Whole Genome and Tandem Duplicate Retention Facilitated Glucosinolate Pathway Diversification in the Mustard Family

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Abstract

Plants share a common history of successive whole-genome duplication (WGD) events retaining genomic patterns of duplicate gene copies (ohnologs) organized in conserved syntenic blocks. Duplication was often proposed to affect the origin of novel traits during evolution. However, genetic evidence linking WGD to pathway diversification is scarce. We show that WGD and tandem duplication (TD) accelerated genetic versatility of plant secondary metabolism, exemplified with the glucosinolate (GS) pathway in the mustard family. GS biosynthesis is a well-studied trait, employing at least 52 biosynthetic and regulatory genes in the model plant *Arabidopsis*. In a phylogenomics approach, we identified 67 GS loci in *Aethionema arabicum* of the tribe Aethionemae, sister group to all mustard family members. All but one of the *Arabidopsis* GS gene families evolved orthologs in *Aethionema* and all but one of the orthologous sequence pairs exhibit synteny. The 45% fraction of duplicates among all protein-coding genes in *Arabidopsis* was increased to 95% and 97% for *Arabidopsis* and *Aethionema* GS pathway inventory, respectively. Compared with the 22% average for all protein-coding genes in *Arabidopsis,* 52% and 56% of *Aethionema* and *Arabidopsis* GS loci align to ohnolog copies dating back to the last common WGD event. Although 15% of all *Arabidopsis* genes are organized in tandem arrays, 45% and 48% of GS loci in *Arabidopsis* and *Aethionema* descend from TD, respectively. We describe a sequential combination of TD and WGD events driving gene family extension, thereby expanding the evolutionary playground for functional diversification and thus potential novelty and success.

Key words: comparative genomics, systems biology, whole-genome duplication, functional diversification, Brassicaceae.

Introduction

Gene duplication has played an important evolutionary role in angiosperm adaptation and success, for example, by contributing to regulatory and enzymatic pathways involved in generating more than 200,000 diverse biochemical plant secondary metabolites found in the Angiosperm lineage (Hartmann 2007). Functional diversification refers to processes of gene duplication followed by sub- or neofunctionalization of the enzymes encoded by duplicate copies (Ohno 1970; Roth et al. 2007; Wang et al. 2011c), mediating specificities to extended classes of substrates or catalysis of novel reactions (Stehle et al. 2008). Fast expansion of gene copy number occurs in various ways. In this study, we focus on whole-genome duplication (WGD), tandem duplication (TD), and gene transposition duplication (GTD). For example, approximately 45% of the *Arabidopsis* nuclear protein-coding genes have been affected by such processes (Bowers et al. 2003; Rizzon et al. 2006; Huang et al. 2012). In this study, we investigated the impact of gene duplication to the diversification of plant secondary metabolites exemplified with glucosinolate (GS) biosynthesis. GS biosynthesis is a well-studied key trait shared by all Brassicales including the mustard family (Brassicaceae) crown group (Schranz et al. 2011) and its sister lineage Aethionemae. Comparative genomics analysis unraveled a history of successive paleopolyploidy events commonly shared by almost all Angiosperms (Bowers et al. 2003). The *Arabidopsis* lineage underwent at least five polyploidy events in the history of life, two preceding and three following Angiosperms radiation (Bowers et al. 2003; Jiao et al. 2011). The most recent WGD is commonly referred to as At-α and occurred approximately 30–60 Ma in the ancestor of all Brassicaceae, including the sister group Aethionemae (Edger PP, Pires Jc, submitted for publication). As a result, pairwise

syntenic regions are scattered throughout the genome (genomic blocks), defined as copies of consecutive ohnologs derived from At-α (Bowers et al. 2003). It is known that polyploidy is succeeded by a genome-wide process of biased fractionation, preferentially targeting one subgenome to retain clusters of dose-sensitive genes organized in functional modules (Thomas et al. 2006). Furthermore, several studies have established a potential link of polyploidy to natural variation due to differential expression of ohnolog copies (Wang et al. 2011c), seed and flower origin and diversification (De Bodt et al. 2005; Irish and Litt 2005; Jiao et al. 2011), morphological complexity (Freeling and Thomas 2006), and survival of plant lineages at the Cretaceous-Tertiary extinction event (Fawcett et al. 2009). In this study, we provide solid evidence for the link of WGD to pathway expansion of a distinct key trait relevant for herbivore defense and hence highly connected to fitness. Interestingly, polyploidy also affects other kinds of duplication, creating networks of factors with mutual influence. Recent studies have shown an interaction between polyploidy and the fractionation rate of tandem duplicate copies in both *Arabidopsis* and *Brassica rapa* (having undergone an additional genome triplication). Hence, we analyzed short-sequence duplications to utilize the evolutionary significance of different duplication classes.

TD of short sequences can be caused by unequal crossing-over or template slippage during DNA repair, producing tandem arrays (TARs) of homologous genes in close genomic vicinity (Kane et al. 2010). Depending on the number of allowed gene spacers, TAR genes include about 10–15% of the *Arabidopsis thaliana* genome (0 and 10 spacers, respectively) (Rizzon et al. 2006). Comparison of TARs in *Arabidopsis* and rice revealed enrichment of genes encoding membrane proteins and function in biotic and abiotic stress (Rizzon et al. 2006). Notably, the impact of TD to trait evolution has been elucidated in multiple taxa, including disease resistance in Solanaceae (Parniske et al. 1999) and Brassicaceae (Leister 2004). Likewise, TD played a role in the evolution of signal transduction, for example, the expansion and functional diversification of the F-box type transcriptional activator gene family in Fabaceae (Bellieny-Rabelo et al. 2013). Moreover, TD is an important factor for increasing versatility of defense response in Brassicaceae. In GS biosynthesis, subfunctionalization of TAR genes is evident for 2-oxoglutarate-dependent dioxygenases (AOP) (Kliebenstein et al. 2001), flavin-monooxygenases (FMO_{GSOX}) (Li et al. 2008) and methylthioalkylmalate synthases (MAM) (Kroymann et al. 2003; Heidel et al. 2006; Textor et al. 2007). In this study, we integrate previous findings to dissect the influence of polyploidy with TD and GTD in the last 30–60 Ma of GS pathway expansion since *Aethionema* and *Arabidopsis* lineage divergence.

Duplicate gene copies can move to a new genomic location. The observed frequency of gene movements explains the observed erosion of synteny between plant genomes during evolution (Wicker et al. 2010), defining the limits of synteny-based approaches for ortholog detection. Gene movements are often caused by transposition. GTD events occur when a single nontransposon gene relocates to a new position, and segregants contain duplicates (Freeling 2009). Although transposable elements (TEs) account for approximately 10% of the *Arabidopsis* genome (Huang et al. 2012) and show nonrandom association to syntenic blocks (Hughes et al. 2003), 14% of all protein-coding genes in *Arabidopsis* transposed at least once during Rosid evolution (Freeling et al. 2008; Woodhouse et al. 2011). Importantly, a novel genomic context of the transposed copy potentially influences rates of gene expression (Wang et al. 2013) and might thereby contribute to the phenotypic consequences of the duplication event (Kliebenstein 2008). Accordingly, TE activity was shown to foster variation of NBS-resistance proteins in grape (Malacarne et al. 2012) as well as natural growth variation and expansion of ERF family transcriptional regulators in *Arabidopsis* (Nakano et al. 2006; Vlad et al. 2010). In contrast, evolutionary dynamics of GTD events affecting genetic versatility of plant secondary metabolism has not yet been investigated.

GS comprise a class of secondary plant metabolites derived from amino acids and sugars, part of a two-component chemical defense against herbivory in Brassicales (Rodman 1998; Windsor et al. 2005; Beekwilder et al. 2008). Myrosinase enzymes are the other component of the defense system and confer GS hydrolysis activity. They are released from the vacuole upon tissue damage, producing a plethora of GS degradation products such as nitriles, isothiocyanates, thiocyanates, and ephithioalkanes with various bioactivities (Rask et al. 2000; Bones and Rossiter 2006). GS are of particular interest for human health because they can inhibit carcinogen activation (Hecht 2000; Nakajima et al. 2001) and carcinogenesis by triggering cell cycle arrest and stimulating apoptosis (Wittstock et al. 2003; Hayes et al. 2008). The observed variation in GS biochemistry across Brassicales is due to the differences in biochemistry among their amino acid precursors (Fahey et al. 2001; Windsor et al. 2005) and allows GS grouping to four distinct classes. Oxidative deamination of Phe and Tyr initiates biosynthesis of indolic GS (I); Trp is the substrate for indolic GS production (II); Ala, Val, Leu, and Ile are precursors for biosynthesis of aliphatic GS (III) (Mithen et al. 2010). Although aromatic and aliphatic GS have been detected in other eudicot families including Phytolaccaceae, Euphorbiaceae, and Pittosporaceae (Rodman et al. 1996; Fahey et al. 2001), indolic GS are Brassicales specific. Met-derived GS form a fourth class of GS (IV), referring to a subset of aliphatic GS specific to the Brassicales crown group, including the sister group Aethionemae. The utilization of Trp- and Met-derived amino acids for GS production may be tied to pathway expansion caused by ancient WGD events (Schranz et al. 2011).

The genus *Aethionema* of the tribe Aethionemae is an ideal group for comparative genomics of polyploidy and GS pathway evolution. First, it shares the composite GS chemotype

observed in the larger and more diverse Brassicaceae crown group (Schranz et al. 2012). Second, phylogenetic analysis highly support the tribe Aethionemae as the earliest diverged clade and extant sister to the crown group Brassicaceae (Couvreur et al. 2010) with an estimated split of the two lineages approximately 30–60 Ma. However, a high degree of interspecies synteny (see Results) is maintained. Third, the most recent WGD event identified in the lineage of *Arabidopsis* (referred to as At-α) predated the divergence of *Arabidopsis* and *Aethionema*. Furthermore, it was not succeeded by an additional species-specific genome polyploidation, preventing additional fractionation of synteny (Bowers et al. 2003; Haudry et al. 2013) (Edger PP, Pires Jc, submitted for publication). In contrast, *B. rapa* underwent an additional genome triplication event (Wang et al. 2011b), complicating efforts to analyze the potential impact of At-α on the evolution of the GS pathway inventory.

Materials and Methods

Aethionema arabicum Genome Assembly and Set of Annotated Genes

Sequence assembly and annotation of the *Aethionema arabicum* genome was obtained from Haudry et al. (2013).

