Functional Divergence in the *Arabidopsis* β-1,3-Glucanase Gene Family Inferred by Phylogenetic Reconstruction of Expression States

Andrew C. Doxey,[1] *Mahmoud W. F. Yaish,*[1,2] *Barbara A. Moffatt, Marilyn Griffith, and Brendan J. McConkey*

Department of Biology, University of Waterloo, Waterloo, Ontario Canada

Plant β-1,3-glucanases (β-1,3-Gs) (E.C. 3.2.1.39) comprise large, highly complex gene families involved in pathogen defense as well as a wide range of normal developmental processes. In spite of previous phylogenetic analyses that classify β-1,3-Gs by sequence relatedness, the functional evolution of β-1,3-Gs remains unclear. Here, expression and phylogenetic analyses have been integrated in order to investigate patterns of functional divergence in the *Arabidopsis* β-1,3-G gene family. Fifty β-1,3-G genes were grouped into expression classes through clustering of microarray data, and functions were inferred based on knowledge of coexpressed genes and existing literature. The resulting expression classes were mapped as discrete states onto a phylogenetic tree and parsimony reconstruction of ancestral expression states was performed, providing a model of expression divergence. Results showed a highly nonrandom distribution of developmental expression states in the phylogeny ($P = 0.0002$) indicating a significant degree of coupling between sequence and developmental expression divergence. A weaker, yet significant level of coupling was found using stress response data, but not using hormone-response or pathogen-response data. According to the model of developmental expression divergence, the ancestral function was most likely involved in cell division and/or cell wall remodeling. The associated expression state is widely distributed in the phylogeny, is retained by over 25% of gene family members, and is consistent with the known functions of β-1,3-Gs in distantly related species and gene families. Consistent with previous hypotheses, pathogenesis-related (PR) β-1,3-Gs appear to have evolved from ancestral developmentally regulated β-1,3-Gs, acquiring PR function through a number of evolutionary events: divergence from the ancestral expression state, acquisition of pathogen/stress-responsive expression patterns, and loss of the C-terminal region including the glycosylphosphatidylinisotol (GPI)-anchoring site thus allowing for extracellular secretion.

Introduction

β-1,3-glucanases (glucan endo-1,3-glucosidases, β-1,3-Gs, E.C. 3.2.1.39) are a class of hydrolytic enzymes that catalyze the cleavage of 1,3-ß-D-glucosidic linkages in β-1,3-glucans and are found in bacteria, fungi, metazoa (Bachman and McClay 1996), and viruses (Sun et al. 2000). β-1,3-Gs form highly complex and diverse gene families in plants, where they are involved in a wide range of physiological and developmental processes (Jin et al. 1999; Leubner-Metzger and Meins 1999). β-1,3-Gs have received a considerable amount of attention due to their role in plant pathogen defense. As members of the PR-2 group of pathogenesis-related (PR) proteins, β-1,3-Gs are induced by pathogen infection and play an active antifungal role in hydrolyzing β-1,3-glucan, a major structural component of fungal cell walls (Leubner-Metzger and Meins 1999). The degradation of β-1,3-glucan in cell walls is thought to contribute toward fungal cell wall destabilization as well as release of cell wall associated immune elicitors that further stimulate defense responses (Leubner-Metzger and Meins 1999). Antifungal activity has been confirmed both in vitro (Sela-Buurlage et al. 1993) and in vivo (Jach et al. 1995) through genetic transformation studies. Another intriguing role of β-1,3-Gs associated with PR function is their involvement in cold response (Griffith and Yaish 2004; Yaish et al. 2006). Although research on β-1,3-Gs to date has focused primarily on their PR functions, β-1,3-Gs also play critical roles in normal developmental plant processes. Callose (plant β-1,3-glucan) is found as a transitory material in the cell plate during cell division (Fulcher et al. 1976; Longly and Waterkeyn 1977; Samuels et al. 1995), as a major component of pollen mother cell walls, pollen tubes, plasmodesmatal canals (Stone and Clarke 1992), and seed-covering structures of several dicot species (Leubner-Metzger 2003). Thus, in addition to their roles in pathogen defense, β-1,3-Gs have been implicated in cell division, pollen development and tube growth, regulation of plasmodesmata signaling, cold response, seed germination, and maturation (see Hoj and Fincher 1995; Leubner-Metzger and Meins 1999; Leubner-Metzger 2003, for reviews).

The diversity of β-1,3-G function is mirrored by its large gene family size (Jin et al. 1999). Previous attempts to classify β-1,3-Gs into a manageable set of subclasses have relied on isoelectric point, sequence similarity, and other sequence features. As a result, a number of distinct sequence classes have been defined in tobacco (Linthorst et al. 1990), barley (Hoj and Fincher 1995), soybean (Jin et al. 1999), and Arabidopsis (Dong et al. 1991; Hird et al. 1993; Delp and Palva 1999). The biological significance of β-1,3-G gene multiplicity and the relationship between sequence and function, however, remains unclear. Ultimately, although a phylogenetic analysis based on sequence alone provides a means to infer the molecular evolution of a gene family, it does not necessarily reflect divergence of function. Through acquisition or loss of regulatory *cis*-elements, the expression profiles of duplicate genes may diverge independently of coding sequence allowing genes to acquire new or modified functions. Therefore, the incorporation of expression data into a phylogenetic analysis is crucial in the construction of a proper model of functional divergence.

Arabidopsis thaliana is an ideal model organism for the study of expression divergence, an area of considerable interest in current evolutionary studies (see Li et al. 2005 for

[1] Equal contribution to this work.

[2] Present address: Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada.

Key words: β-1,3-glucanase, molecular evolution, expression analysis, functional divergence, pathogenesis-related proteins.

E-mail: mcconkey@uwaterloo.ca.

Mol. Biol. Evol. 24(4):1045–1055. 2007
doi:10.1093/molbev/msm024
Advance Access publication February 1, 2007

review) for a number of reasons (Blanc and Wolfe 2004). Arabidopsis has undergone a number of rounds of whole-genome duplication, providing a large number of polyploid genes. Full genomic information is also available, so it is possible to obtain all members of a particular gene family. Lastly, publicly available Arabidopsis microarray resources provide a means to classify and functionally annotate genes based on their expression patterns.

Here, we have integrated phylogenetic and expression analyses to develop a model of functional divergence of the β-1,3-G gene family in Arabidopsis. Microarray expression data are used to cluster genes into different expression categories, and functions are attached to the expression clusters using knowledge of coexpressed genes as well as existing literature. The expression clusters are subsequently mapped onto a phylogenetic tree, and ancestral expression states are inferred using parsimony. Finally, a random permutation test is performed to compare the observed parsimony score against a random distribution from which a *P* value is calculated. The combined analysis of protein sequence, expression, and phylogeny is demonstrated as a powerful approach for investigating functional divergence in large gene families.

