Subclade of Flavin-Monoxygenases Involved in Aliphatic Glucosinolate Biosynthesis

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Glucosinolates (GSLs) are amino acid-derived secondary metabolites with diverse biological activities dependent on chemical modifications of the side chain. We previously identified the flavin-monoxygenase FMOGS-OX1 as an enzyme in the biosynthesis of aliphatic GSLs in Arabidopsis (Arabidopsis thaliana) that catalyzes the S-oxygenation of methylthioalkyl to methylsulfinylalkyl GSLs. Here, we report the fine mapping of a quantitative trait locus for the S-oxygenating activity in Arabidopsis. In this region, there are three FMOs that, together with FMOGS-OX1 and a fifth FMO, form what appears to be a crucifer-specific subclade. We report the identification of these four uncharacterized FMOs, designated FMOGS-OX2 to FMOGS-OX5. Biochemical characterization of the recombinant protein combined with the analysis of GSL content in knockout mutants and overexpression lines show that FMOGS-OX2, FMOGS-OX3, FMOGS-OX4, and FMOGS-OX5 have broad substrate specificity and catalyze the conversion from methylthioalkyl GSL to the corresponding methylsulfinylalkyl GSL independent of chain length. In contrast, FMOGS-OX5 shows substrate specificity toward the long-chain 8-methylthiooctyl GSL. Identification of the FMOGS-OX subclade will generate better understanding of the evolution of biosynthetic activities and specificities in secondary metabolism and provides an important tool for breeding plants with improved cancer prevention characteristics as provided by the methylsulfinylalkyl GSL.

Glucosinolates (GSLs) are amino acid-derived secondary metabolites present in the order Brassicales. Upon disruption of plant tissue by, for example, wounding or mastication, GSLs are hydrolyzed by the thioglucosidases, myrosinases, which produce a range of breakdown products, primarily isothiocyanates and nitriles, with diverse biological activities (Halkier and Gershenzon, 2006; Zhang et al., 2006b). GSLs (typically their hydrolysis products) act as antimicrobials against pathogens, feeding deterrents toward generalist herbivores, and as attractants for specialist herbivores (Kliebenstein et al., 2001a; Tierens et al., 2001). For humans, the well-studied sulforaphane, which is derived from 4-methylsulfinylbutyl (4-MSB) GSL and the isothiocyanates derived from 7-methylsulfinylethyl (7-MSH) GSL and 8-methylsulfinylloctyl (8-MSO) GSL, are considered very potent cancer-preventive agents because of their strong induction of xenobiotic phase II detoxification enzymes in animals (Zhang et al., 1992; Rose et al., 2000). These isothiocyanates can decrease the risk of developing different cancers, such as breast cancer (Rose et al., 2005), gastric cancer (Fahey et al., 2002), and skin cancer (Talalay et al., 2007). The potent cancer-preventive property of the hydrolysis product of methylsulfinylalkyl (MS) GSLs makes it desirable to characterize the genes that encode for the enzymes that catalyze the S-oxygenation reaction from methylthioalkyl (MT) to MS GSLs.

Biosynthesis of MS GSLs can be divided into three separate phases (i.e. Met chain elongation, GSL core synthesis, and, finally, an S-oxygenating reaction [Fig. 1]). An Arabidopsis (Arabidopsis thaliana) flavin-monoxygenase FMOGS-OX1 was recently shown to catalyze the S-oxygenation of MT to MS GSLs (Hansen et al., 2007). The presence of MS GSLs in FMOGS-OX1 T-DNA knockout mutants indicated that additional genes catalyzing this reaction are present (Hansen et al., 2007).

The conversion of MT to MS GSLs was originally studied via genetic means using natural variation in Arabidopsis and Brassica napus as defined by the GSL S-oxygenation (GS-OX) quantitative trait loci (QTLs; Giamoustaris and Mithen, 1996; Kliebenstein et al., 2001a, 2001b, 2002). These studies identified several independent GS-OX loci that mapped near FMOGS-OX1, although they did not overlap with the physical position of FMOGS-OX1. These GS-OX loci even showed

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[1] The online version of this article contains Web-only data.
structural specificity such that some affected all aliphatic GSLs, whereas others were specific to subsets of substrates. The QTL study suggests that the GS-OX loci near the physical position of FMOGS-OX1 may contain the candidate genes for the additional MT to MS-oxygenating enzyme activity previously suggested (Hansen et al., 2007).

In this article, we identify four FMOGS-OX1-related genes encoding for enzymes that catalyze the MT to MS-oxygenation reaction. By fine genetic mapping analysis, we found that three FMOGS-OX1 homologs, At1g62540, At1g62570, and At1g62560, mapped to a 0.2-Mb area containing a GS-OX QTL in multiple Arabidopsis populations. The five FMO genes At1g65860 (FMOGS-OX1), At1g62540 (FMOGS-OX2), At1g62560 (FMOGS-OX3), At1g62570 (FMOGS-OX4), and At1g12140 (FMOGS-OX5) were found within a subclade of the FMO phylogeny that (at least presently) consists of genes from only cruciferous species. Furthermore, we characterize the Arabidopsis enzymes in this subclade and show that FMOGS-OX2 to FMOGS-OX4 are able to catalyze the S-oxygenation independent of chain length, as was observed for FMOGS-OX1, and that FMOGS-OX5 is specific for 8-methylthiooctyl (8-MTO) GSL.