RNA Isolation and Sequencing

Aethionema arabicum RNA was isolated from fresh apical meristematic tissue or very young leaves using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Samples were kept on liquid nitrogen before RNA isolation. The optional step of heating the lysis solution to 65 °C was used to maximize RNA yield. RNA was eluted into a final volume of 100 μl RNase-free water. Total mass of RNA and quality was estimated using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Samples were deemed acceptable if RIN (RNA integrity number) scores were greater than 8.0. A minimum of 20 μg of total RNA was required for library building and sequencing. RNA-seq (Wang et al. 2009) paired-end libraries with average fragment lengths of 250 bp were constructed, and each library was sequenced on a single lane of an Illumina GAIIX sequencer flow cell (Illumina, San Diego, CA) to generate a minimum of 3 gigabases of 75 bp, paired-end sequences.

Database of *A. thaliana* Genes Retaining an Ohnologous Copy Dating Back to the At-α WGD Event

First, we generated a spreadsheet with information on all 33,323 *A. thaliana* nuclear genes annotated in the TAIR database v10, including 1) *Arabidopsis* gene identifiers (AGIs), 2) locus type, 3) locus name, and 4) short description of encoded function. Second, we integrated optional affiliation to 5) syntenic block and 6) ohnolog copy dating back to At-α WGD event as described previously (Freeling and Thomas 2006). The corresponding authors did not account for every gene in their analysis and inferred the genomic location of ohnolog blocks dating back to At-α using the TIGR *Arabidopsis* genome annotation v5 from 2005. Third, we added an additional column (i), to indicate coverage of the gene in Feeling's study (yes/not considered/not present in TIGR5).

Database for GS Biosynthetic Gene Identification in *A. thaliana* and *Aet. arabicum*

Files containing the coding sequences representing the complete set of GS biosynthetic and regulatory (AtGS) genes in *A. thaliana* (Sonderby et al. 2010) were acquired from the TAIR database v10 (www.arabidopsis.org, last accessed November 9, 2013). We highlighted the AGIs in the spreadsheet covering all nuclear genes in *Arabidopsis* (see Materials and Methods).

Interpolation of Novel Putative GS Biosynthetic Genes and Retained At-α Ohnolog Pairs in *A. thaliana*

We utilized similarity among ohnologous gene copies. We employed the spreadsheet containing information on genomic location of AtGS genes and α-blocks with optional retained duplicates therein. We visually screened for all ohnolog copies of AtGS genes not sharing annotation as AtGS genes themselves. Differential expression of ohnolog pairs was tested using the botany array resource (http://bar.utoronto.ca/welcome.htm, last accessed November 8, 2013). We included all ohnolog copies for our analysis to create an extended AtGS gene set. For the spreadsheet, see supplementary table S1, Supplementary Material online. Previous approaches of ohnologous gene pair identification did not consider every protein-coding gene (Bowers et al. 2003; Thomas et al. 2006). To minimize resulting errors for analysis of AtGS loci, we performed a BlastP screen without a cut-off *e* value, querying all AtGS genes with nonretained At-α ohnologs against all other *Arabidopsis* genes with nonretained At-α ohnologs. Highest-scoring sequence pairs sharing genomic location within converse copies of the same α-block were tested for synteny and positives defined as additional pairs of retained At-α ohnolog copies (marked as "our addition/OA" in table 1 and supplementary table S1, Supplementary Material online).

Database of *A. thaliana* Tandem Arrayed Genes

A database of *A. thaliana* coding sequences organized in TARs was generated for the TAIR annotation v10 as described previously (Rizzon et al. 2006), using a low-stringency approach with a number of $N = 10$ allowed gene pacers. Information was updated to TAIR10 and included to the spreadsheet covering all *Arabidopsis* nuclear genes (supplementary table S1, Supplementary Material online).

Database of *A. thaliana* GS Genes Affected by GTD

A database of the epoch-independent positional history of all *Arabidopsis* genes was generated as described previously for

Table 1

Retained At-α Ohnolog Duplicate Gene Pairs in *Arabidopsis* and *Aethionema* GS Pathway Inventory

Protein Name[a]	AGI	α-Block	Evident SSD[b]	AabID[c]	Syntelog	% Identity	Col-0 → Aab[d]
Core-structure formation							
UGT74C1	AT2G31790	A02N051	TD	Aab37175	Yes	79.44	6 → 10
[UGT like]	AT1G05670	A02N051	TD	Aab31930	Yes	81.11	6 → 10
FMO-GSOX-2	AT1G62540	A03N117	TD	Aab10869	Yes	76.6	11 → 8
[FMO like]	AT1G12130	A03N117	TD	Aab13543	Yes	65.09	11 → 8
CYP79F2	AT1G16400	A05N062	TD	—	—	—	8 → 9
CYP79C1	AT1G79370	A05N062	GTD	Aab34143	Yes	76.8	8 → 9
SOT16	AT1G74100	A05N186	TD	Aab14278	Yes	91.07	3 → 3
SOT17	AT1G18590	A05N186		Aab19675	Yes	86.05	3 → 3
SUR1	AT2G20610	A10N194		Aab30136	Yes	89.15	3 → 2
[SUR like]	AT4G28420	A10N194	TD	Aab31155	Yes	57.93	3 → 2
CYP79B2	AT4G39950	A10N257	GTD	Aab17805	Yes	81.38	8 → 9
CYP79B3	AT2G22330	A10N257	GTD	Aab19477	Yes	81.4	8 → 9
GGP1	AT4G30530	A10N314	TD	Aab24374	Yes	87.6	5 → 5
[GGP like]	AT2G23960	A10N314	TD	Aab11021	Yes	61.38	5 → 5
GSTF11	AT3G03190	A12N102		Aab14996	Yes	77.1	4 → 4
[GSTF12]	AT5G17220	A12N102		Aab14791	Yes	77.84	4 → 4
Cosubstrate pathways							
[AAO3]	AT2G27150	A02NOA1	GTD	Aab27016		77.67	2 → 2
AAO4	AT1G04580	A02NOA1		Aab24896	Yes	79.58	2 → 2
APK1	AT2G14750	A10NOA2		Aab32150	Yes	83.75	2 → 2
APK2	AT4G39940	A10NOA2	GTD	Aab17804	Yes	86.05	2 → 2
Side-chain elongation							
BCAT4	AT3G19710	A08N074		Aab21007	Yes	75	6 → 6
[BCAT7]	AT1G50090	A08N074	TD	Aab22548	Yes	76.9	6 → 6
IPMI1	AT3G58990	A11N226		Aab13092	Yes	83	3 → 3
[IPMI like]	AT2G43090	A11N226	TD	Aab19619	Yes	85.99	3 → 3
BCAT3	AT3G49680	A19N002		Aab33782	Yes	76.02	6 → 6
[BCAT5]	AT5G65780	A19N002	TD	Aab23605	Yes	75.3	6 → 6
BAT5	AT4G12030	A20N095		Aab32285	Yes	76.21	2 → 2
[BAT like]	AT4G22840	A20N095		Aab23321	Yes	91.82	2 → 2
TF regulation							
OBP2	AT1G07640	A02N142		Aab18330	Yes	80.28	2 → 2
[OBP like]	AT2G28810	A02N142		Aab24559	Yes	70.03	2 → 2
MYB122	AT1G74080	A05N185		Aab14276	Yes	57.56	6 → 4
MYB51	AT1G18570	A05N185		Aab19683	Yes	59.89	6 → 4
IQD1	AT3G09710	A14N046		Aab18852	Yes	65.59	2 → 2
[IQD2]	AT5G03040	A14N046		Aab18368	Yes	77.39	2 → 2
MYB28	AT5G61420	A26N034		Aab12163	Yes	67.39	6 → 4
MYB29	AT5G07690	A26N034	TD	Aab33585	Yes	65.13	6 → 4
	36% TD (13/36)			29% TD (10/35)		∅ 76.58	
	14% GTD (5/36)			14% GTD (5/35)			

[a]Squared brackets indicate ohnolog copies of GS biosynthetic genes without GO!-annotation to GS biosynthetic process.
[b]TD refers to members of TARs and GTD refers to a history of transposition in *Arabidopsis*.
[c]Predicted *Aethionema* CDS.
[d]Change of gene family locus count in *Arabidospsis* → *Aethionema* order.

TAIR9 (Woodhouse et al. 2011). We updated all putative GTD copies to TAIR10. Woodhouse et al. scored gene duplicates as transposed based on a function of synteny across taxa in the direction *A. thaliana* → *A. lyrata* → *Carica papaya* → *Populus trichocarpa* → *Vitis vinifera*. For analysis of Brassicaceae genome evolution, methodical restrictions apply due to the low resolution within that clade, covered by only two tribes. Thus, we screened the genomic context of AtGS genes within a narrow window of 3 kb for flanking TE-like sequences, using the GEvo function from the CoGe comparative genomics platform (http://genomevolution.org/CoGe/GEvo.pl, last accessed November 8, 2013) (Lyons and Freeling 2008). Graphical

highlights of TE-like sequences have been customized by choosing "show other features" in the "results visualization" screen. By that means, we confirmed AtGS genes that transposed at least once during lineage evolution as defined by Woodhouse et al. and identified further GTDs missed by that approach due to lack of synteny data (i.e., GTDs predating *Vitis* speciation and recent GTDs of Brassicaceae-specific genes; marked by asterisks in table 2). Information on GTD events was added to an additional column in supplementary table S1, Supplementary Material online.

Analysis of Putative GS Genes Not Affected by TD, GTD, or At-α Ohnolog Retention in *Arabidopsis*

We performed additional analysis of AtGS loci beyond the aforementioned types of duplication by considering more ancient WGD events. Information on *Arabidopsis* genome-wide distribution of ohnolog duplicate pairs dating back to the At-ß and At-γ WGD events (Bowers et al. 2003) were added to an additional column in supplementary table S1, Supplementary Material online. GS genes were referenced accordingly. Remnants did not show significant similarities to any other locus in *Arabidopsis* by definition, and evolutionary stability was confirmed using the *Arabidopsis* transpositional history database (http://nature.berkeley.edu/freelinglab, last accessed November 8, 2013) and data on AtGS syntelogs in *B. rapa* (Wang et al. 2011a).