Materials and Methods

Sequence Retrieval and Phylogenetic Analysis

A Blast (Altschul et al. 1997) search using a representative β-1,3-G sequence (AGI locus identifier: At3g57270) was performed to screen the Arabidopsis protein database accessible at The *Arabidopsis thaliana* Information Resource (TAIR, http://www.arabidopsis.org). The top 50 hits had *E* values $\leq$ 1e-32, whereas subsequent hits had *E* values $\geq$ 0.084. All sequences were annotated as or similar to "glycosyl hydrolase family 17" by TAIR. A number of additional sequences are incorrectly annotated as glycosyl hydrolase family 17 by virtue of sharing homology with the C-terminal domain (Henrissat and Davies 2000) and were not included the data set. The set of identified glucanases was verified by position-specific iterated Blast (Altschul et al. 1997) using the National Center for Biotechnology Information nonredundant database restricted to Arabidopsis with an inclusion threshold of E $\leq$ 1e-30. No additional glucanases were identified. A multiple alignment (supplementary fig. SF1 and supplementary data file SD1, Supplementary Material online) of the sequences encoding the conserved glycosyl hydrolase family 17 domain was constructed with MUSCLE v. 3.52 (Edgar 2004), and gaps and poorly aligned sections were removed using Seaview (Galtier et al. 1996) prior to phylogenetic analysis. Using ProtTest version 1.3 (Abascal et al. 2005), the best fitting model for amino acid evolution was determined to be WAG + I + G under the Akaike Information Criterion and all other available statistical frameworks. Using this model and its associated parameters (gamma shape and proportion of invariant sites), a Bayesian phylogenetic tree was constructed using MrBayes version 3 (Ronquist and Huelsenbeck 2003). Convergence was reached after 60,000 generations, sampling trees every 10 generations. The first 1,500 trees were discarded as "burn-in" and a majority-rule consensus tree was built with posterior probabilities calculated for each clade. Using the same model, maximum likelihood (ML) analysis was performed with PHYML (Guindon and Gascuel 2003) and bootstrap values determined from a population of 100 replicates. A Neighbor-Joining (NJ) tree was also generated and bootstrapped (1000 replicates) using ClustalX (Chenna et al. 2003). Conserved introns were identified by mapping intron positions onto the protein sequence alignment (supplementary fig. SF1, Supplementary Material online). For prediction of GPI-anchor attachment sites, the GPI-SOM algorithm was used (Fankhauser and Mäser 2005).

Expression Analysis

Microarray data were retrieved from 2 online microarray data repositories: the Botany Array Resource (BAR), accessible at http://bbc.botany.utoronto.ca/ (Toufighi et al. 2005), and Genevestigator, accessible at https://www.genevestigator.ethz.ch (Zimmermann et al. 2004). Four data sets were downloaded from BAR including the AtGenExpress tissue, stress, hormone, and pathogen series composed of microarray data from a wide variety of experimental sources. The corresponding AtGenExpress IDs are listed in figure SF2 in the Supplementary Material online. More information on these microarray experiments can be found at http://www.weigelworld.org. The Digital Northern tool within Genevestigator was used to retrieve additional microarray data concerning response to ozone, salicylic acid, ethylene, and a number of fungal pathogens (Genevestigator experiment numbers 13, 25, 85, 108, 113, 146, 147, and 161). For experiments involving multiple time points, the Genevestigator protocol was followed and fold-change values were averaged into a single representative value for each gene.

All expression data have been previously collected using the ATH1 22K Affymetrix GeneChip (Hennig et al. 2003). Only experiments using wild-type plants were included, and all microarray data were processed in the same manner. First, in order to reduce background noise, data points less than 50 were set to a threshold value of 50. Expression values were calculated as the ratio of the average of replicate treatments relative to the average of the appropriate controls. The choice of controls and the calculations for the 4 AtGenExpress data sets and the Genevestigator data set were identical to those specified in the BAR resource and Genevestigator, respectively. The ratio expression data were $\log_2$ transformed, and each of the 5 data sets was separately clustered using a 2-way hierarchical procedure (centroid linkage, centered/Pearson correlation). In order to define clusters, a clustering threshold was set to a Pearson correlation coefficient of 0.5. Cluster version 3 (Eisen et al. 1998) was used for microarray data clustering and results were visualized with Treeview version 1.012. Both Cluster and Treeview are available via http://rana.lbl.gov/EisenSoftware.htm.

Genes outside the β-1,3-G family with expression profiles correlated to β-1,3-G genes were identified using ATTED (*Arabidopsis thaliana trans*-factor and *cis*-element prediction database, http://www.atted.bio.titech.ac.jp/), which uses microarray expression data obtained from the public AtGenExpress project. The ATTED database uses

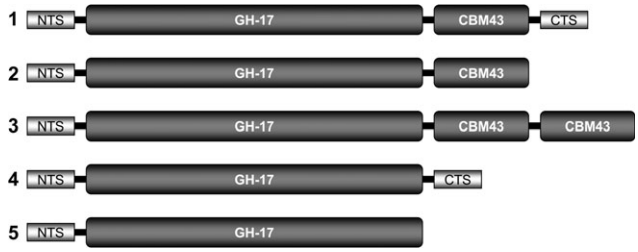

FIG. 1.—Protein domain architectures observed in the Arabidopsis β-1,3-G gene family. The 5 architectural classes are based on the presence/absence of an N-terminal sequence (NTS), a cellulose binding module (CBM43), and hydrophobic C-terminal sequence (CTS), in addition to the core glycosyl hydrolase family 17 domain (GH-17).

Pearson correlation coefficients to determine coexpressed genes. Coexpressed gene pairs were defined as those having $r \geq 0.8$.

Ancestral State Reconstruction and Statistical Analysis

Ancestral state reconstruction and reshuffling was performed with Mesquite version 1.06 (Maddison WP and Maddison DR 2005). The expression classes were discretized by treating each cluster as a separate character state. Ancestral states were then reconstructed by parsimony using an unordered model in which all state changes are treated equally. If the resulting distribution of expression states on the phylogenetic tree is nonrandom and exhibits conserved, clade-specific patterns, one would expect the parsimony score (number of state changes in the tree) to be significantly lower than that of a random data set. Therefore, to evaluate the statistical likelihood of an observed parsimony score, the data were randomly permutated by reshuffling the character matrix 10,000 times and calculating the parsimony score for each reshuffled matrix. From the resulting distribution of parsimony scores, a *P* value was calculated as the fraction of random scores less than or equal to the observed score.

Results and Discussion

Sequence Characterization and Phylogenetic Analysis

Fifty β-1,3-G sequences were identified as members of the Arabidopsis β-1,3-G family. All sequences contain an N-terminal signal peptide and a glycosyl hydrolase family 17 (β-1,3-G) domain. A variable C-terminal domain, first characterized as the X8 domain (Henrissat and Davies 2000), is present in just over half (27) of the sequences. Recently, the X8 domain has been defined as a new class of carbohydrate-binding modules (carbohydrate-binding modules family 43, CBM43) responsible for binding β-1,3-glucan (Barral et al. 2005). A C-terminal hydrophobic sequence, which may encode a transient transmembrane domain involved in GPI-anchor attachment (Henrissat and Davies 2000; Borner et al. 2002) or possibly a vacuolar targeting peptide in other cases (Leubner-Metzger and Meins 1999), is found in 25/50 of the sequences. The presence/absence of these features define 5 protein domain architectural groups (fig. 1).