RESULTS

Phylogenetic Tree of Plant FMOs in Proposed Clade III

Previous phylogenetic analysis had shown three plant FMO clades from the genomic sequence of rice (Oryza sativa), Arabidopsis, and poplar (Populus tremuloides; Hansen et al., 2007). Clade III is the putative S-oxygenation clade and contains what appears to be crucifer-specific radiation of FMOs, which includes the biochemically characterized FMOGS-OX1 (Hansen et al., 2007). In the intervening time, several additional plant genomic sequences became available and allowed for retesting of this FMO clade’s specificity to crucifers. To do this, we obtained all of the FMOs from the genomic sequences of poplar, rice, Medicago truncatula, grape (Vitis vinifera), and Physcomitrella patens and created a complete phylogeny to identify those FMOs present in clade III, which has a single ancestor present in P. patens. To further extend this analysis, we utilized the FMOGS-OX and related Arabidopsis sequences to identify all ESTs that were at least 80% identical at the amino acid level. These ESTs identified one potato (Solanum tuberosum) FMO, two Citrus clementine FMOs, and a set of crucifer FMOs from Raphanus, Brassica, and Thalaginella ssp. (Fig. 2; Supplemental Table S1). Phylogenetic analysis showed that only genomic and EST sequences from the Cruciferae clustered within the FMOGS-OX subclade, leading us to hypothesize that this is a crucifer-specific clade of FMOs (Fig. 2). Because GSLs are also unique to these plants, these crucifer-specific FMOs provide candidate genes for the residual MT to MS-S-oxygenating enzyme activity present in the FMOGS-OX subclade (Hansen et al., 2007). Interestingly, this clade is marked by numerous species-specific duplications as shown by the grape and rice radiations (Fig. 2).

Fine-Scale Mapping of GS-OX

Previous quantitative genetics analysis mapped a GS-OX locus on Arabidopsis chromosome I in crude proximity to the characterized FMOGS-OX1 (Kliebenstein et al., 2001b; Hansen et al., 2007). This GS-OX locus
controlled differences in the ratio of MT to MS GSLs in the Landsberg erecta (Ler) × Columbia-0 (Col-0) recombinant inbred line (RIL) population (Kliebenstein et al., 2001a). We have conducted a detailed analysis of the existing data in the larger Ler × Cape Verde Islands (Cvi) and Bay-0 × Shahdara (Sha) RIL populations. This showed that the GS-OX locus was in fact two closely linked loci that epistatically control the ratio of MT to MS GSLs (Supplemental Tables S2 and S3; Kliebenstein et al., 2001a; Wentzell et al., 2007). One of

**Figure 2.** Phylogenetic analysis of FMO<sub>GS-OX</sub> homologous sequences. At1g12140, At1g62580, and At1g65860 were used to search the available Genomic and EST databases for homologous FMO sequences in clade III (Hansen et al., 2007). The genomic sequences were utilized as full-length proteins. The EST sequences were aligned to identify the minimum unigene set. Clade A, Subclade that appears to be crucifer specific.
these two loci, as shown by the GH157 and F5I14 markers in the Ler × Cvi and Bay-0 × Sha populations, respectively, was tightly linked to FMOGS-OX1 (At1g65860; Fig. 3). Further, the At1g65860 transcript had a significant cis-expression QTL in the Bay-0 × Sha population that agreed with FMOGS-OX1 likely being the functional basis of one of the two GS-OX loci in this region (Wentzell et al., 2007; West et al., 2007). However, this FMOGS-OX1 QTL was not at the genetic position of the originally identified GS-OX locus (Kliebenstein et al., 2001b; Fig. 3).

To better resolve the molecular genetic basis of the originally identified GS-OX locus, we generated a F2 population by crossing the Ler and Wei-0 accessions, which have a strong difference in the GS-OX phenotype, but have the same allelic status at the epistatic GS-Elong and GS-AOP QTLs (Kliebenstein et al., 2001b; Wentzell et al., 2007). This population thereby allowed us to focus on GS-OX variation. We genotyped and HPLC phenotyped 400 F2 individuals and fine-scale mapped the GS-OX QTL in this population to a 200-kb region between 23.1 and 23.3 Mb on Arabidopsis chromosome I. This is in the same position as the original GS-OX locus from the Ler × Col-0 population, as well as proximal to the nga128 and HH375 markers from the Ler × Cvi and Bay-0 × Sha populations (Fig. 3; Loudet et al., 2002). This fine-scale map position did not include the FMOGS-OX1 gene, but did include three tandem duplicates that are the closest homologs of FMOGS-OX1, At1g62540, At1g62560, and At1g62570 (Fig. 2). Additionally, all three genes had cis-expression QTLs in the available Bay × Sha QTL data, suggesting that gene expression rather than enzyme activity variation in this tandem gene family underlies the GS-OX QTL linked to the nga128 and HH375 markers in multiple Arabidopsis populations (West et al., 2007). No recombination events between these genes could be identified. The phylogenetic relationship of these FMOs to a gene encoding an enzyme that converts MT to MS GSLs, their physical proximity to a locus controlling the conversion of MT to MS GSLs, as well as the presence of expression diversity, led us to hypothesize that these genes are FMOGS-OXs within Arabidopsis. We then proceeded to directly test this biochemical hypothesis on these FMOs and on At1g12140, which belongs to the proposed FMOGS-OX clade (Fig. 2).