Orthologous Gene Identification of *Arabidopsis* GS Biosynthetic Genes in *Aet. Arabicum*

We considered multiple lines of evidence for identification of orthologs between *A. thaliana* GS loci and *Aet. arabicum*. We defined orthologous pairs of *A. thaliana* and *Aet. arabicum* GS loci as reciprocal best hits (RBHs) within a given region of gene colinearity (synteny). First, we screened for regions in the

Table 2
GTDs in *Arabidopsis* and *Aethionema* GS Pathway Inventory

Protein Name	AGI[a]	α-Block	AabID[b]	Syntelog	% Identity	Lineage Specific?	Col-0 → Aab[c]
GS genes with retained α-ohnolog							
[AAO3]	AT2G27150	A02NOA1	Aab27016	Yes	77.67	No	2 → 2
CYP79C1	AT1G79370	A05N062	Aab34143	Yes	76.8	No	8 → 9
APK2	AT4G39940	A10NOA2	Aab17804	Yes	86.05	No	2 → 2
CYP79B2	AT4G39950	A10N257	Aab17805	Yes	81.38	No	8 → 9
CYP79B3	AT2G22330	A10N257	Aab19477	Yes	81.4	No	8 → 9
GS genes with tandem duplicate copy							
AOP1	AT4G03070	A01	Aab37231	Yes	70.03	No	2 → 1
AOP3	AT4G03050	A01	—	—	—	*Arabidopsis*	2 → 1
CYP79F1	AT1G16410	A05	Aab27579	Yes	72.79	*Aethionema*	8 → 9
CYP79C2	AT1G58260	A03	Aab17711	Yes	71.85	*Aethionema*	8 → 9
		A11	Aab22600	No	61.8	*Aethionema*	8 → 9
CYP83A1	AT4G13770	A15	Aab32506	Yes	69.67	No	2 → 3
		—	Aab30975	No	82.8	*Aethionema*	2 → 3
CYP81F2	AT5G57220	A22	—	—	—	*Arabidopsis*	2 → 1
GS genes without α-ohnolog or tandem duplicate copy							
UGT74B1	AT1G24100*	A05	Aab07826	Yes	70.35	No	6 → 10
		A05	Aab07827	Yes	80.65	*Aethionema*	6 → 10
IMD3	AT1G31180	A06	—	—	—	*Arabidopsis*	2 → 1
CYP83B1	AT4G31500	A10	Aab12019	No	92.73	No	2 → 3
GSL-OH	AT2G25450	A10	—	—	—	*Arabidopsis*	1 → 0
IMD1	AT5G14200	A12	Aab14760	Yes	89.5	No	2 → 1
CYP79A2	AT5G05260*	A14	Aab36760	Yes	73.37	No	8 → 9
CHY1	AT5G65940*	A19	Aab05851	Yes	80.75	No	2 → 2
FMO-GSOX-1	AT1G65860*	A25	Aab30109	Yes (minimum)	58.45	No	11 → 8
	24% TD (4/17) 29% retained α-ohnolog (5/17)		18% TD (3/17) 29% retained α-ohnolog (5/17)		∅ 76.78%		

[a]Asterisks mark GTDs inferred by flanking TE-like sequences using GEVo.
[b]Predicted *Aethionema* CDS.
[c]Change of gene family locus count in *Arabidopsis* → *Aethionema* order.

Aet. arabicum genome displaying synteny to genomic regions in *A. thaliana* harboring GS loci, using the "Synfind" function with standard parameters from the CoGe comparative genomics system (www.genomeevolution.org, last accessed November 8, 2013) (Tang and Lyons 2012). Second, we determined RBHs between *A. thaliana* GS genes and *Aet. arabicum* genes within the syntenic regions from (i), using BlastP with a minimum query coverage of $N = 0.5$ and a cut-off *e*-value of 1E−10. Third, we queried all putative *Aet. arabicum* GS loci against the *Aet. arabicum* genome in a BlastP screen with a cut-off *e*-value of 1E−30. We screened for subject sequences not sharing the query sequence scaffold and identified syntenic regions in *A. thaliana*. If a GS biosynthetic gene was present in syntenic *A. thaliana* region (BlastP with a cut-off *e* value of 1E−30), we defined the aligned *Aet. arabicum* subject sequence as ortholog to the *A. thaliana* query sequence.

Tandem Arrayed Gene Copy Identification of Putative GS Biosynthetic Genes in *Aet. arabicum*

We queried all putative *Aet. arabicum* GS loci against the *Aet. arabicum* genome in a BlastP screen with a cut-off *e*-value of 1E−30. For identification of TDs, GS query sequences were grouped with the subset of respective subject sequences located within a window of $N = 10$ allowed gene spacers to form *Aet. arabicum* superfamilies of putative TAR genes. TDs were visualized using the MAFFT package (http://mafft.cbrc.jp/alignment/software/, last accessed November 8, 2013) (Katoh et al. 2002). We further confirmed *Aet. arabicum* GS genes expression by querying the RNA-seq data. Transcriptome data were mined for expression of GS genes using TBlastX with a cut-off *e*-value of 1E−10 (data not shown).

Identification of Lineage-Specific GS GTDs Comparing Putative GS Biosynthetic Genes in *Aet. arabicum* and *A. thaliana*

We queried all putative *Aet. arabicum* GS loci against the *Aet. arabicum* genome in a BlastP screen with a cut-off *e*-value of 1E−30. For identification of GTDs following divergence of these lineages, we screened for subject sequences not sharing the query sequence scaffold and identified syntenic regions in *A. thaliana*. If GS biosynthetic gene is absent in syntenic *A. thaliana* region (BlastP with a cut-off *e*-value of 1E−30), we defined the aligned *Aet. arabicum* subject sequence as lineage-specific GTD copy.

Phylogenetic and Similarity Analysis

A number of *Arabidopsis* flavin-monooxygenases involved in GS biosynthesis (FMO GS-OX) are encoded in clusters consisting of retained ohnolog copies as well as both tandem- and gene transposition duplicates. To visualize the evolution of *FMO*-like sequences in Brassicales, *Carica. papaya,* and *Tarenaya hasslerania* (Cheng et al. 2013), *FMO* orthologs from these species were obtained using the CoGe comparative genomics system. A phylogenetic tree was constructed using the maximum-likelihood method with PhyML 3.1 software (Guindon et al. 2010), employing the Le/Gascuel (LG) model for amino acid substitution. Protein sequence similarity analysis were performed using the Needle program from the EMBOSS software package (http://emboss.sourceforge.net/, last accessed November 8, 2013) (Rice et al. 2000).

Genome Data Visualization and Statistics

Fisher's exact test for count data was performed using the R package for statistical computing (www.r-project.org, last accessed November 8, 2013). Circular visualization of genome data was performed using the circos package (www.circos.ca, last accessed November 8, 2013) (Krzywinski et al. 2009) and graphically edited with the GIMP-package (www.gimp.org, last accessed November 8, 2013).

Results

The Influence of the At-α WGD Event to GS Pathway Evolution in *Arabidopsis*

We first updated the genomic location of all ohnolog blocks dating back to the At-α WGD event (α-blocks thereafter) in *A. thaliana* from the TIGR5 to the TAIR10 annotation, leading to minor changes in the list published by Thomas et al. (2006) (supplementary table S1, Supplementary Material online). As a first step to understanding the dynamics of GS pathway evolution, we divided the 52 to-date known AtGS genes into three groups: first, genes with a retained At-α ohnolog copy (table 1). Second, genes with lost At-α ohnolog copy, but a genomic location covered by α-blocks (table 3). Third, genes located outside the genomic borders of α-blocks (table 4). For the original set of AtGS genes published by Sonderby et al. (2010), we found an increased retention rate of 49% (24/49) for retained ohnolog copies dating back to the At-α WGD event (fig. 1), compared with a 22% average observed for all *Arabidopsis* protein-coding genes (fig. 2*A* and *B*). These 24 canonical AtGS genes group to six ohnolog pairs with annotation to GS metabolism and 12 loci lacking annotation of one ohnolog copy to GS biosynthesis (figs. 1 and 3). Notably, the 12 ohnolog pairs sharing GS annotation and the six ohnolog pairs lacking GS annotation of one member (forming18 AtGS ohnolog copy pairs in total) either display high degrees of pairwise similarity and/or show similar tendencies in gene expression following treatment with methyljasmonic acid, an organic volatile important for plant defense signaling (Cheong and Choi 2003) (tables 5 and 6). Therefore, we inferred functional redundancy of ohnolog copies due to structural homology. We propose a significant contribution to GS metabolism and consistently include all 12 ohnolog copies lacking GS annotation to our analysis, forming 12 pairs of

Table 3

Genes with Nonretained At-α Ohnolog Duplicate Gene Copy in *Arabidopsis* and *Aethionema* GS Pathway Inventory

Protein Name	AGI	α-Block	Evident SSD[a]	AabID[b]	Syntelog	% Identity	Col-0 → Aab[c]
Core-structure formation							
AOP1	AT4G03070	A01	TD/GTD	Aab37231	Yes	70.03	2 → 1
AOP3	AT4G03050	A01	TD/GTD	—	—	—	2 → 1
UGT74-like	Aab specific	A02	TD	Aab37178	Yes	82.05	6 → 10
UGT74-like	Aab specific	A02	TD	Aab37179	Yes	77.63	6 → 10
UGT74-like	Aab specific	A02	TD	Aab37180	Yes	78.33	6 → 10
GSTF10	AT2G30870	A02	TD	Aab28612	Yes	91.59	4 → 4
FMO-GSOX-3	AT1G62560	A03	TD	Aab10867	Yes	71.9	11 → 8
FMO-GSOX-4	AT1G62570	A03	TD	Aab10866	Yes	55.2	11 → 8
FMO-GSOX-5	AT1G12140	A03	TD	Aab13546	Yes	71.9	11 → 8
CYP79C2	AT1G58260	A03	TD	Aab17711	Yes	71.85	8 → 9
	Aab specific	A11		Aab22600	No	61.8	8 → 9
CYP79F1	AT1G16410	A05	TD	Aab27579	Yes	72.79	8 → 9
SOT18	AT1G74090	A05	TD	Aab14277	Yes	83.9	3 → 3
GSTU20	AT1G78370	A05	TD	Aab7000	Yes	67.29	5 → 6
	Aab specific			Aab6995	Yes	48.86	5 → 6
UGT74B1	AT1G24100	A05	GTD	Aab07826	Yes	70.35	6 → 10
	Aab specific			Aab07827	Yes	80.65	6 → 10
CYP83B1	AT4G31500	A10	GTD	Aab12019	No	92.73	2 → 3
GSL-OH	AT2G25450	A10	GTD	—	—	—	1 → 0
CYP79A2	AT5G05260	A14	GTD	Aab36760	Yes	73.37	8 → 9
CYP83A1	AT4G13770	A15	GTD	Aab32506	Yes	69.67	2 → 3
	Aab specific			Aab30975	No	82.8	2 → 3
CYP81F2	AT5G57220	A22	TD/GTD	—	—	—	2 → 1
FMO-GSOX-1	AT1G65860	A25	GTD	Aab30109	Yes	58.45	11 → 8
Cosubstrate pathways							
CHY1	AT5G65940	A19	GTD	Aab05851	Yes	80.75	2 → 2
GSH1	AT4G23100	A20	NA	Aab22781	Yes	91.81	2 → 2
BZO1	AT1G65880	A25	TD	Aab31601	Yes	70.04	2 → 4
			TD	Aab31602	Yes	69.4	2 → 4
Side-chain elongation							
IMD3	AT1G31180	A06	GTD	—	—	—	2 → 1
IPMI2	AT2G43100	A11	TD	Aab19630	Yes	78.71	3 → 3
IMD1	AT5G14200	A12	GTD	Aab14760	Yes	89.5	2 → 1
IIL1	AT4G13430	A15	NA	Aab18132	Yes	93.9	1 → 1
TF regulation							
MYB76	AT5G07700	A26	TD	—	—	—	6 → 4
	60% TD (15/25)			57% TD (16/28)		∅ 76.46%	
	48% GTD (12/25)			39% GTD (11/28)			

Note.—NA, not applicable.