As shown in the multiple alignment (supplementary fig. SF1, Supplementary Material online), the 2 catalytic glutamic residues, which correspond to E231 and E288 in barley isoenzyme GII (Varghese et al. 1994), are highly conserved (present in 49/50 and 46/50 glucanases, respectively). Other residues previously implicated in β-1,3-glucan binding and hydrolysis (Varghese et al. 1994) are also highly conserved (fig. SF1, Supplementary Material online). Thus, β-1,3-glucan hydrolysis activity is likely a common characteristic among nearly all putative β-1,3-Gs. Interestingly, several sequences with changes to highly conserved residues, including At3g55780 and At1g33220, also have weak expression levels and represent possible pseudogenes.

ML, Bayesian, and NJ phylogenetic analysis (fig. 2*a*) partitioned the 50 β-1,3-G sequences into 3 major clades (denoted α, ß, and γ). Generally, the tree topology is well supported by all 3 methods, with the exception of several higher order branches of clade α, which display lower ML and NJ bootstrap values. In order to independently test the reliability of the tree topology, protein domain architecture and intron/exon structure were mapped onto the tree (fig. 2*b*). Conserved intron positions are located predominantly at the end of the N-terminal signal sequence (NTS) and at several different sites within the CBM43 domain (fig. SF1, Supplementary Material online). The introns are highly clade specific, with introns I2 and I7 present only in clade ß, and I3, I4, I5, I6, I8, and I9 present only in clade α. The finer structure of the tree is also supported by the intron data, with a few minor exceptions. For example, the distribution of intron I5 would support a tree grouping At3g15800 closer to At2g39640 and At3g55430, though this is not supported by sequence data. Presence of the CBM43 domain and/or a hydrophobic C terminus is also largely clade dependent which reflects ancestral gains/losses of these sequence features (i.e., clade ß lacks the CBM43 domain completely whereas all members of clade γ and over half the members of clade α contain the CBM43 domain). Because these features were not used in the construction of the tree, the clade-specific patterns of intron and protein domain conservation provide additional support for the proposed phylogeny.

Clustering of Tissue and Growth Stage Expression Data Reveals Distinct Developmental Classes

Presumably, the addition of gene expression data to phylogenetic analysis should provide a more complete model of functional divergence than can be achieved using sequence information alone. Clusters derived from microarray expression data tend to be "significantly enriched for specific functional categories" and thus can be used for inferring the function of unknown genes (D'haeseleer 2005). For the purposes of expression clustering and functional assignment, the AtGenExpress developmental expression data set (Schmid et al. 2005) was selected as a source of microarray data, which facilitates a classification of genes based on the patterns of gene expression in a range of tissues/organs and developmental stages. If the enzymatic function of the protein family is known (as is the case with β-1,3-Gs), spatial/temporal expression data for the corresponding gene should be particularly informative in determining its biological/physiological role.

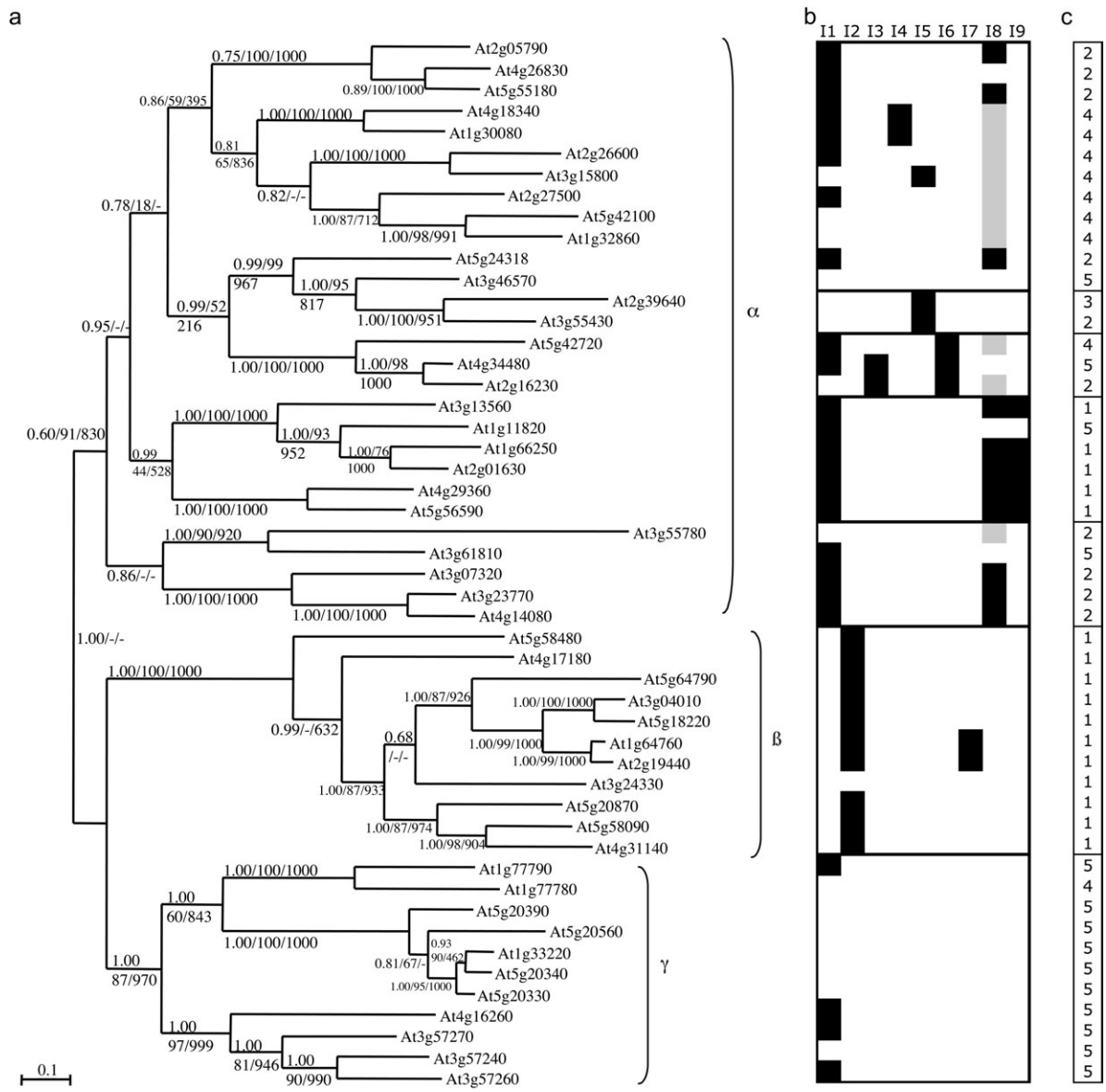

FIG. 2.—(*a*) Majority-rule consensus tree generated by Bayesian inference of phylogeny. Bayesian posterior probabilities (/1), maximum likelihood support (/100) and NJ bootstrap values (/1000) are indicated above the clades where the clade is present in the respective tree. (*b*) Presence/absence of conserved introns and protein domain architecture mapped onto the phylogenetic tree. The locations of introns (labeled I1–I9) are shown in the multiple sequence alignment (fig. SF1, Supplementary Material online). Black boxes indicate presence of introns, white indicates absence, and gray boxes indicate introns that are located in the C-terminal domain but do not align well with I8. (*c*) Protein domain architectural class as defined in figure 1.