**Recombinant FMOGS-OXs Catalyze the Conversion of MT to MS GSLs**

We previously showed that FMOGS-OX1 S-oxygenates desulfo and intact MT GSLs, but not other precursors in the Met-derived GSL biosynthesis pathway in Arabidopsis (Hansen et al., 2007). Therefore, we utilized desulfo MT GSLs as substrate to test whether the other Arabidopsis proteins in the FMOGS-OX subclade catalyzed the S-oxygenation of MT to MS GSLs. FMOGS-OX1, FMOGS-OX2, FMOGS-OX3, FMOGS-OX4, and FMOGS-OX5 were individually expressed in Escherichia coli. Spheroplasts of *E. coli* expressing these FMOGS-OX proteins were incubated with desulfo 4-methylthiobutyl (4-MTB) GSL for 1 h, and desulfo GSLs were analyzed by HPLC. Recombinant protein of FMOGS-OX1, FMOGS-OX2, and FMOGS-OX4 all catalyzed the production of desulfo 4-MSB GSL (Fig. 4, C–E) in levels comparable to the FMOGS-OX1 (Fig. 4B). Control spheroplasts transformed with empty vector did not show a significant production of desulfo 4-MSB (Fig. 4A). Recombinant protein of the most distant member of this subclade, FMOGS-OX5, did not convert 4-MTB into 4-MSB (Figs. 2 and 4F). This indicated that either FMOGS-OX5 cannot catalyze the S-oxygenation of MT GSLs or it may have substrate specificity for other MT GSLs than 4-MTB.
The aliphatic, Met-derived GSLs are divided into classes of different side chain lengths: short chain, propyl (C3) and butyl (C4); middle chain, pentyl (C5) and hexyl (C6); and long chain, heptyl (C7) and octyl (C8). Because 4-MTB is the only commercially available MT GSL, we extracted desulfo GSLs from seeds of the Arabidopsis accession Col-0 to use as substrates in the FMOGS-OX enzyme assay. 4-MTB and 8-MTO GSLs are the dominant GSLs in these seeds. This allowed us to test whether any of the FMOGS-OXs catalyzes the S-oxygenation of MT GSLs with chain lengths other than 4-MTB. Spheroplasts of E. coli expressing FMOGS-OX1, FMOGS-OX2, FMOGS-OX3, FMOGS-OX4, and FMOGS-OX5 were incubated with the desulfo GSLs derived from seeds, followed by HPLC analyses. Consistent with the previous work, FMOGS-OX1, FMOGS-OX2, FMOGS-OX3, and FMOGS-OX4 catalyzed the conversion of 4-MTB to 4-MSB (Fig. 5, B–F). Interestingly, all five recombinant proteins converted 8-MTO to 8-MSO (Fig. 5, B–F). Thus, FMOGS-OX1, FMOGS-OX2, FMOGS-OX3, and FMOGS-OX4, but not FMOGS-OX5, catalyzed the conversion of 4-MTB to 4-MSB (Fig. 5, B–F). Interestingly, all five recombinant proteins converted 8-MTO to 8-MSO (Fig. 5, B–F). Thus, FMOGS-OX1, FMOGS-OX2, FMOGS-OX3, and FMOGS-OX4 catalyze the S-oxygenating reaction for both short-chain and long-chain MT GSL, whereas FMOGS-OX5 has a more limited substrate specificity as indicated by its specificity for 8-MTO. This suggests that this cluster of FMOs is involved in GSL biosynthesis and that the enzymes have evolved different substrate specificities.

GSL Analyses of FMOGS-OX Knockout Mutants

To validate the S-oxygenation activities of these FMOs in planta, we obtained two independent T-DNA knockout mutants for, respectively, FMOGS-OX2 and FMOGS-OX4 and one T-DNA knockout mutant for, respectively, FMOGS-OX1 and FMOGS-OX3. These T-DNA mutants were confirmed as having no detectable transcript for the corresponding FMOGS-OX by reverse transcription (RT)-PCR (Supplemental Fig. S1). For each FMOGS-OX knockout mutant, we measured GSL content in leaves and seeds of segregating progeny obtained from a heterozygous parent (Supplemental Table S4). By analyzing the GSL content in wild-type and homozygous knockout plants in a segregating population derived from a single heterozygous parent, we minimize the influence of potential maternal effects. From the HPLC data, the ratio of MT GSL to the sum of MT and MS GSL, which represents the S-oxygenation activity for the conversion from MT GSL to MS GSL, was calculated for each chain length. For FMOGS-OX2 and FMOGS-OX4, there was no statistically significant difference between the MT:(MT + MS) ratios of the two independent T-DNA knockout mutants of the same gene, and the data from the mutants were pooled. In agreement with predicted biochemistry for FMOGS-OX2, its homozygous knockout mutants showed an increased ratio of MT:(MT + MS) for the butyl, pentyl, heptyl, and octyl Met-derived GSLs in both leaves and seeds in comparison with wild-type plants (Table I). A homozygous knockout mutant in the proposed long-chain-specific FMOGS-OX5 had an increased MT:(MT + MS) for C8 GSLs in seeds, but also for other chain lengths (Table II). This agrees with the observation that only 8-MTO was found to be a substrate for recombinant FMOGS-OX5. The observed changes in the knockout mutants are likely not absolute due to compensatory function present in the other
functioning FMOGS-OXs. In contrast, the T-DNA knockout mutant of FMOGS-OX4 did not show an increase in MT:(MT + MS) in either leaves or seeds (Table III). This may be due to low expression or low in planta activity in the Col-0 accession, in which case a mutant phenotype will be hidden by functional redundancy with the other FMOGS-OXs.

The only available T-DNA knockout mutant for FMOGS-OX3 is in the Ler accession. In contrast to the Col-0 accession, Ler has predominantly propyl C3 instead of butyl C4 Met-derived GSLs (Kliebenstein et al., 2001b). Another difference between the two ecotypes is that Ler expresses a functional AOP3 (2-oxoglutarate-dependent dioxygenase; Kliebenstein

![Figure 5. Analysis of S-oxygenation activity of FMOGS-OX1 to FMOGS-OX5 for methylthioalkyl GSL. The activity of each recombinant FMOGS-OX was detected in assays using desulfo GSL extracts from Arabidopsis seed with a final concentration of 2 mM total desulfo GSL as substrate. Spheroplasts of E. coli expressing empty vector were used as negative control. A, Negative control; B, assay of FMOGS-OX1; C, assay of FMOGS-OX2; D, assay of FMOGS-OX3; E, assay of FMOGS-OX4; F, assay of FMOGS-OX5. Ratio of 4-MTB:(4-MTB + 4-MSB) was calculated to represent the S-oxygenating activity for short-chain GSL. The significance of the difference between the ratio 4-MTB:(4-MTB + 4-MSB) of the respective FMOGS-OX and the negative control was determined by ANOVA. For FMOGS-OX1 to FMOGS-OX4, P < 0.001; for FMOGS-OX5, P = 0.304. Ratio of 8-MTO:(8-MTO + 8-MSO) was calculated to represent the S-oxygenating activity for long-chain GSL. The significance of the difference between the ratio 8-MTO:(8-MTO + 8-MSO) of the respective FMOGS-OX and the negative control was determined by ANOVA. For FMOGS-OX1 to FMOGS-OX5, P < 0.001.]