[a]TD (Tandem Dupicate) refers to members of tandem arrays and GTD (Gene Transposition Duplication) refers to a history of transposition in *Arabidopsis*.

[b]Predicted *Aethionema* CDS

[c]Change of gene family locus count in Arabidospsis → Aethionema order

two ohnolog copies each. We thereby created an extended set of 64 putative AtGS genes (figs. 1 and 3). Among genes located within α-block boundaries, we found an At-α ohnolog retention rate of 59% (36/61) for the extended AtGS set (fig. 1), which is more than double of the observed 22% average rate for ohnolog retention among all *Arabidopsis* protein-coding loci harbored within the boundaries of α-blocks (fig. 2*B*).

Quantification of TD Influence to GS Pathway Evolution in *Arabidopsis*

In the next step, we quantified the impact of TD to GS pathway versatility in *Arabidopsis*. Minor changes were made in the list of *Arabidopsis* TAR genes by Rizzon et al. (2006) due to the gene updates to TAIR10 (supplementary table S1, Supplementary Material online). We mined the 1,497 *Arabidopsis* TARs comprising 4,034 duplicate gene copies for AtGS

Table 4

Genes Not Covered by α-Blocks in *Arabidopsis* and *Aethionema* GS Pathway Inventory

Protein Name[a]	AGI	α-Block	Evident SSD[b]	AabID[c]	Syntelog	% Identity	Col-0 → Aab[d]
Side-chain elongation							
MAM1	AT5G23010	—	TD	Aab12229	Yes	72.31	2 → 4
				Aab12230	Yes	71.5	2 → 4
MAM-L	AT5G23020	—	TD	Aab12225	Yes	70.67	2 → 4
				Aab12226	Yes	68.36	2 → 4
TF regulation							
MYB34	AT5G60890	—	NA	—	—	—	6 → 4
	66% TD (2/3)			100% TD (4/4)		∅ 70.71%	
	0% GTD			0% GTD			

[a]Squared brackets indicate ohnolog copies of GS biosynthetic genes without GO!-annotation to GS biosynthetic process.
[b]TD refers to members of TARs and GTD refers to a history of transposition in *Arabidopsis*.
[c]Predicted *Aethionema* CDS.
[d]Change of gene family locus count in *Arabidospsis* → *Aethionema* order.

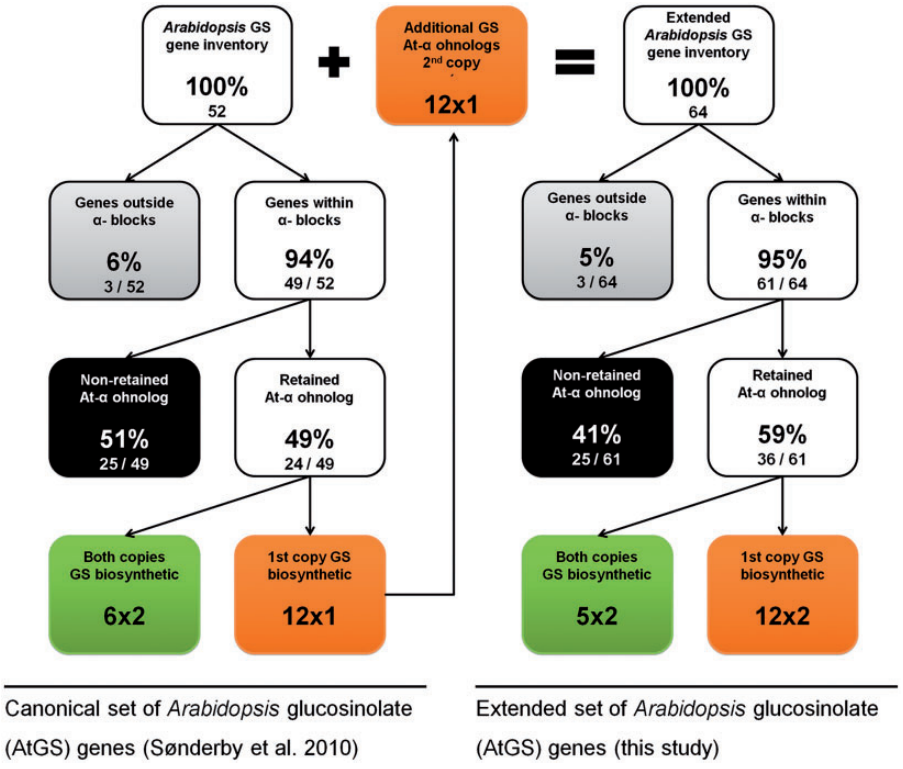


Fig. 1.—Distribution of GS pathway inventory relative to At-α WGD event. AtGS genes are shown before (left) and after (right) interpolation of ohnolog duplicate copies. We hypothesize functional redundancy of 12 additional ohnologs to canonical GS biosynthetic genes.

genes. Forty-five percent (29/64) of AtGS genes are members of TARs, compared with a genome-wide average of 15% (figs. 2*B* and *D*). Contribution of GTDs to GS pathway evolution in *Arabidopsis*

We quantified the influence of GTD to GS pathway evolution in *Arabidopsis*. Initially, a list of 4,575 genes with putative origin due to a GTD was proposed for TAIR9 (Woodhouse et al. 2011). Our update to TAIR10 retained 4,539 loci clearly referenced to transposition events (supplementary table S1, Supplementary Material online), illustrating a 14% average for GTD genes among protein-coding loci in *Arabidopsis* (fig. 2*B*). Among those, we confirmed all 13 references to AtGS loci (table 2), using the GEvo function from the CoGe platform (see Materials and Methods). We thereby discovered

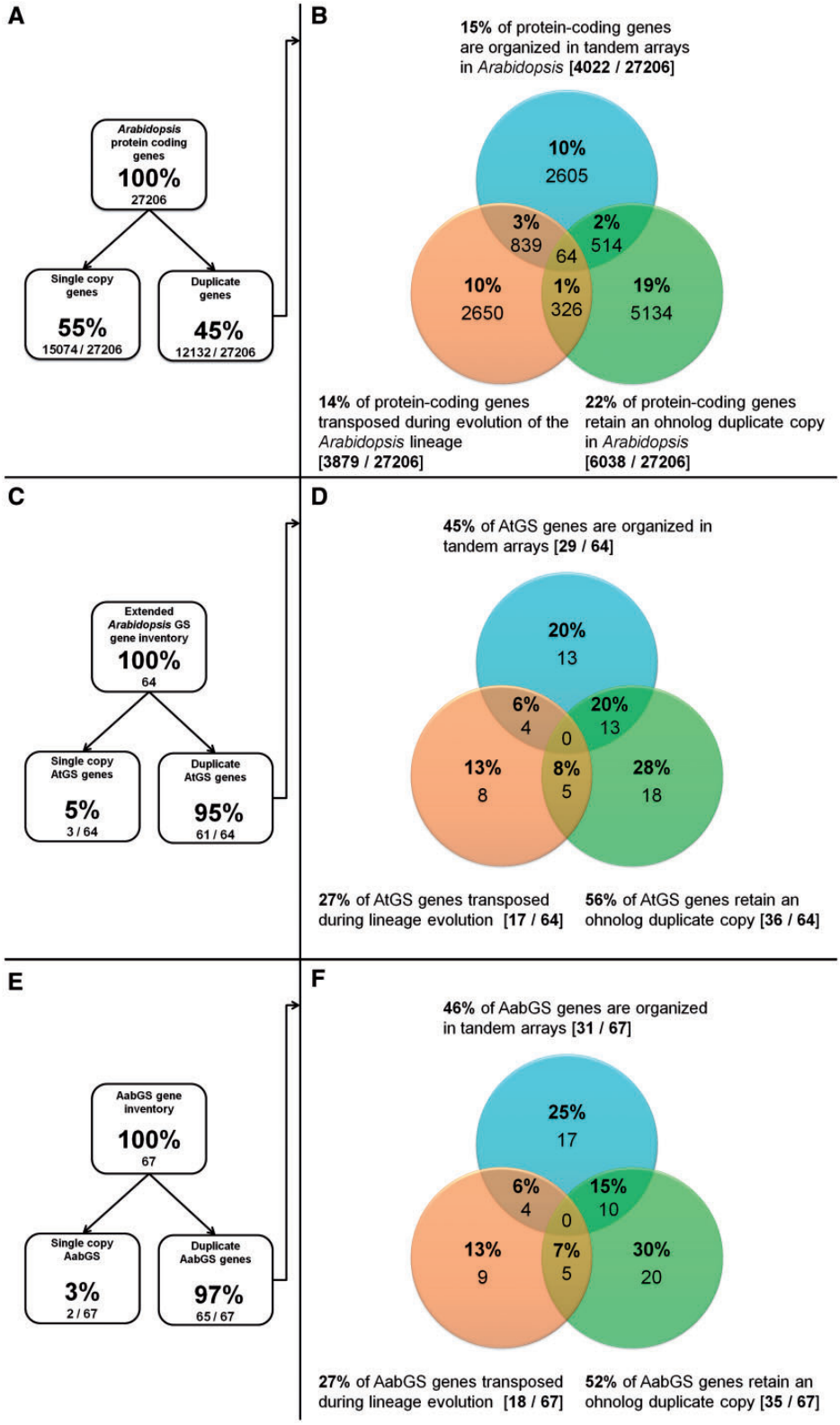


Fig. 2.—Duplicate distribution among (*A*, *B*) *Arabidopsis* protein-coding genes compared with (*C*, *D*) AtGS and (*E*, *F*) *Aethionema* GS loci. Shown are retained ohnologs (green), tandem duplicates (blue), and gene transposition duplicates (orange). GS metabolic versatility resulted from a combination of increased ohnolog retention and TD rates.