Within the developmental data set, the genes At1g11820 and At5g24318 were not found in the lookup table and At2g19440 and At1g64760 were recognized by the same probe set (267335_s_at) and were therefore excluded from subsequent analysis. At3g46570 and At1g77790 were also removed, as all expression values were beneath the noise floor threshold. The remaining 44 genes grouped into 13 expression clusters denoted A–M including 6 singleton clusters (fig. 3*a*). As demonstrated by the highly conserved developmental expression patterns (fig. 3*a*), a large portion of the β-1,3-G gene family appears to be under a set of several distinct developmental programs. Clustering of hormone, stress-, and pathogen-response expression data was also performed, but a large number of genes formed singleton clusters (fig. SF3, Supplementary Material online) and, as discussed later, the clusters produced marginally significant or nonsignificant parsimony scores when mapped onto the phylogenetic tree. This may be attributed to the presence of highly divergent expression profiles or absence of phylogenetically relevant characteristics in the microarray data.

In addition to clustering gene expression profiles within the β-1,3-G family, identification of coexpressed genes outside of the β-1,3-G family may provide information useful for functional assignments. The ATTED provides pairwise correlation of expression data for Arabidopsis genes using data obtained from the AtGenExpress project, and was used to identify genes significantly correlated

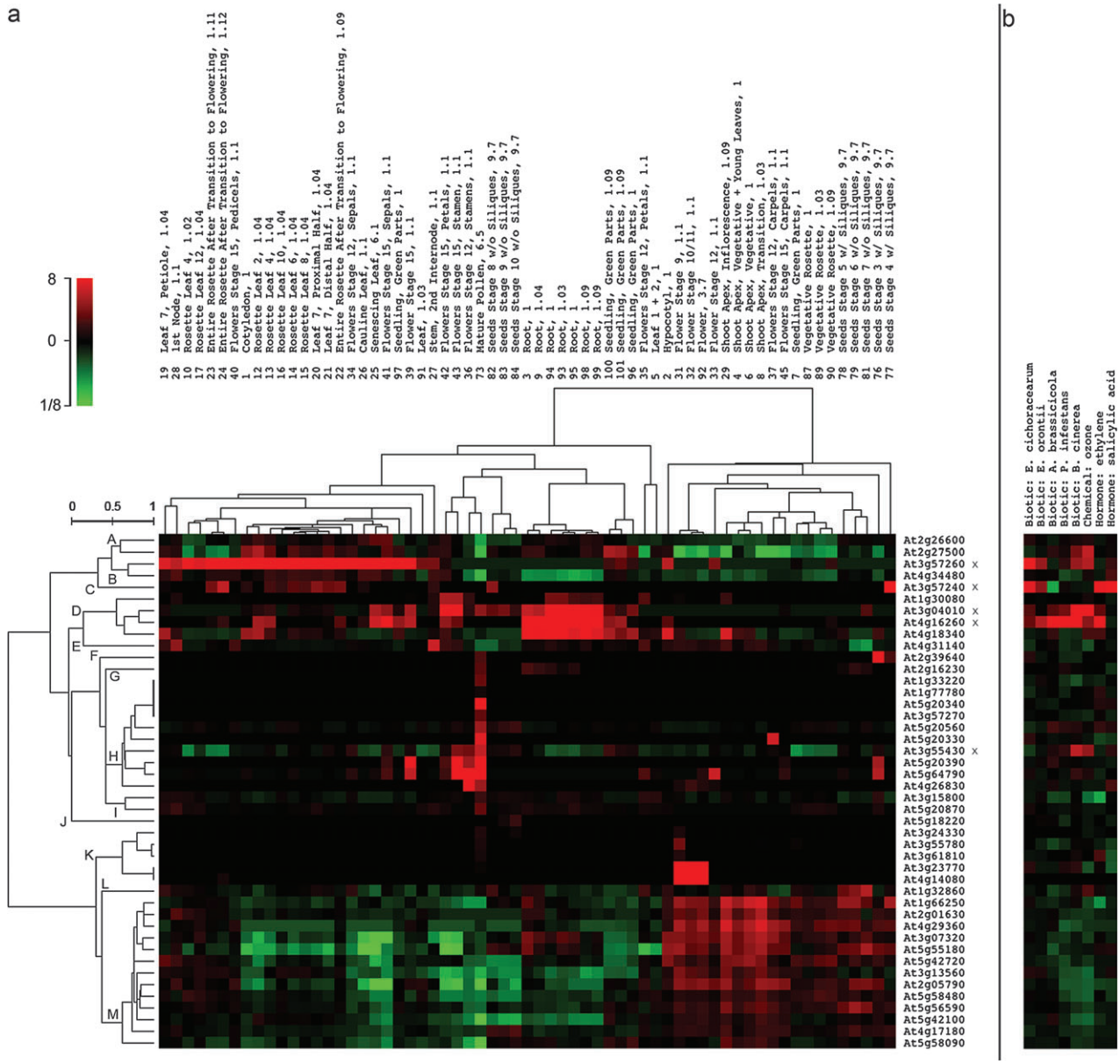

FIG. 3.—Clustering of microarray expression data. (*a*) Developmental expression clusters (labeled A–M) were assigned using Pearson correlation coefficients with a centroid correlation threshold of 0.5. The experiments are labeled according to their AtGenExpress sample ID, tissue, Boyes growth stage (Boyes et al. 2001). Stress response expression data from fungal pathogen treatments and selected chemical stresses are also shown in (*b*) but were not used in clustering the developmental data set. Ozone, salicylic acid, and ethylene response expression data were collected via Genevestigator (experiments 13, 113, and 17, respectively). Pathogen response microarray data sources are described in table 1, and PR-responsive glucanases are indicated with an X. Differential regulation is shown as a scale from 8-fold down regulated (1/8, green) to 8-fold up regulated (8, red).

(pairwise expression profiles with $r \geq 0.8$) with β-1,3-G genes (see supplementary data file SD2, Supplementary Material online). Using the known functions of correlated genes, tissue/developmental and stress response expression data, and existing literature, putative functions were assigned to the expression clusters.