Table I. Altered GS-OX activity in the FMOGS-OX2 T-DNA mutant

GSL content in seeds and leaves was analyzed. All plants were segregants derived from a parental line heterozygous for the T-DNA knockout allele. MT:(MS + MT) represents the S-oxygenation activity for the conversion from MT GSL to MS GSL. This T-DNA mutant is in a wild-type Col-0 background.

<table>
<thead>
<tr>
<th>MT:(MS + MT)</th>
<th>Leaf Tissue</th>
<th>Seed Tissue</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous</td>
<td>Homozygous</td>
</tr>
<tr>
<td></td>
<td>Knockout (n = 52)</td>
<td>Wild Type (n = 36)</td>
</tr>
<tr>
<td>Propyl GSL (C3)</td>
<td>0.12 ± 0.008</td>
<td>0.13 ± 0.011</td>
</tr>
<tr>
<td>Butyl GSL (C4)</td>
<td>0.33 ± 0.011</td>
<td>0.24 ± 0.013</td>
</tr>
<tr>
<td>Pentyl GSL (C5)</td>
<td>0.24 ± 0.013</td>
<td>0.17 ± 0.015</td>
</tr>
<tr>
<td>Hexyl GSL (C6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heptyl GSL (C7)</td>
<td>0.24 ± 0.009</td>
<td>0.22 ± 0.016</td>
</tr>
<tr>
<td>Octyl GSL (C8)</td>
<td>0.10 ± 0.005</td>
<td>0.09 ± 0.008</td>
</tr>
</tbody>
</table>

*Number of individual lines measured per genotype class. **Mean is the mean value of MT:(MS + MT). ***Standard error for the mean value. ****P value for GSL differences between the two genotypes as determined by ANOVA. *****Nonsignificant P values (P > 0.05). Given GSL was not detectable; therefore, no statistical analyses were conducted.
et al., 2001c), which catalyzes the conversion from MS GSL to hydroxyl GSL (OH) GSL (Fig. 1). This results in accumulation of OH GSL instead of MS GSL in Ler. Therefore, the ratio of MT GSL to the sum of MT and OH GSLs was used as a measure for GS-OX activity in Ler. Significant increase of 3-MT:3-(MT + 3-OH) was observed in both leaves and seeds in homozygous knockout mutants compared to wild-type plants (Table IV). No significant difference was found for other MT GSLs in this knockout mutant, which suggests a preference of FMOGS-OX for short-chain MT GSLs in Ler. In our data, there is agreement between the in vitro and in planta activities for the majority of FMOGS-OX.

**Table II. Altered GS-OX activity in the FMOGS-OX T-DNA mutant**

GSL content in seeds and leaves was analyzed. All plants were segregants derived from a parental line heterozygous for the T-DNA knockout allele. MT:(MS + MT) represents the S-oxygenation activity for the conversion from MT GSL to MS GSL. This T-DNA mutant is in a wild-type Col-0 background.

<table>
<thead>
<tr>
<th>MT:(MS + MT)</th>
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<td>Homozygous Knockout (n = 35)</td>
<td>Homozygous Wild Type (n = 29)</td>
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<td></td>
<td>Mean&lt;sup&gt;b&lt;/sup&gt;</td>
<td>se&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propyl GSL (C3)</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Butyl GSL (C4)</td>
<td>0.20</td>
<td>0.020</td>
</tr>
<tr>
<td>Pentyl GSL (C5)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Hexyl GSL (C6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heptyl GSL (C7)</td>
<td>0.20</td>
<td>0.020</td>
</tr>
<tr>
<td>Octyl GSL (C8)</td>
<td>0.27</td>
<td>0.040</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of individual lines measured per genotype class. <sup>b</sup>Mean value of MT:(MS + MT). <sup>c</sup>Standard error for the mean value. <sup>d</sup>p value for GSL differences between the two genotypes as determined by ANOVA. <sup>e</sup>Nonsignificant P values (P > 0.05). <sup>f</sup>Given GSL was not detectable; therefore, no statistical analyses were conducted.

**Table III. Altered GS-OX activity in the FMOGS-OX T-DNA mutant**

GSL content in seeds and leaves was analyzed. All plants were segregants derived from a parental line heterozygous for the T-DNA knockout allele. MT:(MS + MT) represents the S-oxygenation activity for the conversion from MT GSL to MS GSL. This T-DNA mutant is in a wild-type Col-0 background.

<table>
<thead>
<tr>
<th>MT:(MS + MT)</th>
<th>Leaf Tissue</th>
<th>Seed Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous Knockout (n = 40)</td>
<td>Homozygous Wild Type (n = 37)</td>
</tr>
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<td>Mean&lt;sup&gt;b&lt;/sup&gt;</td>
<td>se&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propyl GSL (C3)</td>
<td>0.26</td>
<td>0.020</td>
</tr>
<tr>
<td>Butyl GSL (C4)</td>
<td>0.41</td>
<td>0.020</td>
</tr>
<tr>
<td>Pentyl GSL (C5)</td>
<td>0.23</td>
<td>0.020</td>
</tr>
<tr>
<td>Hexyl GSL (C6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heptyl GSL (C7)</td>
<td>0.25</td>
<td>0.010</td>
</tr>
<tr>
<td>Octyl GSL (C8)</td>
<td>0.11</td>
<td>0.010</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of individual lines measured per genotype class. <sup>b</sup>Mean value of MT:(MS + MT). <sup>c</sup>Standard error for the mean value. <sup>d</sup>p value for GSL differences between the two genotypes as determined by ANOVA. <sup>e</sup>Nonsignificant P values (P > 0.05). <sup>f</sup>Given GSL was not detectable; therefore, no statistical analyses were conducted.
VII). For C4 GSLs, a very slight, but statistically significant, increase of MT:(MT+MS) was detected, indicating a possible repression of the conversion from 4-MTB to 4-MSB. As with the other FMOGS-OX overexpressor data, the 35S::FMOGS-OX4 data were derived from two independent, segregating T1 transgenic overexpression lines that exhibited a significant decrease of the MT:(MT+MS) ratio in comparison to wild-type plants (Supplemental Fig. S2; Supplemental Table S6). This suggests that FMOGS-OX4 catalyzes the sulfur-oxygenation reaction and that the 35S::FMOGS-OX4 transgene was silenced in the T2 generation.