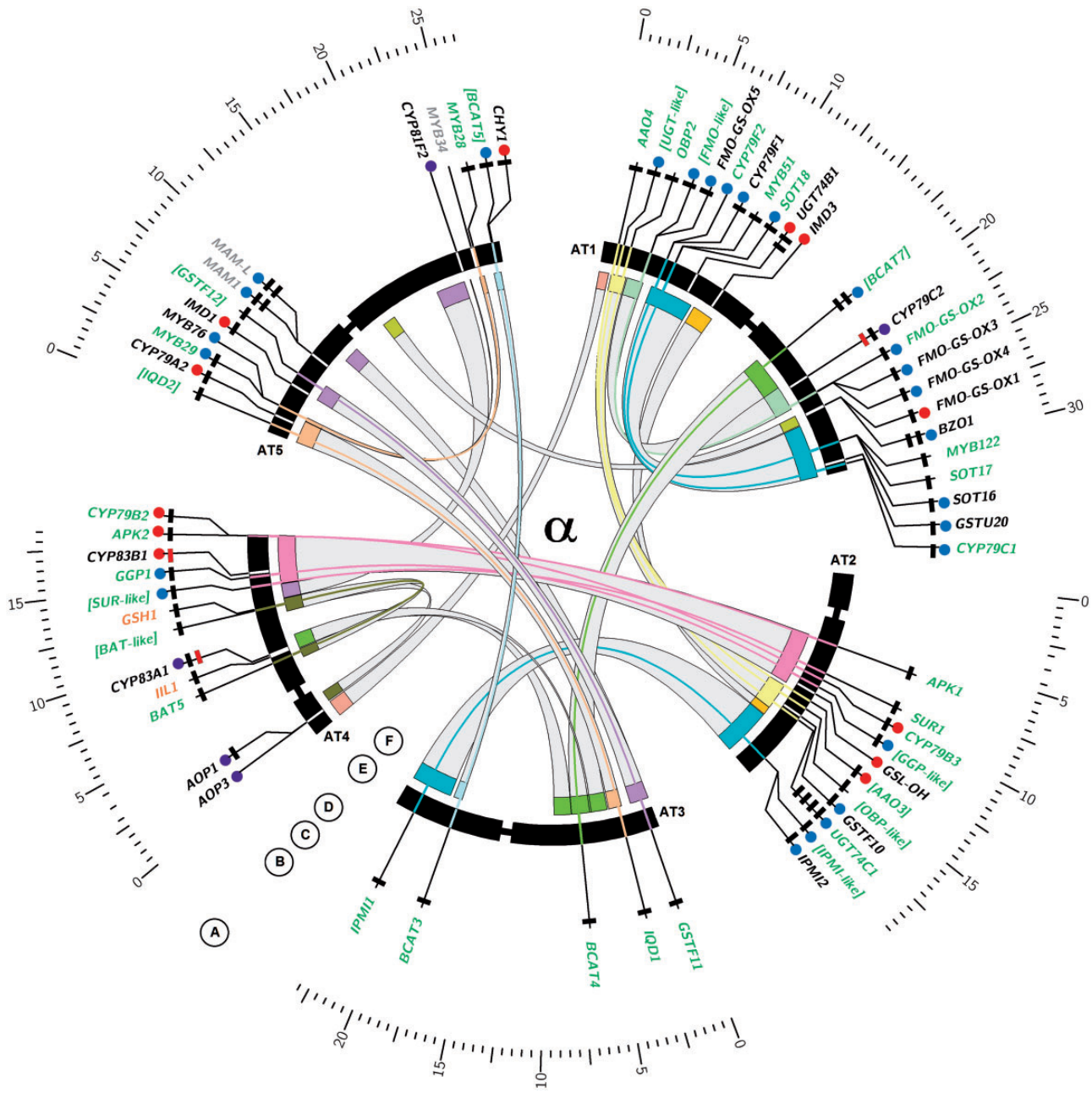
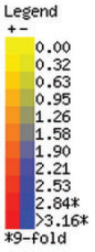


Fig. 3.—Ideogram of *Arabidopsis thaliana* chromosomes with GS biosynthetic genes. Circos plot visualizing the evolutionary contribution of different duplication types to GS pathway inventory in *Arabidopsis* and *Aethionema*. (*A*) Inner chromosome scale (Mb). (*B*) *Arabidopsis thaliana* GS biosynthetic genes. Gray text indicates genomic location outside ohnolog blocks. Black text indicates genomic location within ohnolog blocks but nonretained ohnolog copy. Green text indicates retained pairs of ohnolog copies with missing GO annotation to GS biosynthetic process shown in edged brackets. Orange text indicates single copy genes without clear paralogs in both species. (*C*) Blue circles indicate genes organized in TARs (i). Red circles indicate genes with transpositional history (ii). Purple circles indicate loci sharing (i) and (ii). (*D*) Number of rectangles indicates number of homologs present in the *Aethionema arabicum* draft genome (0–4). Color of rectangles indicates presence (black) or absence (red) of synteny between *A. thaliana* and *Aet. arabicum* in the genomic context of the target gene. (*E*) *Arabidopsis thaliana* chromosomes with labels showing GS biosynthetic genes. Bands for genes retained in ohnolog pairs are connected with colors of corresponding ohnolog blocks, as defined by Bowers et al. (2003). (*F*) Genomic location of ohnolog block copies harboring GS biosnthetic genes in *A. thaliana*, connected by gray bands. All ranges are in scale.

Table 5

Intraspecies Protein Similarities for At-α Ohnolog Pairs Sharing GS Annotation, Shown with Differential Expression in *Arabidopsis* Following MeJA Treatment

α-Block	Protein Name[a]	GO![b]	AGI	Similarity in Col-0 (%)	AabID[c]	Similarity in Aab (%)	Expression change[d]		
Both ohnologs with annotation to GS biosynthetic process							Time -> 0.5	1	3
A05N062	CYP79C1	Yes	AT1G79370	60.90	Aab34143	NA	0	0.3	0.5
	CYP79F2	Yes	AT1G16400[b]		—		0.8	0.3	1.1
A05N185	MYB122	Yes	AT1G74080	68.80	Aab14276	61.50	0.6	3.1	0.7
	MYB51	Yes	AT1G18570		Aab19683		–0.8	–0.6	0
A05N186	SOT17	Yes	AT1G18590	83.50	Aab19675	83.20	1	1.1	0.8
	SOT16	Yes	AT1G74100		Aab14278		0.8	1.2	1.4
A10NOA1	APK1	Yes	AT2G14750	67.50	Aab32150	62.10	0.7	1.4	1.6
	APK2	Yes	AT4G39940		Aab17804		1.3	1.9	1.6
A10N257	CYP79B3	Yes	AT2G22330	92.10	Aab19477	29.50	0.9	2	2.6
	CYP79B2	Yes	AT4G39950		Aab17805		0.5	1.4	2.2
A26N034	MYB29	Yes	AT5G07690	72.30	Aab33585	66.00	0.1	0.2	–0.6
	MYB28	Yes	AT5G61420		Aab12163		–0.2	–0.3	–0.7
				Ø 74.18		Ø 60.46			



NOTE.—MeJA, methyljasmonic acid; NA, not applicable.

[a]Squared brackets indicate ohnolog copies of GS biosynthetic genes without GO! annotation to GS biosynthetic process.

[b]GO! column indicates if gene is part of canonical GS pathway inventory set (Sonderby et al. 2010).

[c]Predicted *Aethionema* CDS.

[d]Whole WT plant averages of log-transferred expression change in *Arabidopsis*.

that four additional AtGS genes (marked by asterisks in table 2) lost all syntenic anchor genes in genomic proximity (±1,000 kb) but are surrounded by TE-like sequences. Thus they may have transposed following the At-α WGD event (marked by asterisks in table 2). These additional genes may be Brassicaceae specific but lost secondarily in *A. lyrata*. This might explain their absence in the *Arabidopsis* gene transpositional history database (that mainly scores pre-α GTD events due to the lack of further Brassicaceae synteny data necessary for scoring of post-α GTDs). Hence, the total fraction of GTD copies among AtGS genes sums up to 27% (18/67) (fig. 2*D*).

Analysis of GS Genes Not Affected by TD, Ohnolog Retention, or Transposition

In addition, we performed a more in-depth analysis of the three AtGS genes lacking TD, retained At-α ohnolog copy or evidence for transposition during evolution of the *Arabidopsis* lineage (table 7). Among those, *MYB34* is the only locus retaining an ohnologous copy dating back to the At-ß WGD event, leaving two putative nonduplicate genes in AtGS pathway inventory: *GSH1* and *IIL1*, functioning in GS cosubstrate pathways and side-chain elongation, respectively (table 3). To confirm the observed evolutionary stability of these genes, we identified syntelogs in *V. vinifera* (*IIL1*) and *B. rapa* (*GSH1*), respectively. Syntelogs in *Vitis* proof that *IIL1* did not transpose since the birth of the Rosids. Therefore, this gene represents a very ancient unigene. In case of duplication before *Vitis* lineage evolution, all copies were lost subsequently before radiation of the Rosid clade. In contrast, *GSH1* may be Brassicaceae-specific unigene that likewise lost all duplicates with above-threshold similarity.

GS Biosynthetic Gene Identification from Draft *Aet. arabicum* Genome

On the basis of the *Aet. arabicum* genome v1.0 and 37,839 annotated genes (Haudry et al. 2013), we identified homologs of *A. thaliana* loci coding for GS biosynthetic and regulatory genes. Combining reciprocal best BlastP hits with LAST screens for large scale gene colinearity/synteny (100 kb–1.2 Mb) (employed by the Synfind algorithm, see Materials and Methods), we found putative *Aet. arabicum* orthologs covering 57 of the 64 proposed AtGS genes with an observed nucleotide sequence identity of 45–94% (tables 1, 3, and 4). Among those, seven loci gave rise to an additional 10 further paralogs due to TD and GTD in *Aethionema* (see later), thereby extending the copy number of six multigene families to a total of 67 putative AabGS genes. The mRNA sequencing data for *Aet. arabicum* supported the evidence that all 67 putative AabGS genes were expressed (data not shown).