β-*1,3-Gs in Cell Wall Morphogenesis and Cell Division*

The largest expression cluster (group M) contains 13 genes and displays a somewhat nonspecific expression pattern with abundant expression in a variety of tissues/organs including the flower, seeds, shoot apex, and in some cases the root and hypocotyl (fig. 3*a*). The minimum expression correlation for a group M member with the centroid of the cluster is 0.62, despite the large group size and widespread expression throughout the plant. In general, group M genes display only a minimal response to most stresses and hormones (fig. SF2, Supplementary Material online) and a somewhat negative response to biotic stress with the exception of the tumorigenic stressor *Agrobacterium tumefaciens* (Genevestigator experiment number 8, data not shown), making a PR role improbable. Given the expression in multiple tissues and particularly the high relative expression in

the shoot apex, involvement of group M genes in a constitutive biological process such as cell division/cell wall remodeling is more likely. The deposition and removal of callose during cell plate formation in active meristems has been noted in previous studies (Fulcher et al. 1976; Longly and Waterkeyn 1977; Samuels et al. 1995) and suggests a role for β-1,3-Gs in cytokinesis. β-1,3-Gs have also been implicated in the maintenance of callose-free plasmodesmata during cell differentiation and signaling (Rinne et al. 2001). In addition, recent computational and proteomic studies have revealed a large number of Arabidopsis β-1,3-Gs to be GPI-anchored, a feature that is strongly associated with cell wall remodeling (Borner et al. 2002, 2003; Elortza et al. 2003). As determined using the ATTED database, group M genes were found to be significantly coexpressed with a number of cell division and cell wall remodeling related genes including At3g02210 (COBRA cell expansion protein), At1g02730 (cellulose synthase family protein), At5g18580 (TONNEAU 2, a cell morphology control protein), At4g34160 (cyclin delta 3), and number of cell division control and cell division cycle family proteins (see supplementary data file SD2, Supplementary Material online). Arabidopsis *COBRA* genes also encode GPI-anchored proteins involved in cell wall expansion and control of cell wall morphogenesis (Roudier et al. 2002). In the previous study by Borner et al. (2002), 9 of 13 of the genes in group M were predicted as GPI-anchored proteins, 4 of which were confirmed in a subsequent study (Borner et al. 2003). As predicted by the GPI-SOM algorithm, all GPI-attachment sites are located in the far C-terminal region following the hydrolytic domain and CBM43 domain when present (fig. SF1, Supplementary Material online). One interesting question regarding group M genes is why so many appear to have very similar developmental expression profiles. One possibility is that these genes have undergone expression divergence in categories that were not included in the microarray data set. If expression patterns appear highly similar on a tissue/organ macro level, it is still possible that they are dissimilar on a smaller spatial or temporal scale, which may indeed be the case for genes involved in cell wall remodeling.

Flower-Specific β-1,3-Gs and Possible Roles in Microsporogenesis and Pollen Tube Growth

Expression groups H and K include β-1,3-G genes highly specific to flower/reproductive organs (fig. 3*a*). Although the roles of β-1,3-Gs in plant flowering are not entirely clear (Delp and Palva 1999), several specific functions have been identified. In pollen development, before microspores can be released into the anther locule for pollen maturation, a thick callose wall surrounding the tetrad must be degraded. In Arabidopsis, this function is associated with 2 characterized "anther specific" Arabidopsis β-1,3-G genes (At4g14080 and At3g23770) (Hird et al. 1993), both found in expression group K along with 3 other somewhat weakly expressed genes. A second function of β-1,3-Gs in flower development involves dissolution of callose in the stylar matrix during pollen tube growth, a function proposed both for glucanases expressed in the style (Delp and Palva 1999) as well as glucanases found in pollen grain itself (Huecas et al. 2001; Takeda et al. 2004). This possible function has been described for a previously identified Arabidopsis gene At5g20330 (*BG4*) found in group H, which was shown by previous RNA blots and in situ hybridization results to be expressed in the style and septum of the ovary (Delp and Palva 1999). In the same study, the tandemly linked gene At5g20340 (*BG5*), also found in group H, was not preferentially expressed in the style or ovary, but displayed a flower-specific expression pattern (Delp and Palva 1999). The expression data is consistent with both of these previous results (fig. 3*a*). Other genes highly expressed in the pollen and/or stamen are found in cluster H and may play similar roles in pollen tube growth. However, it must be noted that several of these genes are very weakly expressed and thus the small peak in pollen-specific expression observed for these genes is less convincing. A putative PR β-1,3-G, At3g57270 (*BG1*) (Dong et al. 1991), falls into this category. These genes may appear to be weakly expressed due to the lack of an appropriate experiment in the microarray data set capable of inducing mRNA expression above background levels.

The proposed functions of expression groups H and K in microsporogenesis and pollen development are further substantiated by results obtained from the ATTED database (supplementary data file SD2, Supplementary Material online). At3g23770 is coexpressed with MS2 ($r = 0.978$), a male sterility protein expressed in the tapetum during the release of tetrad microspores, thus further supporting its role microsporogenesis (Aarts et al. 1997). Furthermore, 2 neighboring group H, pollen/stamen-specific genes (At5g20390 and At5g64790) expressed in the final stages of Arabidopsis development (fig. 3) are coexpressed with the tyrosine phosphatase gene *AtPTEN1*. This gene is also expressed exclusively in pollen grains during the late stage of pollen development and is an essential gene for pollen maturation (Gupta et al. 2002). Lastly, the expression of *BG4* is significantly correlated with that of the inner no outer gene, which encodes a transcription factor essential in the determination of abaxial–adaxial patterning in ovule development (Villanueva et al. 1999). Future studies of these genes would be valuable for further understanding the role of β-1,3-Gs in microsporogenesis and pollen development.

Despite the flower-specific expression of the abovementioned genes, several genes tend to show some expression in particular stages of seed development as well (e.g., At5g64790 and At5g20390). These genes, along with some other genes including At2g39640, At5g18220, and At3g57240, may reflect the established role of β-1,3-Gs in seed development and after ripening (Leubner-Metzger and Meins 2000; Leubner-Metzger 2003, 2005).

Root and Leaf β-1,3-Gs

In addition to the pollen development and cell division–related expression classes that account for the majority of β-1,3-Gs in Arabidopsis, expression clustering also revealed a class of β-1,3-Gs highly specific to the root (group D) and leaves (groups A–C). As illustrated in supplementary figure SF2 (Supplementary Material online), genes found in these groups also displayed the most

Table 1
β-1,3-G Genes with Greater than a 3-Fold Change in Expression Level Following Fungal Pathogen Treatment

AGI ID	*A. brassicicola*	*B. cinerea*	*E. cichoracearum*	*E. orontii*	*P. infestans*
At4g16260	22.48	10.15	2.38	4.35	53.44
At3g57260[a]	0.77	3.47	8.02	3.50	1.55
At3g57240[a]	0.26	0.89	6.32	3.16	1.14
At3g04010	2.06	10.52	2.05	1.08	2.59
At3g55430	1.30	4.81	0.62	0.91	1.56

NOTE.—Values indicate the change in expression relative to controls.