In agreement with its predicted substrate specificity, 35S::FMOGS-OX5 lines had a significant decrease of MT:(MT+MS) for only octyl C8 GSL in seeds compared to wild type (Table VII). Interestingly, 7-methylthioheptyl (7-MTH) GSL, another long-chain MT substrate, exhibited a similar concentration as 8-MTO in seeds of the wild-type plant, but we did not detect any 7-MSH GSL, indicating that no significant conversion from 7-MTH to 7-MSH occurred in 35S::FMOGS-OX5 (Supplemental Table S5). This confirmed that FMOGS-OX5 is specific for the 8-MTO GSL substrate.

### DISCUSSION

Modifications of the GSL side chain are of particular importance because the biological activity of the GSL hydrolysis products is determined to a large extent by the structure of the side chain. We identified four new FMO genes encoding for enzymes capable of sulfur-oxygenating aliphatic GSLs. Three of these FMOs, FMOGS-OX2, FMOGS-OX3, and FMOGS-OX4, are oxygenated all available MT GSLs and the previously characterized FMOGS-OX1 in a subclade that appears to be crucial specific. FMOGS-OX2, FMOGS-OX3, and FMOGS-OX4-S-oxygenated all available MT GSLs as did FMOGS-OX1, whereas FMOGS-OX5 showed high substrate specificity to 8-MTO GSL.

### Substrate Specificity of FMOGS-OX

In animals, there are five functionally expressed FMO genes that detoxify a vast spectrum of xenobiotics. This vast spectrum is due to broad substrate specificity.
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Table VI. Altered GS-OX activity in the FMO<sub>GS-OX3</sub> overexpression lines
GSL content in seeds and leaves was analyzed. All plants were segregants derived from two independent T<sub>1</sub> generation 35S::FMO<sub>GS-OX3</sub> lines. MT::(MS + MT) represents the S-oxygenation activity for the conversion from MT GSL to MS GSL.

<table>
<thead>
<tr>
<th>MT::(MS + MT)</th>
<th>Leaf Tissue</th>
<th>Seed Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S::FMO&lt;sub&gt;GS-OX3&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 19)</td>
<td>35S::FMO&lt;sub&gt;GS-OX3&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 12)</td>
</tr>
</tbody>
</table>
|                     | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup>
| Propyl GSL (C3)     | 0.05 ± 0.000 | 0.12 ± 0.010 | <0.001 | 0.01 ± 0.000 | 0.03 ± 0.000 | <0.001 |
| Butyl GSL (C4)      | 0.02 ± 0.000 | 0.22 ± 0.020 | <0.001 | 0.35 ± 0.020 | 0.74 ± 0.020 | <0.001 |
| Pentyl GSL (C5)     | 0.03 ± 0.010 | 0.31 ± 0.030 | <0.001 | 0.48 ± 0.030 | 0.83 ± 0.020 | <0.001 |
| Hexyl GSL (C6)      | ND<sup>a</sup> | ND | ND | 0.13 ± 0.020 | 0.33 ± 0.030 | <0.001 |
| Heptyl GSL (C7)     | 0.07 ± 0.020 | 0.29 ± 0.040 | <0.001 | 0.35 ± 0.020 | 0.63 ± 0.020 | <0.001 |
| Octyl GSL (C8)      | 0.04 ± 0.010 | 0.18 ± 0.020 | <0.001 | 0.15 ± 0.010 | 0.33 ± 0.010 | <0.001 |

<sup>a</sup>Number of individual lines measured per genotype class.  <sup>b</sup>Mean value of MT::(MS + MT).  <sup>c</sup>Standard error for the mean value.  <sup>d</sup>p-value for GSL differences between the two genotypes as determined by ANOVA.  <sup>e</sup>Given GSL was not detectable; therefore, no statistical analyses were conducted.

Table VII. Altered GS-OX activity in the FMO<sub>GS-OX4</sub> overexpression lines
GSL content in seeds and leaves was analyzed. All plants were segregants derived from two independent T<sub>1</sub> generation 35S::FMO<sub>GS-OX4</sub> lines. MT::(MS + MT) represents the S-oxygenation activity for the conversion from MT GSL to MS GSL.

<table>
<thead>
<tr>
<th>MT::(MS + MT)</th>
<th>Leaf Tissue</th>
<th>Seed Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S::FMO&lt;sub&gt;GS-OX4&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 66)</td>
<td>35S::FMO&lt;sub&gt;GS-OX4&lt;/sub&gt; (&lt;i&gt;n&lt;/i&gt; = 13)</td>
</tr>
</tbody>
</table>
|                     | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup> | Mean<sup>b</sup> st<sup>c</sup>
| Propyl GSL (C3)     | 0.13 ± 0.010 | 0.13 ± 0.010 | NS<sup>e</sup> | ND | ND |
| Butyl GSL (C4)      | 0.26 ± 0.010 | 0.25 ± 0.010 | 0.001 | 0.75 ± 0.040 | 0.74 ± 0.050 | 0.030 |
| Pentyl GSL (C5)     | 0.22 ± 0.010 | 0.22 ± 0.010 | NS | ND | ND |
| Hexyl GSL (C6)      | ND<sup>a</sup> | ND | ND | ND | ND |
| Heptyl GSL (C7)     | 0.26 ± 0.010 | 0.25 ± 0.010 | NS | 0.37 ± 0.040 | 0.30 ± 0.080 | NS |
| Octyl GSL (C8)      | 0.14 ± 0.010 | 0.14 ± 0.010 | NS | 0.21 ± 0.030 | 0.18 ± 0.050 | NS |

