 million years ago, either Arabidopsis is the only species in the entire clade to have maintained the ancestral 2-hydroxyenzyme activity or Arabidopsis revolved this activity on its own. Comparison of GSL-OH sequences between these species should distinguish between these possible explanations.

**Future Work**

The isolation of the gene responsible for converting but-3-enzyme glucosinolate to 2-hydroxybut-3-enzyme glucosinolate provides a key molecular tool for studying the function and formation of 2-hydroxybut-3-enzyme glucosinolate. The GSL-OH gene creates new opportunities for the modification of glucosinolate-containing agricultural species. 2-Hydroxybut-3-enzyme glucosinolate is goiterogenic because the hydroxyl substituent promotes the formation of the cyclic 5-vinyl-oxazolidine-2-thione (Fenwick et al., 1983) from myrosinase hydrolysis-generated isothiocyanate. This substance inhibits iodine metabolism in the thyroid, causing goiter disease in most mammals. This problem has been previously dealt with by generating lines low in total glucosinolate content. The identification of At2g25450 could allow for specific suppression of the 2-hydroxyenzyme activity in certain Brassica crops, allowing for their widespread use as animal food while maintaining their endogenous glucosinolates for insect defense. The gene also enables specific transgenic or genetic modification of the ability to produce 2-hydroxybut-3-enzyme glucosinolate in nearly isogenic lines to allow testing of the specific effects of this glucosinolate on herbivores, pathogens, and competing plant species.

**MATERIALS AND METHODS**

**Accession Analysis**

All Arabidopsis (Arabidopsis thaliana) plant lines were obtained from the Arabidopsis Stock Center. Ten plants were grown in 3.25×3.25×2.25-inch pots at 18 pots to a flat for 3 weeks in a standard soil-vermiculite mixture at 26°C. They were placed 10 inches from four 60-W cool-white GE bulbs and four 60-W wide-spectrum bulbs in a 16-h-light/8-h-dark photoperiod. This study utilized a collection of 37 accessions, as listed in Supplemental Table S1.

**Sample Preparation and HPLC**

Samples were extracted and analyzed by HPLC as described previously (Kliebenstein et al., 2001c). Specific glucosinolates were identified by comparison of retention times and UV absorption spectra with purified standards. All glucosinolate absorption data (measured at 229 nm) were converted to nanomoles per gram dry weight using response factors determined from the purified standards for each of the glucosinolates (Kliebenstein et al., 2001c; Reichelt et al., 2002).

**Microsatellites**

DNA was isolated with the 96-well DNA prep described previously. The primers listed in Supplemental Table S2 were utilized for fine-scale mapping in both the Ler × Cvi and Cvi × Tac populations. Five microsatellites of the 96-well DNA prep was added to 20 μL of PCR mixture (2.5 μM MgCl2, 200 μM primers, and 0.5 units of Taq) and run with the following cycle program (95°C for 3 min; 40 cycles of 95°C for 20 s, 56°C for 20 s, and 72°C for 1 s; 72°C for 3 min; and 4°C final). The microsatellites were then scored on 4% agarose.

**Genetic Mapping of GSL-OH**

G glucosinolates were extracted and analyzed from 5 mg of seeds from each Ler × Cvi RIL to phenotype the GSL-OH activity. Scoring of the Ler × Cvi RILs was done by analyzing the seeds for the presence of the 2R- and 2S-2-hydroxybut-3-enzyme glucosinolate as well as for the benzylated derivatives 2R- and 2S-2-benzoxyl-but-3-enzyme glucosinolate. Lines that produced these glucosinolates were given a score of 1. Lines that did not contain any of the detectable products were given a score of 0. QTL mapping was done with QTL Cartographer using composite interval mapping and 1,000 permutations to estimate the 0.05 significance threshold (Basten et al., 1999). Mapmaker version 3 and the RILs that accumulated but-3-enzyme glucosinolates were used to refine the chromosome II map position (Lander et al., 1987).

For the Cvi × Tac mapping experiment, homozygous Cvi and Tac were crossed and the resulting F1 progeny were selfed to generate an F2 population. A total of 192 Cvi × Tac F2 plants were simultaneously scored for the T1D16 and F13D4 microsatellites (Supplemental Table S2) and the GSL-OH phenotype by HPLC. They were phenotyped by planting a single F2 seed per cell in a 96-well flat. The seeds were allowed to germinate and grown for 4 weeks under a 9-h-light/15-h-dark photoperiod. After 4 weeks, two to three leaves were harvested for glucosinolate extraction and HPLC analysis, and one to two leaves were taken for DNA purification. After confirming that the GSL-OH locus was between T1D16 and F13D4, an additional 388 F2 progeny were genotyped with these microsatellites to identify recombinant progeny. All recombinant progeny were then scored for the T19L18#2, T22F11, and F17H15 microsatellites (Supplemental Table S2), and their HPLC phenotypes were scored in the F3 generation.

**In Vivo Assay for But-3-enzyme Glucosinolate-2-hydroxyenzyme Activity**

But-3-enzyme glucosinolate was obtained from 30 g of freeze-dried Cvi and extracted with 200 mL of methanol for 4 h. Cvi contains but-3-enzyme glucosinolate and has no detectable accumulation of 2-hydroxybut-3-enzyme glucosinolate (Kliebenstein et al., 2001c). The extract was centrifuged to remove insoluble material, dried, resuspended in 15 mL of water, centrifuged to remove any precipitate, and stored at 4°C until needed. Plants to be assayed were grown for 4 weeks as described above, and 10 leaves with petioles were removed and placed into small petri plates with 30 mL of water such that the leaf was lying on the water with the cut petiole end completely submerged.