[a] Previously characterized PR-genes (Dong et al. 1991). The corresponding experiments used by Genevestigator are *Alternaria brassicicola* (experiment 161); *Botrytis cinerea* (experiment 147); *Erysiphe cichoracearum* (experiments 85); *Erysiphe orontii* (experiment 146); and *Phytophthora infestans* (experiment 108).

significant responses to fungal pathogens, and therefore these groups are most likely to contain possible PR-genes. Groups B and C contain 2 known PR-glucanases from *Arabidopsis*, At3g57260 and At3g57240, respectively, which are both highly expressed in leafy tissues. Genes in groups A–D were also generally more stress responsive than other clusters (fig. SF2, Supplementary Material online). This likely reflects common pathways involved in regulating stress responses and responses to pathogen attack.

Identification of PR β-1,3-Gs Using Biotic Stress Expression Data

In order to more quantitatively determine which β-1,3-Gs are likely to be PR-proteins, β-1,3-G genes exhibiting a greater than 3-fold increase in expression in response to any of the 5 fungal phytopathogens for which data was available were identified and are shown in table 1. Three genes had a greater than 3-fold change in expression in more than one pathogen category and 2 genes were responsive to 1 pathogen only. The genes identified using this procedure (marked by X's in figs. 3 and 4) include 2 known PR-glucanases (At3g57260, *BG2*, PR-2; At3g57240, *BG3*, PR-3) as well as 3 more potential PR-genes (2 root specific and 1 pollen/stamen specific). At4g16260 (a root-specific β-1,3-G) was highly expressed following treatment with ethylene (fig. 3*b*), displayed a significant expression response to the largest number of pathogens (4) and was most highly induced by pathogen stress of all the PR-genes (22- and 53-fold induction by *Alternaria brassicicola* and *Phytophthora infestans*, respectively). A previous study also reported this gene as being significantly induced by the bacterium *Pseudomonas syringae* (Mahalingam et al. 2003). This gene represents an excellent target for future studies of Arabidopsis PR-proteins. Salicylic acid and ethylene, 2 major hormonal regulators of PR-responses (Leubner-Metzger and Meins 1999), are associated with a greater than 3-fold induction in 1 (At3g57240) and 2 cases

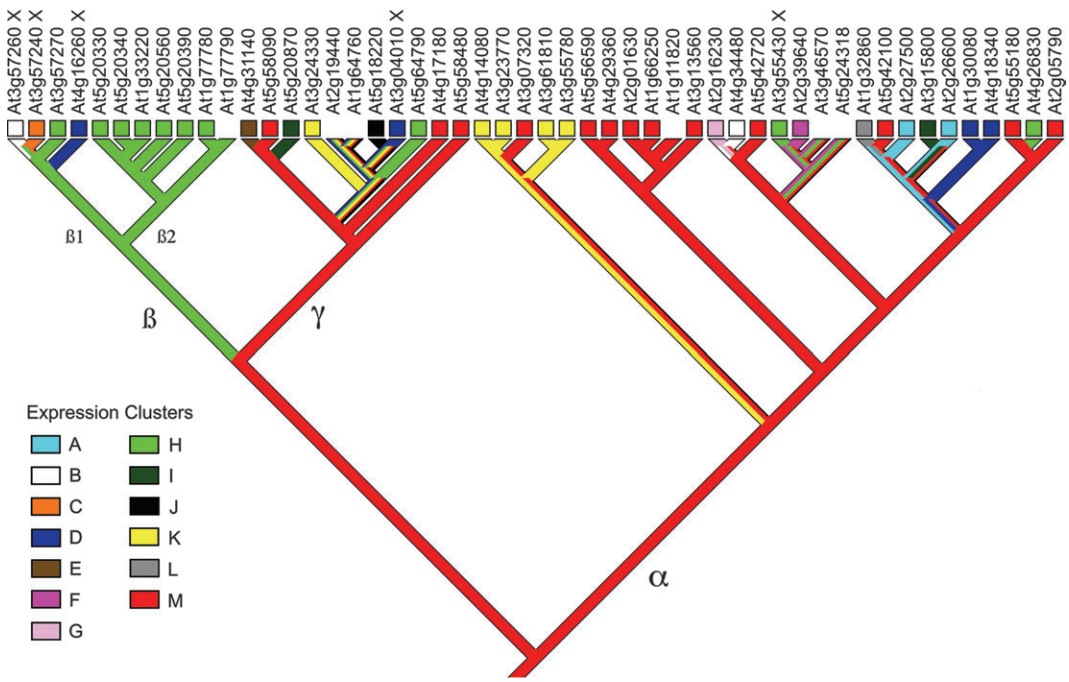

FIG. 4.—Phylogenetic reconstruction of ancestral expression states using parsimony. Colored boxes are shown at the terminal branches of genes included in the expression clustering. Genes with absent expression profiles (no box) are associated with null states in the reconstruction. The colors corresponding to each expression group are shown in the legend, and multicolored branches are associated with ambiguous (multiple possible) states. PR-glucanases identified through fungal stress response expression analysis are marked with an X.

(At3g57240 and At4g16260), respectively (fig. 3*b*). Treatment with ozone, another well-known elicitor of plant PR-proteins and defense responses (Langebartels et al. 2000), resulted in greater than 3-fold induction for 4 of the 5 genes. Ozone is known to induce mRNA levels of a PR β-1,3-G in tobacco as well (Ernst et al. 1992).

Results obtained from the ATTED database provided additional support for the possible role of these genes in pathogen defense (data file SD2, Supplementary Material online). A known PR-glucanase (*BG2*) is significantly coexpressed with At2g14610 (pathogenesis-related protein 1 [PR-1]) (Buell and Somerville 1997). Furthermore, At4g36010 (a PR-thaumatin protein) and At3g12500 (basic endochitinase, ATHCHIB, and PR-3) are correlated with 2 previously uncharacterized, potential PR-glucanases, At3g04010 and At4g16260, respectively. Coexpression of β-1,3-Gs with other PR-proteins has been shown to provide a synergistic antifungal effect in vivo (Jach et al. 1995).

Reconstruction of Ancestral Expression States Reveals Functional Divergence

In comparing expression data and sequence-based phylogeny, several related questions arise: do closely related β-1,3-G genes exhibit closely related expression profiles, or is evolution of sequence and expression uncoupled? If sequence and expression divergence are interrelated, which functional/expression classes are ancestral and which are derived? In order to investigate these questions, the functional classes obtained from expression clustering and analysis can be combined with the phylogenetic tree, and ancestral reconstruction of expression states can be used to build a model of functional divergence.

Using the program Mesquite (Maddison WP and Maddison DR 2005), the expression clusters were mapped onto the phylogenetic tree as single states, and the ancestral expression states were reconstructed by parsimony (fig. 4). The colors of the boxes at the terminal branches represent the expression/function classes of the corresponding genes. Genes with absent profiles were given null states. A change in color between 2 interconnected branches signifies a putative expression divergence event. As seen in figure 4, a considerable number of neighboring genes, likely having arisen from relatively recent duplication events, share expression states (e.g., At3g23770 and At4g14080).