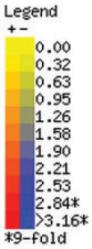
GS Gene Families with Expanded Copy Number in *Aethionema*

Among the 10 novel paralogs, eight were identified as descendants from TD events. Intriguingly, the *Arabidopsis* methylthiomalate synthase array *MAM1/MAM-L* underwent

Table 6

Intraspecies Protein Similarities for At-α Ohnolog Pairs Not Sharing GS Annotation, Shown with Differential Expression in *Arabidopsis* Following MeJA Treatment

α-Block	Protein Name[a]	GO![b]	AGI	Similarity in Col-0 (%)	AabID[c]	Similarity in Aab (%)	Expression change[d]		
One hnologs without annotation to GS biosynthetic process							Time ->	0.5	1	3
A02NOA2	AAO4	Yes	AT1G04580	84.10	Aab24896	79.90		1.7	−0.2	−0.4
	[AAO3]	No	AT2G27150		Aab27016			0.8	−0.2	−0.1
A02N051	UGT74C1	Yes	AT2G31790	22.30	Aab37175	19.50		0.4	0.4	0.3
	[UGT-like]	No	AT1G05670		Aab31930			0.3	0.1	0
A02N142	OBP2	Yes	AT1G07640	68.50	Aab18330	34.30		−0.1	0.1	−0.2
	[OBP-like]	No	AT2G28810		Aab24559			−0.1	−0.4	−0.2
A03N117	FMO-GSOX-2	Yes	AT1G62540	75.90	Aab10869	70.60		−0.3	0.3	0.4
	[FMO-like]	No	AT1G12130		Aab13543			−0.8	−0.6	2.1
A08N074	BCAT4	Yes	AT3G19710	72.20	Aab21007	76.80		0.1	0.2	0.5
	[BCAT7]	No	AT1G50090		Aab22550			0.4	0	0.7
A10N194	SUR1	Yes	AT2G20610	84.00	Aab31155	69.90		0.6	1	0.8
	[SUR-like]	No	AT4G28420		Aab30136			0.9	0.6	−2.5
A10N314	GGP1	Yes	AT4G30530	84.90	Aab24374	83.40		0.8	1	1.4
	[GGP-like]	No	AT2G23960		Aab11021			−0.2	−0.7	−0.7
A11N226	IPMI1	Yes	AT3G58990	80.60	Aab13092	73.20		0.2	0.6	0.9
	[IPMI-like]	No	AT2G43090		Aab19619			0	0.1	0.2
A12N102	GSTF11	Yes	AT3G03190	83.60	Aab14996	41.80		1.4	1.7	1.4
	[GSTF12]	No	AT5G17220		Aab14791			−0.5	0.2	1.1
A14N046	IQD1	Yes	AT3G09710	67.10	Aab18852	52.60		−0.2	−0.3	−0.1
	[IQD2]	No	AT5G03040		Aab18368			−0.2	−0.2	−0.4
A19N002	BCAT3	Yes	AT3G49680	8.20	Aab33782	79.90		0	−0.1	0
	[BCAT5]	No	AT5G65780		Aab23605			−0.1	−0.2	0
A20N095	BAT5	Yes	AT4G12030	78.90	Aab32285	63.30		0.4	−0.1	0.4
	[BAT-like]	No	AT4G22840		Aab23321			0	0	0.6
				Ø 67.52		Ø 62.10				



NOTE.—MeJA, methyljasmonic acid.

[a]Squared brackets indicate ohnolog copies of GS biosynthetic genes without GO! annotation to GS biosynthetic process.

[b]GO! column indicates if gene is part of canonical GS pathway inventory set (Sonderby et al. 2010).

[c]Predicted *Aethionema* CDS.

[d]Whole WT plant averages of log-transferred expression change in *Arabidopsis*.

Table 7

Putative Single-Copy Genes in *Aethionema* and *Arabidopsis* GS Pathway Inventory

AGI	Name	α-Block	Retained At-ß/-γ Ohnolog	Most Ancient Syntelog	Closest Paralog	BlastP *e* Value	Name	α-Block	Retained At-ß/-γ Ohnolog
AT4G13430	*IIL1*	A15	No	*Vitis vinifera*	AT4G26970	1.00E−16	*ACO2*	A22N121	No
AT4G23100	*GSH1*	A20	No	*Brassica rapa*	AT1G19220[a]	0.19	*ARF11*	A05	No
AT5G60890[b]	*MYB34*	—	B20N001	*V. vinifera*	AT1G74080	4.00E−62	*MYB122*	A05N185	B20N004

[a]Gene transpositional duplicate copy.

[b]Absent in *Aethionema*.

a further duplication in *Aethionema*, retaining four *MAM*-like loci (supplementary fig. S1, Supplementary Material online). Likewise, we observed a further TD of the *Arabidopsis* benzoate-CoA ligase array *BZO1/BZO*-like in *Aethionema,* adding two paralogs to the set of putative AabGS genes (table 8). Notably, both clusters encode functions in GS side-chain elongation (MAM) or cosubstrate pathways (BZO), indicating the connection of TD to metabolic versatility in both *Arabidopsis* and *Aethionema*. Furthermore, TD extended the gene inventory of GS core-structure modification in two cases. First, we detected one additional duplicate of the *Arabidopsis* tau-type gluthation-s-transferase array *GSTU19-23* in *Aethionema* (table 8). Second, we identified extension of the *UGT*-like superfamily that is present with five members in *Arabidopsis* and organized in two TARs of distant genomic location (fig. 3*B*). Intriguingly, both regions represent the sister copies of α-block a02 (fig. 3*F*). Furthermore, both *UGT*-like TARs comprise neighboring pairs of the At-α

Table 8

Tandem Duplicate Genes in *Arabidopsis* and *Aethionema* GS Pathway Inventory

Protein Name[a,b]	AGI	α-Block[c]	AabID[d]	Syntelog	% Identity[e]	Lineage Specific?	Col-0 → Aab[f]
Tandem duplicates of syntenic anchor genes retaining an At-α ohnolog							
UGT74C1	AT2G31790	A02N051	Aab37175	Yes	79.44	No	6 → 10
			Aab37178	Yes	82.05	*Aethionema*	6 → 10
			Aab37179	Yes	77.63	*Aethionema*	6 → 10
			Aab37180	Yes	78.95	*Aethionema*	6 → 10
UGT74D1_oa	AT2G31750	A02N053	Aab37181	Yes	78.33	(no GS gene)	6 → 10
[UGT-like]	AT1G05670	A02N051	Aab31930	Yes	81.11	No	6 → 10
UGT-like_oa	AT1G05675	A02	Aab31932	Yes	71.4	(no GS gene)	6 → 10
UGT74E2_oa	AT1G05680	A02N053	Aab31933	Yes	59.8	(no GS gene)	6 → 10
FMO-GSOX-2	AT1G62540	A03N117	Aab10869	Yes	76.6	No	11 → 8
FMO-GSOX-3	AT1G62560	A03	Aab10867	Yes	71.9	No	11 → 8
FMO-GSOX-4	AT1G62570	A03	Aab10866	Yes	55.2	No	11 → 8
FMO-like_oa	AT1G62580	A03	—	—	—	(no GS gene)	10 → 7
FMO-like_oa	AT1G62600	A03	—	—	—	(no GS gene)	10 → 7
FMO-like_oa	AT1G62620	A03	—	—	—	(no GS gene)	10 → 7
[FMO-like]	AT1G12130	A03N117	Aab13543	Yes	65.09	No	11 → 8
FMO-GSOX-5	AT1G12140	A03	Aab13546	Yes	71.9	No	11 → 8
FMO-like_oa	AT1G12200	A03	Aab13549	Yes	66.66	(no GS gene)	10 → 7
CYP79F2	AT1G16400	A05N062	—	—	—	*Arabidopsis*	8 → 9
CYP79F1	AT1G16410	A05	Aab27579	Yes	72.79	*Arabidopsis*	8 → 9
SOT16	AT1G74100	A05N186	Aab14278	Yes	91.07	No	3 → 3
SOT18	AT1G74090	A05	Aab14277	Yes	83.9	No	3 → 3
GSTU20	AT1G78370	A05	Aab07000	Yes	67.29	No	5 → 6
GSTU23_oa	AT1G78320	A05	Aab06994	Yes	81.74	(no GS gene)	5 → 6
GSTU22_oa	AT1G78340	A05	Aab06997	Yes	71.1	(no GS gene)	5 → 6
GSTU21_oa	AT1G78360	A05	Aab06998	Yes	76.71	(no GS gene)	5 → 6
GSTU19_oa	AT1G78380	A05N104	Aab06999	Yes	83.41	(no GS gene)	5 → 6
[BCAT7]	AT1G50090	A08N074	Aab22548	Yes	69	No	6 → 6
BCAT-like_oa	AT1G50110	A08	Aab22550	Yes	78.12	(no GS gene)	6 → 6
[SUR-like]	AT4G28420	A10N194	Aab31155	Yes	47.42	No	3 → 2
		A10	Aab31154	Yes		*Aethionema*	
SUR-like_oa	AT4G28410	A10	Aab31153	Yes	63.96	(no GS gene)	3 → 2
GGP1	AT4G30530	A10N314	Aab24374	Yes	87.6	No	5 → 5
GGP-like_oa	AT4G30540	A10	Aab24373	Yes	75	(no GS gene)	5 → 5
GGP3_oa	AT4G30550	A10	Aab24372	Yes	82	(no GS gene)	5 → 5
[GGP-like]	AT2G23960	A10N314	Aab11021	Yes	69.67	No	5 → 5
GGP-like _oa	AT2G23970	A10	Aab11018	Yes	83.6	(no GS gene)	5 → 5
[IPMI-like]	AT2G43090	A11N226	Aab19619	Yes	85.99	No	3 → 3
IPMI2	AT2G43100	A11	Aab19630	Yes	78.71	No	3 → 3
[BCAT5]	AT5G65780	A19N002	Aab23605	Yes	75.3	No	6 → 6
LINC4_oa	AT5G65770	A19	Aab23607	Yes	70.08	(no GS gene)	6 → 6
MYB29	AT5G07690	A26N034	Aab33585	Yes	65.13	*Arabidopsis*	6 → 4
MYB76	AT5G07700	A26	—	—	—	*Arabidopsis*	6 → 4
Tandem duplicates of genes inside the boundaries of α-blocks with nonretained At-αohnolog							
AOP1	AT4G03070	A01	Aab37231	Yes	70.03	*Arabidopsis*	2 → 1
AOP3	AT4G03050	A01	—	—	—	*Arabidopsis*	2 → 1
GSTF10	AT2G30870	A02	Aab28612	Yes	91.59	No	4 → 4
GSTF9_oa	AT2G30860	A02	Aab28613	Yes	89.76	(no GS gene)	4 → 4
CYP79C2	AT1G58260	A03	Aab17711	Yes	71.85	No	8 → 9
CYP-like_oa	AT1G58265	A03	Aab17712	Yes	60.71	(no GS gene)	8 → 9
UGT74B1	AT1G24100	A05	Aab07827	Yes	80.65	*Aethionema*	6 → 10
		A05	Aab07826	Yes	70.35	*Aethionema*	6 → 10
CYP81F2	AT5G57220	A22	—	—	—	*Arabidopsis*	2 → 1

(continued)