<sup>a</sup>Number of individual lines measured per genotype class.  <sup>b</sup>Mean value of MT::(MS + MT).  <sup>c</sup>Standard error for the mean value.  <sup>d</sup>p-value for GSL differences between the two genotypes as determined by ANOVA.  <sup>e</sup>Nonsignificant P values (<i>P</i> > 0.05).  <sup>f</sup>Given GSL was not detectable; therefore, no statistical analyses were conducted.

specification that makes the mammalian FMOs capable of oxidizing thousands of plant natural products as well as thousands of synthetic therapeutics drugs (Krueger and Williams, 2005; Cashman and Zhang, 2006). Plants, on the other hand, have many more FMO genes, possibly with more restricted substrate specificity. In Arabidopsis, there are 29 genes with homology to known FMOs (Schlaich, 2007). The enzyme FMO<sub>GS-OX1</sub> was shown to have substrate specificity in that it required an S-Glc group on its substrates (Hansen et al., 2007). FMO<sub>GS-OX1</sub> S-oxygenated desulfoglucosinolates and intact GSLs, but not the structurally related Met analogs that do not contain an S-Glc group. In this article, we show additional substrate specificity in plant FMOs because we found that FMO<sub>GS-OX3</sub> is specific for long-chain aliphatic GSLs. This indicates that the S-Glc group is not the only structural requirement for the substrate of FMO<sub>GS-OX3</sub>. Size restriction might be another factor determining substrate specificity whereby FMO<sub>GS-OX3</sub> excludes the short-chain GSLs.

It is reported that when there is no available substrate, the FMO proteins exist as 4<sup>α</sup>-hydroperoxy flavin, which is a precharged complex containing a reduced form of fatty acid dehydrogenase and NADPH that is ready to act on substrates (Eswaramoorthy et al., 2006). The 4<sup>α</sup>-hydroperoxy flavin complex of FMO<sub>GS-OX5</sub> may have a tertiary structure in the binding site that only fits with 8-MTO and prevents shorter chain-length MT GSLs from either entering or being properly held by the enzyme. However, the mechanism of the conformational and chemical complementarities between the MT GSLs and FMO<sub>GS-OX</sub> enzymes remains to be shown.

The phylogenetic tree of plant FMOs in rice, poplar, and Arabidopsis contains three clades (Hansen et al., 2007). Our phylogenetic analysis of FMO<sub>GS-OX</sub> homologs (Fig. 2) is based on the genomic sequences present in clade III and EST sequences with more than 80% identity to at least one of the three FMOs used for database searching. In this proposed S-oxygenation clade III (Fig. 2), FMO<sub>GS-OX1</sub> to FMO<sub>GS-OX5</sub> are the only characterized genes. Given that poplar, rice, alfalfa (Medicago sativa), grape, tomato (Solanum lycopersicum), lemon (Citrus limon), and moss do not produce GSLs, the FMOs in this clade may be involved in S-oxygenation for a diversity of sulfur-containing flavin-Monoxygenases in Glucosinolate Biosynthesis

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Table VIII. Altered GS-OX activity in the FMOGS-OX5 overexpression lines

<table>
<thead>
<tr>
<th>MT:(MS + MT)</th>
<th>Leaf Tissue</th>
<th>Seed Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S::FMOGS-OX5</td>
<td>Col-0 (n = 36)</td>
<td>p&lt;sub&gt;gene&lt;/sub&gt;</td>
</tr>
<tr>
<td>MT::(MS + MT)</td>
<td>Mean&lt;sup&gt;b&lt;/sup&gt;</td>
<td>st&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Butyl GSL (C4)</td>
<td>0.22</td>
<td>0.000</td>
</tr>
<tr>
<td>Propyl GSL (C3)</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Octyl GSL (C8)</td>
<td>0.11</td>
<td>0.000</td>
</tr>
<tr>
<td>Butyl GSL (C4)</td>
<td>0.11</td>
<td>0.000</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of individual lines measured per genotype class.
<sup>b</sup>Mean value of MT:(MS + MT).
<sup>c</sup>Standard error for the mean value.
<sup>d</sup>NS = Not significant.
<sup>e</sup>Given GSL was not detectable; therefore, no statistical analyses were conducted.

Possible Biological Function of FMOGS-OXs

We have shown that the biochemical function of the FMOGS-OXs is to catalyze the 3-oxygenation of the endogenous substrate MT GSL to MS GSL. The physiological function of FMOGS-OXs depends on the biological activity of the hydrolysis products of the MT GSLs and of further modified GSLs. For humans, the isothiocyanate hydrolysis products of 4-MSB, 7-MSH, and 8-MSO GSLs have been shown to be strong inducers of phase II enzymes and thereby function as cancer-preventing agents (Rose et al., 2000; Fahey et al., 2002). In planta, MS GSLs have been shown to play a role in protection against insects (Rohr et al., 2006), and the isothiocyanate derived from 4-MSB GSL has been shown to play a role in protecting Arabidopsis against pathogens (Tiersen et al., 2001). This indicates that FMOGS-OXs are important for plant defense responses. In some Arabidopsis accessions, MS GSLs can be converted to OH, alkelyl, hydroxylalkenyl, and benzoyloxy GSLs (Fig. 1), adding more layers of complexity to the warfare between Arabidopsis and its enemies (Kliebenstein et al., 2001c; Tiersen et al., 2001). The plants overexpressing FMOGS-OXs showed significant increases in the accumulation of MS GSLs at the expense of the MT GSLs. This indicates that the production of MS GSLs and the further modified GSLs downstream in the pathway (Fig. 1) require the FMOGS-OX proteins. This is an important consideration when FMOGS-OXs are utilized in breeding and genetic engineering toward plants with a high level of cancer-preventive methylsulfinylalkyl isothiocyanates, increased pathogen resistance, or decreased level of the deleterious downstream GSLs, such as progoitrin, 2-hydroxy-but-3-enyl GSL (Kliebenstein et al., 2001a; Halkier and Gershenzon 2006). In addition, when altering plants via breeding or engineering approaches toward isothiocyanates of MS GSLs, it is important to also incorporate genes affecting the breakdown of GSLs into the scheme to ensure that isothiocyanates are produced.