The parsimony reconstruction performed on the entire tree indicates the most likely hypothetical expression states for ancestral genes according to a maximum parsimony model. The root ancestral expression state, shared by the root of clades α, γ, as well as the midpoint root of the entire tree, is the expression state M (fig. 4). Thus, according to parsimony reconstruction, the ancestral β-1,3-G gene most likely had a group M-like expression state and thus a cell division/cell wall remodeling like function, which accounts for the wide distribution of group M genes throughout the tree. A cell division/cell wall remodeling like function for the ancestral β-1,3-G genes also makes sense from an evolutionary perspective because cell division is more primitive than the other β-1,3-G functions and is consistent with the functions of other related proteins and protein families. For example, the endo-ß,1,3-G (eng1p) from yeast functions in degradation of the primary septum during cell separation (Martin-Cuadrado et al. 2003). Other plant glycosyl hydrolases such as xyloglucan endotransglycosylases (Campbell and Braam 1999) and ß-1,4-glucanases (Nicol and Hofte 1998; Zuo et al. 2000) also have well documented roles in cell wall remodeling related functions such as cell wall biogenesis, expansion, and loosening.

According to the model, after several ancient rounds of duplication of a cell division-related β-1,3-G gene, the gene family diverged in expression and thus function producing β-1,3-Gs involved in pollen development, pathogen resistance, and other processes. A particularly interesting result is that, although some expression states are highly clade specific, situations in which the same or similar expression profiles emerge independently at different locations in the tree are also quite common. At5g20390 and At5g64790, for example, are neighbors in the clustering tree as they have highly similar developmental expression profiles ($r = 0.89$) but are found in distant phylogenetic clades, ß and γ, respectively. In cases such as these, the genes may have independently acquired or lost similar tissue/developmental *cis*-regulatory elements. Whatever the mechanism may be, it appears to be a common phenomenon in the evolution of the β-1,3-G gene family.

A major question remaining is, where do the PR β-1,3-Gs lie in the phylogenetic tree? As indicated by X's in figure 4, 2 of the pathogen-induced genes are found within clades α and γ, whereas the major cluster of PR-genes is located in clade ß1, which includes the previously characterized PR-genes (*BG1*, *BG2*, and *BG3*) as well as a suspected PR-gene, At416260. The lack of the C-terminal domain and hydrophobic tail sequence in clade ß indicates that it likely arose from an ancestral β-1,3-G gene which had its C-terminal region deleted. Removal of the C-terminal region including the GPI-anchoring site would have effectively directed the ancestral protein extracellularly. This represents a pivotal evolutionary event in which an ancestral β-1,3-G involved in host cell wall morphogenesis was suddenly free to hydrolyze β-1,3-glucan in the cell walls of potential microbial pathogens.

Because the expression state of group M appears to be tightly developmentally controlled and is not responsive to fungal stress, divergence from the ancestral expression state appears to be an additional requirement for evolution of PR function. All the 5 putative PR-genes have diverged away from the ancestral state M, and are associated with 4 different nonancestral expression states. This also reflects a considerable amount of expression divergence between PR-genes themselves. Given the range of expression states associated with PR-genes, it is not surprising that PR β-1,3-Gs are also highly variable in their responses to hormones and chemicals (supplementary fig. SF2, Supplementary Material online) and different species of fungal pathogens (table 1). Possessing diversity with respect to these characteristics is a widely documented feature of PR β-1,3-Gs and is important for broadening the regulatory response to pathogen attack (Memelink et al. 1990; Ward et al. 1991; Leubner-Metzger 2003). Thus, for a number of reasons, expression divergence appears to be a major factor in the maintenance of duplicated PR β-1,3-G genes in evolution.

Two other genes that display PR-like expression responses are found in clades α and γ, but unlike the PR-genes in clade ß, both contain a C-terminal domain. Furthermore, At3g04010 is a predicted GPI-anchored protein (Borner et al. 2002, 2003) and thus may be cell wall associated, which raises into question its ability to act as a PR protein. Whether these genes encode actual PR-proteins or simply display PR-like expression responses to biotic stress remains to be determined. Nevertheless, the multiple independent origins of PR-like expression patterns at different locations in the phylogenetic tree suggests that acquisition of PR-responsive regulatory elements is not an uncommon occurrence in the evolution of the Arabidopsis β-1,3-G gene family. This, paired with the considerable level of expression divergence among PR-genes and the loss of the C-terminal region containing the hydrophobic C terminus and GPI-anchoring site, provides a mechanism for evolution of β-1,3-Gs involved in pathogen defense.

Statistical Evaluation of Ancestral State Reconstruction

In order to statistically evaluate whether the pattern of expression divergence produced by clustering and ancestral state reconstruction is nonrandom, a permutation test was performed in which the expression states were randomly reshuffled and parsimony reconstruction was performed 10,000 times. The number of steps required in the random distribution ranged from 22 to 31, and the observed parsimony score of 22 steps is indicative of a nonrandom distribution with a permutation P value of 0.0002. This supports a model of β-1,3-G evolution in which divergence of developmental expression patterns is partially coupled with sequence divergence. Only marginally significant or nonsignificant results were obtained using stress-, hormone-, or pathogen-response data, with P values of 0.0429, 0.3165, and 1.0000, respectively (supplementary fig. SF3, Supplementary Material online). One possible reason for this is that β-1,3-G genes may have diverged faster in terms of stress, hormone, and pathogen response than developmental response, and that the rate of expression divergence in these categories outpaced the rate of sequence divergence to a degree where the phylogenetic signal was lost. Alternately, it is possible that the nondevelopmental data sets were more variable due to the large number of microarray data sources and types of stress experiments, or there was an absence of phylogenetically relevant characteristics in the microarray data. In the case of the pathogen-response data, a P value of 1.0000 (2-tailed P value $<$ 0.0001) is of interest as it suggests that similar pathogen-response expression profiles arise independently significantly more often than can be expected by chance. This may be a consequence of a positive selection pressure for increased pathogen response and is an interesting area for future analysis.

Conclusion

The expression classes resulting from expression clustering and analysis are consistent with existing data on individual Arabidopsis genes, successfully reflect the known functions of β-1,3-Gs, and provide a means to putatively categorize uncharacterized β-1,3-Gs. Clustering of tissue/developmental expression data revealed β-1,3-Gs associated with cell wall remodeling as well as pollen development, whereas analysis of fungal stress expression data facilitated identification of known and several putative PR-glucanases. The ancestral function assigned through ancestral state reconstruction was cell division/cell wall remodeling and is consistent with the known functions of β-1,3-Gs in more primitive organisms as well as related gene families. Other interesting results include the independent origin of highly similar developmental expression profiles in more distantly related sequences and the variable degrees of expression divergence in different clades. The considerable level of expression divergence observed in the Arabidopsis β-1,3-G gene family provides a basis for assessing the evolution of β-1,3-G function. Furthermore, the acquisition of PR-like expression responses following divergence from the ancestral expression state combined with the loss of the C-terminal region and GPI-anchoring site (as seen in clade β) facilitates fungal stress-induced extracellular secretion, and thus evolution of β-1,3-Gs involved plant pathogen defense. Ultimately, parsimony ancestral state reconstruction paired with microarray expression clustering and analysis provides a powerful method for investigating evolution of function in large gene families.