Table 8 Continued

Protein Name[a,b]	AGI	α-Block[c]	AabID[d]	Syntelog	% Identity[e]	Lineage Specific?	Col-0 → Aab[f]
CYP71B10 _oa	AT5G57260	A22	Aab25774	Yes	73.21	(no GS gene)	2 → 1
BZO1	AT1G65880	A25	Aab31601	Yes	70.04	No	2 → 4
			Aab31602	Yes	69.4	*Aethionema*	2 → 4
BZO-like_oa	AT1G65890	A25	Aab31603	Yes	68.85	(no GS gene)	2 → 4
			Aab31604	Yes	67.83	(no GS gene)	2 → 4
Tandem duplicates of genes outside the boundaries of α-blocks							
MAM1	AT5G23010	—	Aab12229	Yes	72.31	No	2 → 4
			Aab12230	Yes	71.5	*Aethionema*	2 → 4
MAM-L	AT5G23020	—	Aab12225	Yes	70.67	No	2 → 4
			Aab12226	Yes	68.36	*Aethionema*	2 → 4
	AtGS genes: 45% TD (29/64)		AabGS genes: 46% TD (31/67)		∅ 73.43%		

[a]Squared brackets indicate ohnolog copies of GS biosynthetic genes without GO! annotation to GS biosynthetic process.
[b]The "_oa" suffix indicates tandem duplicate copies without GO! annotation to GS biosynthetic process. These genes have not been considered for the tandem duplicate count of GS loci in both organisms.
[c]Underlined items refer to offspring of one pre-α TD event.
[d]Predicted *Aethionema* CDS.
[e]In case of *Aethionema*-specific TAR expansion, the corresponding *Arabidopsis* sequence for identity comparison was determined based on both genomic location and homology criteria.
[f]Change of gene family locus count in *Arabidospsis* → *Aethionema* order.

ohnologs duplicates A02N051 (*UGT74C1* or AT2G31790/*UGT*-like or AT1G05670) and A02N053 (*UGT74D1* or AT2G31750/*UGT74E2* or AT1G05680) (fig. 3*B*, table 8), indicating a pre-At-α TD event generating both precursors of the above-mentioned *UGT*-like ohnolog pairs. In *Aethionema*, we find a further TD-driven extension of this superfamily, adding three more copies to reach a total number of 8 *UGT*-like sequences (table 8). Therefore, the diversity of *UGT*-like sequences in Brassicaceae is expanded by the combination of WGD with pre- and post-At-α TD events.

GTD accounts for the copy number expansions of two putative AabGS loci. Both cases involve *CYP*-like genes that play a role in GS core-structure formation (Sonderby et al. 2010). In *Aethionema*, the TAR formed by 1) *CYP79C2* (At1G85260) and 2) the *CYP*-like locus AT1G58265 transposed an additional copy of the TAR to a different genomic location (supplementary fig. S2, Supplementary Material online). Likewise, we identified an additional GTD of *CYP83A1* in *Aethionema* (table 3). *CYP83A1* metabolizes oximes in GS biosynthesis, is not redundant to *CYP83B1,* and interestingly also possesses a history of GTD events in *Arabidopsis* (Naur et al. 2003).

Arabidopsis GS Loci without Orthologs in *Aethionema*

In seven cases, RBH- and synteny-based evidence was not sufficient to clearly assign orthologs to AtGS loci in the *Aet. arabicum* draft genome (tables 1, 3, and 4), leading to the contraction of six multigene families and loss of one single-copy gene.

Four of those loci, namely *AOP3*, *CYP79F2*, *IMD3,* and *MYB76,* are likewise absent in the *B. rapa* genome and may therefore be specific to *A. thaliana* and more closely related species (Wang et al. 2011a). *AOP1/AOP3* and*CYP79F1/2* represent two neighboring TARs with evident subfunctionalization in *Arabidopsis* (Kliebenstein et al. 2001; Prasad et al. 2012). Although AOP3 functions in GS side-chain elongation in *Arabidopsis* (Kliebenstein et al. 2001), *CYP79F2* encodes an enzyme involved in core structure formation of long-chain aliphatic GS. Furthermore, overexpression of the MYB76 transcription factor correlates with increased levels of both long-chained and short-chained aliphatic GS in *Arabidopsis* (Gigolashvili et al. 2008). However, experiments with *Arabidopsis myb76* T-DNA insertion lines to date failed to show any significant change in GS chemotype, making a strict requirement of *MYB76* for GS biosynthesis unlikely (Gigolashvili et al. 2008). Moreover, *IMD3* encodes a predicted enzyme with proposed functional redundancy to (as well as strong coexpression with) IMD1, a protein that was shown to be involved in GS accumulation in *Arabidopsis* (Hirai et al. 2007; Wentzell et al. 2007; Gigolashvili et al. 2009; Sawada et al. 2009; He et al. 2010). Therefore, absence of *IMD1 (IMD3)* in *B. rapa* (*Aet. arabicum)* supports the hypothesized capability of mutual phenotype rescue among *IMD1/3* double knock-outs in Brassicaceae, eventually preventing significant alterations of GS chemotype due to fractionation of *IMD*-like genes in *Aethionema*.

The other three of the seven AtGS loci that lack a clear ortholog in *Aethionema* are not found in the *B. rapa* genome: *MYB34, CYP81F2,* and *GSL-OH* (tables 3 and 4). Therefore, they represent *Aethionema* lineage-specific gene losses. MYB34 was shown to control indolic GS biosynthesis in *Arabidopsis* (Celenza et al. 2005). Interestingly, overexpression of *MYB34* in *Arabidopsis* partially rescued the altered GS chemotype caused by *MYB51* knockout (Gigolashvili et al. 2007).

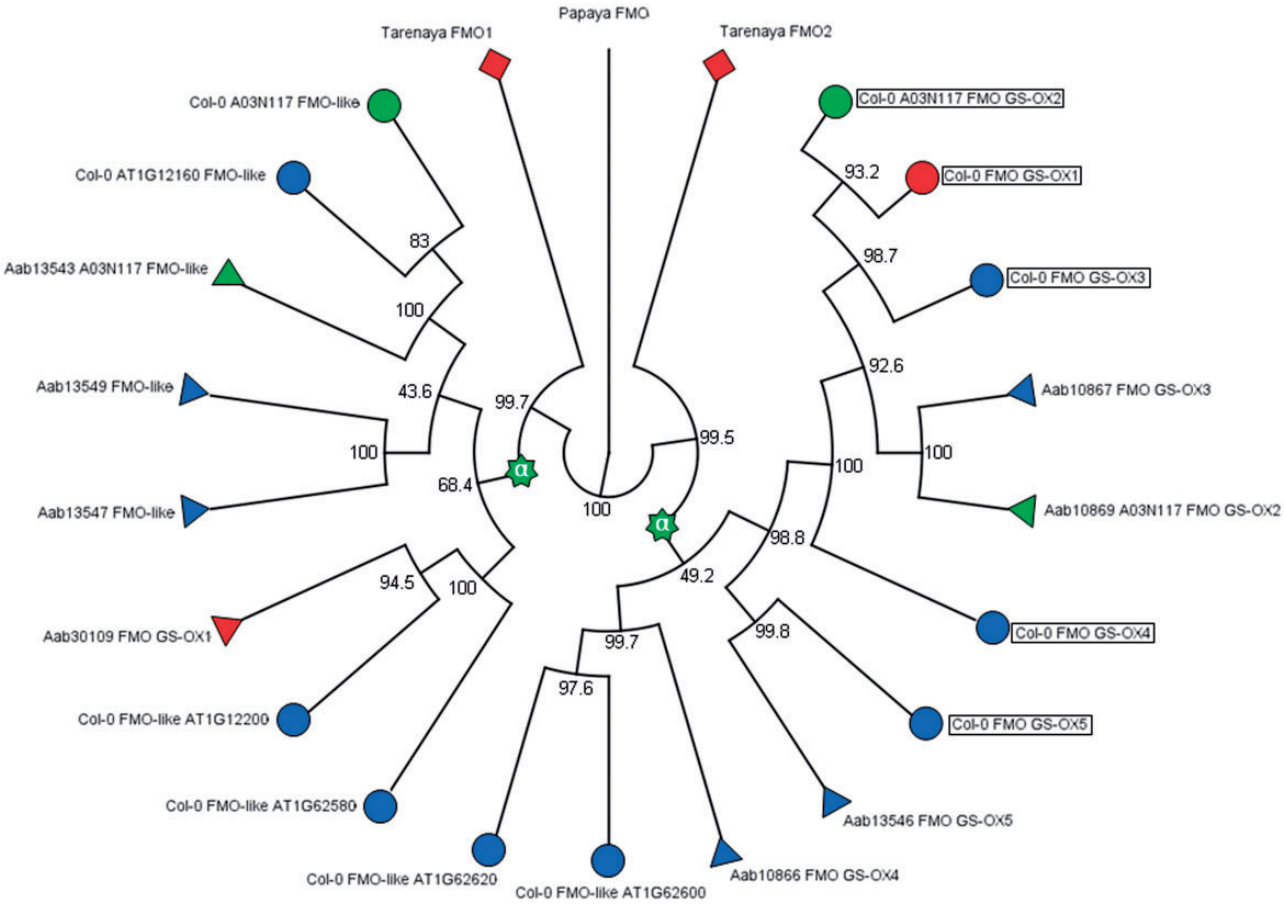


Fig. 4.—Phylogenetic relationships among FMO proteins. Col-0, Aab, and papaya refer to *Arabidopsis thaliana* (circles), *Aethionema arabicum* (triangles), and *Carica papaya* (colorless), respectively. Boxes indicate annotation to GS metabolic activity in *Arabidopsis*. *Tarenaya hasslerania* (diamonds) represents the closest-related outgroup of Brassicaceae, including the sister clade Aethionemae. Stars: At-α WGD event. Blue: proteins encoded by members of TARs. Red: protein encoded by GTD locus. The At-α WGD leads to duplication of a *FMO* locus, resulting in two clades comprising all *FMO*-like sequences of both *Aethionema* and *Arabidopsis*. Thus, FMO versatility has been promoted by a combination of increased degree of ohnolog retention and TD events.

Because of functional redundancy of MYB51/34, the loss of MYB34 likely does not cause significant changes in GS chemotype in *Aethionema* lineages. In contrast, *CYP81F2* and *GSL-OH* encode functions associated with secondary modification of the GS core structures (Sonderby et al. 2010). CYP81F2 has been shown to control the indole GS modifier1 quantitative trait loci in *Arabidopsis*, catalyzing the conversion of indole-3-yl-methyl GS to 4-hydroxy-indole-3-yl-methyl GS (Pfalz et al. 2009). Notably, these metabolites play a significant role in MAMP-triggered immunity in *Arabidopsis* (Bednarek et al. 2009). Among various known cytochrome p450s active in indolic GS biosynthesis, *CYP81F2* is the only locus impairing callose deposition after detection of the non-self-infection in *Arabidopsis* (Clay et al. 2009). On the basis of these findings, we concluded a CYP81F2-specific onset of sub/neofunctionalization from GS biosynthesis toward plant innate immunity after divergence of the *Arabidopsis* and *Aethionema* lineages, thereby mitigating fatal consequences of the absent ortholog for GS chemotype in *Aethionema*.