Approximately 50 μL of the glucosinolate extract was added to the water. The leaves were then incubated in the solutions for 2 or 4 d under 24-h light. Controls incubated in pure water were run simultaneously. The 10 leaves were then divided into two samples of five leaves each, and the glucosinolates were extracted and analyzed by HPLC as described. But-3-enzyme glucosinolate-2-hydroxyenzyme activity was estimated by taking the sum of the
R and 5 forms of 2-hydroxybutyl-3-enyl glucosinolate and dividing this by the total of the but-3-enyl glucosinolate plus R and 5 forms of 2-hydroxybutyl-3-enyl glucosinolate. This was then divided by the number of days for which the sample was incubated to generate a per day activity measure. The least-square means for the average conversion from three independent experiments are presented.

**cDNA and DNA Preparation for Sequencing**

Total RNA was isolated from approximately 100 mg of leaf tissue utilizing the Trizol reagent. Approximately 1 µg of total RNA was utilized for cDNA synthesis as described (Frohman et al., 1988). One to 8 µL of the resulting total cDNA was then added to 25-µL PCR samples with the GSOH-F and GSOH-R primers from Supplemental Table S2 to amplify the At2g25450 cDNA. The resulting product was then separated on a 1.5% agarose gel, the band was removed, and the cDNA was purified with Qagen gel purification columns. The resulting cDNA was sequenced with the primers from Supplemental Table S2 using Perkin-Elmer Big dye terminator chemistry on a Perkin-Elmer 3700 sequencer. The sequences were then analyzed with the DNAsat analysis package. Trees were generated utilizing TREECON for Windows version 1.3b with 1,000 reiterations and neighbor-joining analysis. Genomic DNA for sequencing of the 4.9-kb gene was prepared by designing primers to split the gene into two 2.6-kb pieces, and each piece was independently amplified, cloned, and sequenced as described above. The primers utilized are listed in Supplemental Table S2. The accessions sequenced for the full gene were Col-0, Cvi, Di-1, Ei-2, Hodja, Kondara, L, Mk-0, Mt-0, Tac-0, and Tsu-1.

**Quantitative Reverse Transcription-PCR**

cDNA was generated as described previously. Primers for the RAN cDNA were utilized to standardize for the amount of cDNA present in each of the samples. Each accession utilized in this study was first sequenced as described above. This sequence was then used to generate the GSOHRT-F and GSOHRT-R primers, which are perfectly homologous to all of the accessions tested (Supplemental Table S2). The GSOH and RAN primers were then used to amplify specific cDNAs from 10, 1, and 0.1 µL of starting cDNA. The resulting products were then separated on a gel and detected with an Eagle Eye and ethidium bromide. The resulting files were transferred to a phosphoimager, which was used in densitometric mode to measure the cDNA produced. Each accession was measured in triplicate.

**Analysis of the At2g25450 T-DNA Mutant**

A T-DNA mutation in the second exon of At2g25450, SALK_089807, was obtained from the Arabidopsis Biological Resource Center. This was then crossed three independent times to Col-0 containing a functional AOP2 transgene (Wentzell et al., 2007). At least five homozygous plants for each of the four genotypic classes were obtained per cross and measured via HPLC for glucosinolate content. This was replicated twice, and the homozygous plants were selfed to generate homozygous F3 families. Individual and total aliphatic glucosinolate accumulation was then analyzed via ANOVA using a general linear model. In this model, y denotes the glucosinolate accumulation on Arabidopsis genotype g from cross c in replicate r. The ANOVA model for the glucosinolate accumulation is $y_{grc} = \mu + G_g + C_c + R_r + e_{grc}$ where $e_{grc}$ represents the error and is assumed to be normally distributed with mean 0 and variance $\sigma^2$. Cross and replicate did not show any significance in this analysis. Genotypic means were compared within the model using $t$ tests.

**Trichoplusia ni Herbivory Analysis**

The lines generated using the At2g25450 T-DNA mutation and the functional AOP2 transgene were used for assaying resistance to T. ni herbivory. At least 10 plants for each of the four genotypic classes were obtained per cross. Each plant was in an individual pot, and these pots were physically separated to prevent larval movement. T. ni eggs were obtained from Benzon Research and hatched on artificial medium. After 48 h, two first instar larvae were placed on each plant, and herbivory was measured every 24 h by visual measurements whereby leaf area removed was estimated using a ruled grid. The entire experiment was replicated twice. Herbivory at each time point was independently analyzed via ANOVA using a general linear model. In this model, $y_{grc}$ denotes the herbivory in square centimeters on Arabidopsis genotype g from cross c in replicate r. The ANOVA model for the glucosinolate accumulation is $y_{grc} = \mu + G_g + C_c + R_r + e_{grc}$ where $e_{grc}$ represents the error and is assumed to be normally distributed with mean 0 and variance $\sigma^2$. Cross and replicate did not show any significance in this analysis. Genotypic means were compared within the model using $t$ tests.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1. Expression of At2g25450 in SALK_089807.**

**Supplemental Figure S2. Dendrogram depicting the phylogenetic relationships of Arabidopsis GSL-OH to other closely related Arabidopsis 2-ODDs.**

**Supplemental Figure S3. GSL-OH variation in Arabidopsis accessions.**

**Supplemental Table S1. **

**Supplemental Table S2. **

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