Gene Duplication and Evolution of FMOGS-OXs

The Arabidopsis FMOGS-OXs are present in three gene clusters that appear to have evolved through a combination of local tandem duplication, whole-genome duplications, and a distal duplication (Fig. 2; Vision et al., 2000; Blanc and Wolfe 2004; Rizzon et al., 2006). Interestingly, this group of FMOs may represent crucifer-specific radiation because we have to date only identified cruciferous genes to reside within this FMOGS-OX subclade. Whereas we utilized all available sequence databases, further work is required to fully validate whether this is in fact crucifer-specific radiation associated with the evolution of GSL biosynthesis. Thus, this FMO subclade begins to allow potential detailed analysis of how gene duplicates can either partition the original function (subfunctionalization) or derive entirely new functions (neofunctionalization; Lynch and Conery, 2000). In the Arabidopsis FMOGS-OX part of this FMO subclade, there is evidence for subfunctionalization; however, the true direction of this process will require the identification and biochemical characterization of FMOGS-OX ancestral genes in species basal to the Cruciferae. Phylogenetic analysis suggests that there might be a precursor FMOGS-OX, which first duplicated into FMOGS-OX5 and a FMOGS-OX1 to FMOGS-OX4 precursor. The different substrate specificity between FMOGS-OX5 and FMOGS-OX1 to FMOGS-OX4 is potentially an instance of biochemical subfunction- alization whereby FMOGS-OX5 is likely subfunctionalized for long chain-length substrate, whereas FMOGS-OX1 to FMOGS-OX4 retained broad chain-length specificity from the precursor protein. Further subfunctionalization, potentially from tissue-specific expression patterns, can also be found within FMOGS-OX1 to FMOGS-OX4 and is evidenced by the knockout mutant analysis where both FMOGS-OX1 and FMOGS-OX2 control 8-MSO pro-
duction in planta, whereas FMO\textsubscript{GS-OX}, which is capable of catalyzing this reaction in vitro, does not control this reaction in planta (Tables I and II; Hansen et al., 2007). These slight differences between FMO\textsubscript{GS-OX} genes might be explained by analysis of microarray data (http://www.genevestigator.ethz.ch; Zimmermann et al., 2004) showing that these genes have different expression patterns across organs and growth stages. Specific identification and validation of the tissue expression patterns of these FMO\textsubscript{GS-OX} and the substrates for the other members of this FMO clade could help our understanding of how and when duplicates undergo either biochemical or expression-based sub-functionalization.

**Gene Duplication and Quantitative Genetic Variation**

The association of the duplicated FMO\textsubscript{GS-OX} with independent QTLs for the S-oxygenation reaction in Arabidopsis raises another possible role for duplicated gene families (Fig. 2). Duplicated gene families, while providing redundancy to a system, may also enhance the potential for quantitative variation within a trait. This is illustrated by the fact that each of the FMO\textsubscript{GS-OX} has a large expression polymorphism, yet there is only quantitative variation for this trait rather than qualitative. As such, the large polymorphisms in each independent gene are dampened by the presence of the other genes. While duplicated genes do show enhanced levels of genetic variation as would be expected under this model (Gu et al., 2004; Kliebenstein, 2008), it remains to be seen whether duplicated gene families show any bias in controlling quantitative trait variation.

In summary, identification of S-oxygenating activity of the FMO\textsubscript{GS-OX} may impact both applied and basic research fields. These genes can potentially be applied in genetic engineering for the production of 4-MSB, 7-MSH, and 8-MSO GSLs, or removal of 2-hydroxybut-3-enyl GSL. In addition, the characterization of the FMO\textsubscript{GS-OX} will help to gain more biochemical insight into plant FMO proteins, which we have just begun to learn about, and it may also bring clues for the functions of the noncharacterized plant FMOs. Finally, this well-defined gene family may provide an optimal model for studying neo- versus sub-functionalization following gene duplication.

**MATERIALS AND METHODS**

**Generation of Phylogenetic Tree**

The entire FMO complement from the genomic sequence for *Medicago truncatula*, grape (*Vitis vinifera*), *Physcomitrella patens*, rice (*Oryza sativa*), Arabidopsis (*Arabidopsis thaliana*), and poplar (*Populus tremuloides*) were obtained and translated into their corresponding amino acid sequence. Gene abbreviations are per genome consortium convention or previous description. The sequences were used to construct a complete neighbor-joining tree with 1,000 bootstraps in ClustalX. The sequences were used to define the root for the presented cladogram focused on the short-chain aliphatic GSL phenotype. All of these EST sequences showing at least 80% amino acid identity with one of three FMOs and included these in the phylogenetic analysis. All of these EST sequences clustered within the *P. patens* sequence on an unrooted cladogram containing all sequences and, as such, we utilized the *P. patens* sequence as a root for the presented cladogram focused on the S-oxygenation subclade (Fig. 2). Only those branches with at least 600 of 1,000 bootstrap support are labeled.

**Gene Duplication and Quantitative Genetic Variation**

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In summary, identification of S-oxygenating activity of the FMO\textsubscript{GS-OX} may impact both applied and basic research fields. These genes can potentially be applied in genetic engineering for the production of 4-MSB, 7-MSH, and 8-MSO GSLs, or removal of 2-hydroxybut-3-enyl GSL. In addition, the characterization of the FMO\textsubscript{GS-OX} will help to gain more biochemical insight into plant FMO proteins, which we have just begun to learn about, and it may also bring clues for the functions of the noncharacterized plant FMOs. Finally, this well-defined gene family may provide an optimal model for studying neo- versus sub-functionalization following gene duplication.