Moreover, we determined the absence of the 2-oxoacid-dependend oxygenase activity GSL-OH in *Aethionema* (table 3). GSL-OH is necessary for biosynthesis of 2-hydroxybut-3-enyl GS in *Arabidopsis* (Wentzell et al. 2007; Hansen et al. 2008) and present in the *B. rapa* genome (Wang et al. 2011a). Noteworthy, *GSL-OH* was the only multigene family member within the extended AtGS set whose loss in *Aethionema* was not accompanied by copy number expansion of one or more paralogs (table 3). Accordingly, we concluded an *Aethionema*-specific loss of this locus after divergence from the *Arabidopsis* lineage, creating measurable differences in GS chemotype among *Arabidopsis* and *Aethionema*. Consistently, we could not detect traces of 2-hydroxybut-3-enyl GSs in *Aet. arabicum* root-, leaf-, and seed extract using uHPLC (data not shown).

GS Gene Families with Lower Copy Number in *Aethionema*

Considering the Aethionemae-specific loss of *GSL-OH*, six putative GS-annotated multigene families display a lower copy number in *Aethionema (AOPx, CYP79x, CYP81x, GSLx, IMDx,* and *MYBx)* (tables 1, 3, 4, and 8). In sum, their total gene count increased from 20 genes in *Aethionema* to 27 observed in *Arabidopsis*, thereby mediating a 35% increase (tables 1, 4, and 8).

Although *IMD3* and *GSL-OH* possess GTD copies in *Arabidopsis*, these loci are absent in *Aethionema*. In contrast, *AOP1/2*, *IMD1/3*, *MYB29/76,* and *CYP81F2* with its *CYP*-like neighbor AT1G58265 comprise TARs in *Arabidopsis* but are likewise absent in *Aethionema* (table 8) and *B. rapa* (Wang et al. 2011a).

Therefore, the underlying TD events may be *Arabidopsis* specific. Thus, TD facilitated GS pathway expansion in the *Arabidopsis* lineage after split from the tribe Aethionemae.

Furthermore, we found evidence for *Arabidopsis*-specific TD of three neighboring *FMO*-like loci (*FMO GS-OX*$_{2\text{-}4}$) (figs. 3 and 4), leading to lower copy number in *Aethionema*. These genes were lost in *B. rapa* (Wang et al. 2011a), illustrating a degree of plasticity across Brassicaceae. *FMO*-like loci comprise a multigene family with five members annotated to GS biosynthesis in *Arabidopsis* mapping to three distant genomic locations (fig. 3*B*). Among those, two regions are embedded in ohnolog copies of α-block A02 (fig. 3*E*) and contain the retained α-pair of AT1G62540 (*FMO GS-OX2*) and AT1G12130 (*FMO*-like) (fig. 3*B*). The latter is not annotated to GS biosynthesis in *Arabidopsis*. However, AT1G12130 is member of a *FMO*-like 4-gene TAR with its 3′ neighbor *FMO GS-OX5* involved in aliphatic GS biosynthesis (Li et al. 2008). The third genomic region in *Arabidopsis* harboring a *FMO*-like sequence with encoded function in GS metabolism is defined by AT1G65860 (*FMO GS-OX1*), representing a transposed duplicate gene copy (fig. 4). Interestingly, *FMO GS-OX*$_{1\text{-}4}$ share broad substrate specificity and catalyze the conversion from methylthioalkyl GS to the related methylsulfinylalkyl GS independent of chain length. In contrast, *FMO GS-OX5* shows substrate specificity for 8-methylthiooctyl GS (Hansen et al. 2007; Li et al. 2008). This example is similar to the case of *UGT*-like loci (see earlier) and again illustrates the combination of ohnolog retention with tandem- and GTD leading to increased GS pathway versatility in Brassicaceae (fig. 4).

Deduction of Total Duplicate Frequencies in *Aethionema* and *Arabidopsis* GS Pathway Inventory and Comparison to *Arabidopsis* Genome-Wide Average

We found the fraction of retained ohnolog duplicate gene pairs among *Arabidopsis* (56%) and *Aethionema* (52%) GS biosynthetic and regulatory genes significantly increased compared with the genome-wide average in *Arabidopsis* (22%) (fig. 2, table 9).

Table 9

Statistical Test[a] on Duplicate Fractions in *Arabidopsis* and *Aethionema* GS Pathway Inventory Compared with Genome-Wide Average in *Arabidopsis*

	Arabidopsis Genome	*Arabidopsis* GS Genes[b]	*Aethionema* GS Genes
Protein-coding genes	27,206	64	67
Retained At-α ohnologs	6,038/22%	36/56%	35/52%
P value		3.87E−09	1.26E−07
Tandem duplicates	4,022/15%	29/45%	32/48%
P value		5.71E−09	9.14E−10
GTDs	3,879/14%	17/27%	18/27%
P value		0.01066	0.07462
Sum duplicates	12,132/45%	61/95%	65/97%
P value		2.20E−16	2.20E−16

[a]Fisher's exact test on count data.
[b]Extended set (fig. 1).

Moreover, 46% (31/67) of AabGS genes are organized in TARs (fig. 2*F*), compared with a 22% average for all protein-coding genes in *Arabidopsis,* thereby significantly surpassing the TAR coverage rate of 45% (29/64) observed for AtGS loci (fig. 2*B* and *D*, table 9).

For duplication by gene transposition, we detected 27% (17/67) of affected AabGS loci (fig. 2). In summary, we found no significant enrichment of GTD events among GS pathway inventory in both species (table 9).

Discussion

The Aethionemae/Brassicaceae crown-group/sister-group lineages split about 30–60 Ma shortly after the last common WGD event and independently evolved ever since (Couvreur et al. 2010; Mithen et al. 2010; Schranz et al. 2011). Notably, the radiation process evident for the Brassicaceae lineage created about 3,700 species (Couvreur et al. 2010). In contrast, the species-poor *Aethionema* lineage (Schranz et al. 2012) is well established as most ancient Brassicaceae extant sister and may therefore possess a more "ancient" genome organization when compared with *Arabidopsis*. This facilitates the recognition and quantification of common factors underlying rapid innovation of complex traits shared by both species. We exploit novel genomics resources for evolutionary analysis of the complete GS pathway inventory in both *A. thaliana* and *Aet. arabicum* to utilize the impact of different kinds of duplication classes to diversification of plant secondary metabolites. In a comparative genomics approach, we employ the phylogenetic relation of *Aet. arabicum* and *A. thaliana* to identify key factors driving GS pathway divergence. In this context, we establish GSs genetics/genomics as a scaffold to incorporate further phenotypic data for better understanding the impact of duplication to rapid evolution of novel key traits. In

Arabidopsis, several GS genes retained duplicate gene copies dating back to the last WGD event but lacking annotation to GS metabolic processes (fig. 1). Illustrating high degrees of protein similarities among these ohnolog copy pairs and/or similar responses in gene regulation following GS pathway induction (tables 5 and 6), we identified 12 novel putative *Arabidopsis* genes associated to GS biosynthesis (figs. 1 and 3). Given the fact that these loci remained unknown despite their putative relevance for an experimentally very well-studied trait-like GS biosynthesis, we highlight the importance of considering ohnolog copies when analyzing a plethora of other highly diverged multigene pathways (i.e., terpenoid biosynthesis). We thereby provided an easy-to-follow framework on how to use existing data on WGD in *Arabidopsis* to better understand the networks of functional redundancy, especially involving genes that are targeted for knock-out experiments in functional studies.

Evolutionary analysis of homologous GS loci in *Arabidopsis* and *Aethionema* found a majority (all but two) comprising duplicate groups organized in multigene families (figs. 2 and 3). This underlined the dominant role of duplication for creation and expansion of biochemical diversity in plant (secondary) metabolism.

Clear orthologs of seven *Arabidopsis* GS genes are absent in the *Aethionema* draft genome (due to three *Aethionema*-specific GS gene losses and four *Arabidopsis*-specific TDs). Evolution of 10 additional *Aethionema* paralogs (two due to gene transposition and eight due to TD events, fig. 3) lead to an almost 100% conserved GS pathway inventory across the crown group/sister group system. This sheds light upon the relevance of genome plasticity for key trait maintenance despite of scattered gene losses. To test this hypothesis, we indicate the requirement of further research on additional multigene pathways in a deeper phylogenetic resolution. Identification of *Aethionema* GS gene homologs allowed confirming the increased frequency of duplicates in lineages that diverged more than 30–60 Ma. The absence of lineage-specific polyploidy events in either species facilitated the comparative analysis of genes duplicated due to the common ancient WGD events (particularly At-α) as well as lineage-specific gene tandem and transposition duplications. Partitioning the duplicate genes set in GS pathway inventory revealed significant enrichments of retained At-α ohnologs and tandem duplicates (but not GTD events) in both species compared to the average observed for protein-coding genes in *Arabidopsis* (table 2). We therefore conclude that WGD and TD facilitated the early and continued evolution of GS biosynthesis in the mustard family. To our knowledge, this is the first study providing distinct indications on a genetics level for the connection of WGD to the emergence of key traits in planta.

Various duplicates of different GS gene families code for proteins encoding functions in consecutive steps of GS biosynthesis (Kliebenstein et al. 2001; Hansen et al. 2007). Among GS biosynthetic and regulatory genes, pairs of retained At-α ohnolog duplicates in distant genomic location further expand to TARs (fig. 3). In *Arabidopsis*, the S-oxygenase activity FMO is provided by a pair of retained ohnologs on distant arms on chromosome 1 (figs. 3 and 4). Both copies evolved further tandem duplicates with different substrate specificities (Li et al. 2008). Different groups of substrates are products of SOT-type sulfotransferases provided by another retained ohnolog pair on At1 with additional TD copies sharing annotation to GS production (Bowers et al. 2003; Piotrowski et al. 2004) (fig. 3). The reaction delivering substrates for GS SOT-type sulfotransferases is catalyzed by UGT-type proteins, likewise encoded by a pair of retained ohnologs that evolved multiple tandem and gene transposition duplicates in both *Aethionema* and *Arabidopsis* (fig. 3). It is thus inferred that subfunctionalization of both TD and retained At-α ohnolog pairs caused functional diversification of GS biosynthetic and regulatory elements. Showing mutual influence of ohnolog retention and TD rate across a crown group–sister group system, we describe a complex network of gene duplication fostering the expansion of a composite trait, thereby contributing to the means of mutation and selection to create evolutionary innovation in a limited timeframe. Evidence of the model of evolution by gene duplication can be found in comparative GS pathway analysis. Thus, GS may provide a framework for investigating the expansion of complex traits.

Supplementary Material

Supplementary figures S1 and S2 and table S1 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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