**Heterologous Expression of FMO\textsubscript{GS-OX} Proteins in *Escherichia coli***

The coding sequences of the four FMO\textsubscript{GS-OX} were amplified by RT-PCR. Total plant RNA was isolated with TRIzol (Invitrogen) according to the manufacturer’s recommendations. First-strand cDNA was synthesized using the Script cDNA synthesis kit (Bio-Rad). The coding sequence of FMO\textsubscript{GS-OX} (At1g62540) was amplified from first-strand cDNA with the following primers: 5'-ATGCCAACAGCTCAAAAC-3' and 5'-GGATATGGGAAGGATGGACTAAT-3'. The coding sequence of FMO\textsubscript{GS-OX} (At1g62560) was amplified with the following primers: 5'-ATGGCGACCGTGCTCAGAAC-3' and 5'-TCCATCAGTACAGTGACGAGAG-3'. The coding sequence of FMO\textsubscript{GS-OX} (At1g62570) was amplified with the following primers: 5'-ATGGCCGACCTCTGTAGTCGAT-3' and 5'-TCCATCCGGCTGACTGAATT-3'. The coding sequence of FMO\textsubscript{GS-OX} (At1g62580) was amplified with the following primers: 5'-ATGGCGAGACGTCCGCTCAGAAACG-3' and 5'-GGATATGGGAAGGATGGACTAAT-3'. The coding sequence of FMO\textsubscript{GS-OX} (At1g62580) was amplified with the following primers: 5'-ATGGCGACCGTGCTCAGAAC-3' and 5'-TCCATCAGTACAGTGACGAGAG-3'. The coding sequence of FMO\textsubscript{GS-OX} (At1g62580) was amplified with the following primers: 5'-ATGGCGACCGTGCTCAGAAC-3' and 5'-TCCATCAGTACAGTGACGAGAG-3'.

**Spheroplast Enzymatic Assays**

The enzymatic activity of the four FMO\textsubscript{GS-OX} was analyzed by spheroplast enzymatic assays. A 100-µL volume of assay solution contained spheroplasts corresponding to 50 µg of total E. coli protein, substrate, 0.1 M Tricine (pH 7.9),...
and 0.25 mM NADPH. The reaction mixture was incubated for 1 h at 30°C followed by the addition of 100 μL methanol and centrifugation at 5,000g for 2 min. Supernatant (200 μL) was lyophilized and dissolved in 50 μL water. In the assays using desulfo 4-MTB GSL as substrate, final substrate concentration was 0.25 mM. In the assays using desulfo GSL extracts from Arabidopsis seeds as substrate, final concentration was 2 mM total GSLs.

Plant Growth

Plants were grown in a growth chamber at a photosynthetic flux of 100 μE at 20°C and 70% relative humidity with a 16/8-h photoperiod.

Genotyping and RT-PCR of FMOGS-OX T-DNA Insertion Mutants

Two T-DNA insertion mutants for each of FMOGS-OX2 and FMOGS-OX3 and one T-DNA insertion mutant for FMOGS-OX3 in Col-0 background were obtained. One T-DNA insertion mutant in ecotype Ler background was obtained for FMOGS-OX2.

The insertion mutants for FMOGS-OX2 were the Salk_098986 line (FMOGS-OX3) and Salk_098986 line (FMOGS-OX3; Alonso et al., 2003). Primers for genotyping FMOGS-OX2 were 5′-TTTCCAGCATGACATTTTG-3′, 5′-TGGATCTTTTATAACAGTTC-3′, and the T-DNA-specific primer Lba1 5′-TGGTTTACCTCGAGCCATCG-3′. Primers for genotyping FMOGS-OX5 are 5′-TTTGTTACCTCGAGCCATCG-3′, 5′-ACCACCCGACATACAACTG-3′, and T-DNA-specific primer Lba1. FMOGS-OX5 plants were harvested for GSL analysis and for PCR genotyping.

The insertion mutant for FMOGS-OX3 was GT13906 line (Martienssen, 1998) in Ler background and genotyping primers were 5′-CAACTCTTGTGACTACC-3′, 5′-GCTCTAGACGCAATGTGGACTT-3′, and T-DNA-specific primer Drs5, 5′-GCTTTGGTATATCGCTTCCG-3′.

The insertion mutants for FMOGS-OX4 were Salk-059185 line (FMOGS-OX3) and Salk_078861 line (FMOGS-OX3; Alonso et al., 2003). Genotyping primers for FMOGS-OX4 were 5′-CACATGGCAGAGAAAATACATC-3′, 5′-GAGCTTTGGAATCAGTGTG-3′, and T-DNA-specific primer Lba1. Genotyping primers for FMOGS-OX2 were 5′-TGACGTTTTGATATCCCGTTTCCG-3′, 5′-AACGGTGATCTTCCGAGT-3′, and T-DNA-specific primer Lba1.

The insertion mutants for FMOGS-OX5 were WiscDsLox361H10 line (Wood et al., 2007). Genotyping primers for FMOGS-OX5 were 5′-CCCTGGCCAT-3′, 5′-AAAATCTTCTGCTGTTG-3′, and T-DNA-specific primer 5′-AACGGTGATCTTCCGAGT-3′. Leaves from homozygous FMOGS-OX4-2, FMOGS-OX5-5, and FMOGS-OX5-2 were harvested at 25 d after germination. RNA was extracted with TRizol reagent (Invitrogen) according to the manufacturer’s instructions. First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). The users primed in cloning of the coding sequences of the four genes on all individual GSLs and resultant variables as described previously (Hansen et al., 2007). Seeds were analyzed the same way as described above.

Supplemental Data

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

Supplemental Figure S1. Confirmation of FMOGS-OX T-DNA mutants by RT-PCR.

Supplemental Figure S2. GSL profile of seed in T1 generation of 35S::FMOGS-OX line and wild-type plants.

Supplemental Table S1. FMOGS-OX homologous sequences utilized in analysis.

Supplemental Table S2. Ler × Cvi short-chain aliphatic seed FMOGS-OX analysis.

Supplemental Table S3. Ler × Cvi short-chain aliphatic leaf FMOGS-OX analysis.

Supplemental Table S4. S4. GSL profile of FMOGS-OX T-DNA knockout mutants.

Supplemental Table S5. GSL profiles of 35S::FMOGS-OX overexpression lines and wild-type plants.

Supplemental Table S6. Altered FMOGS-OX activity in the T2 generation of 35S::FMOGS-OX overexpression lines.

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LITERATURE CITED