Supplementary Material

Supplementary figures SF1–SF3 and supplementary data file SD1 and SD2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

This work is supported by the National Sciences and Engineering Research Council of Canada through grants to B.A.M., B.J.M., and M.G., and a Canada Graduate Scholarship to A.C.D. We would like to thank Michael Lynch for helpful discussions on phylogenetic analysis.

Literature Cited

Aarts MG, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ, Scott R, Pereira A. 1997. The Arabidopsis MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. Plant J. 12:615–623.

Abascal F, Zardoya R, Posada D. 2005. ProtTest: selection of best-fit models of protein evolution. Bioinformatics. 21:2104–2105.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Bachman ES, McClay DR. 1996. Molecular cloning of the first metazoan beta-1,3 glucanase from eggs of the sea urchin *Strongylocentrotus purpuratus*. Proc Natl Acad Sci USA. 93:6808–6813.

Barral P, Suarez C, Batanero E, Alfonso C, Alche Jde D, Rodriguez-Garcia MI, Villalba M, Rivas G, Rodriguez R. 2005. An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. Biochem J. 390:77–84.

Blanc G, Wolfe KH. 2004. Functional divergence of duplicated genes formed by polyploidy during Arabidopsis evolution. Plant Cell. 16:1679–1691.

Borner GH, Lilley KS, Stevens TJ, Dupree P. 2003. Identification of glycosylphosphatidylinositol-anchored proteins in Arabidopsis. A proteomic and genomic analysis. Plant Physiol. 132: 568–577.

Borner GH, Sherrier DJ, Stevens TJ, Arkin IT, Dupree P. 2002. Prediction of glycosylphosphatidylinositol-anchored proteins in Arabidopsis. A genomic analysis. Plant Physiol. 129: 486–499.

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Gorlach J. 2001. Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell. 13:1499–1510.

Buell CR, Somerville SC. 1997. Use of Arabidopsis recombinant inbred lines reveals a monogenic and a novel digenic resistance mechanism to *Xanthomonas campestris* pv *campestris*. Plant J. 12:21–29.

Campbell P, Braam J. 1999. Xyloglucan endotransglycosylases: diversity of genes, enzymes and potential wall-modifying functions. Trends Plant Sci. 4:361–366.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31:3497–3500.

Delp G, Palva ET. 1999. A novel flower-specific Arabidopsis gene related to both pathogen-induced and developmentally regulated plant beta-1,3-glucanase genes. Plant Mol Biol. 39:565–575.

D'haeseleer P. 2005. How does gene expression clustering work? Nat Biotechnol. 23:1499–1501.

Dong X, Mindrinos M, Davis KR, Ausubel FM. 1991. Induction of Arabidopsis defense genes by virulent and avirulent Pseudomonas syringae strains and by a cloned avirulence gene. Plant Cell. 3:61–72.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA. 95:14863–14868.

Elortza F, Nühse TS, Foster LJ, Stensballe S, Peck SC, Jensen ON. 2003. Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. Mol Cell Proteomics. 2:1261–1270.

Ernst D, Schraudner M, Langebartels C, Sandermann H Jr. 1992. Ozone-induced changes of mRNA levels of beta-1,3-glucanase, chitinase and 'pathogenesis-related' protein 1b in tobacco plants. Plant Mol Biol. 20:673–682.

Fankhauser N, Mäser P. 2005. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. Bioinformatics. 21:1846–1852.

Fulcher R, McCully M, Setterfield G, Sutherland J. 1976. ß-1,3-Glucans may be associated with cell-plate formation during cytokinesis. Can J Bot. 54:539–542.

Galtier N, Gouy M, Gautier C. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci. 12:543–548.

Griffith M, Yaish MW. 2004. Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci. 9:399–405.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 52:696–704.

Gupta R, Ting JT, Sokolov LN, Johnson SA, Luan S. 2002. A tumor suppressor homolog, AtPTEN1, is essential for pollen development in Arabidopsis. Plant Cell. 14:2495–2507.

Hennig L, Menges M, Murray JA, Gruissem W. 2003. Arabidopsis transcript profiling on Affymetrix GeneChip arrays. Plant Mol Biol. 53:457–465.

Henrissat B, Davies GJ. 2000. Glycoside hydrolases and glycosyltransferases. Families, modules, and implications for genomics. Plant Physiol. 124:1515–1519.

Hird DL, Worrall D, Hodge R, Smartt S, Paul W, Scott R. 1993. The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to beta-1,3-glucanases. Plant J. 4:1023–1033.

Hoj PB, Fincher GB. 1995. Molecular evolution of plant beta-glucan endohydrolases. Plant J. 7:367–379.

Huecas S, Villalba M, Rodriguez R. 2001. Ole e 9, a major olive pollen allergen is a 1,3-beta-glucanase. Isolation, characterization, amino acid sequence, and tissue specificity. J Biol Chem. 276:27959–27966.

Jach G, Gornhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C. 1995. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. 8:97–109.

Jin W, Horner HT, Palmer RG, Shoemaker RC. 1999. Analysis and mapping of gene families encoding beta-1,3-glucanases of soybean. Genetics. 153:445–452.

Langebartels C, Ernst D, Kangasjarvi J, Sandermann H Jr. 2000. Ozone effects on plant defense. Methods Enzymol. 319:520–535.

Leubner-Metzger G. 2003. Functions and regulation of beta-1,3-glucanases during seed germination, dormancy release and after-ripening. Seed Sci Res. 13:17–34.

Leubner-Metzger G. 2005. ß-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. Plant J. 41:133–145.

Leubner-Metzger G, Meins F Jr. 1999. Functions and regulation of plant beta-1,3-glucanases (PR-2). In: Datta SK, Muthukrishnan S, editors. Pathogenesis-related proteins in plants. Boca Raton (FL): CRC Press LLC. p. 49–76.

Leubner-Metzger G, Meins F Jr. 2000. Sense transformation reveals a novel role for class I beta-1,3-glucanase in tobacco seed germination. Plant J. 23:215–221.

Li WH, Yang J, Gu X. 2005. Expression divergence between duplicate genes. Trends Genet. 21:602–607.

Linthorst HJ, Melchers LS, Mayer A, van Roekel JS, Cornelissen BJ, Bol JF. 1990. Analysis of gene families encoding acidic and basic beta-1,3-glucanases of tobacco. Proc Natl Acad Sci USA. 87:8756–8760.

Longly B, Waterkeyn L. 1977. Les stades callosiques de la plaque cellulaire somatique. Cellule. 72:195–224.

Maddison WP, Maddison DR. 2005. Mesquite: a modular system for evolutionary analysis [Internet]. Available from: http://www.mesquiteproject.org. Accessed 28 Aug 2005.

Mahalingam R, Gomez-Buitrago A, Eckardt N, Shah N, Guevara-Garcia A, Day P, Raina R, Fedoroff NV. 2003. Characterizing the stress/defense transcriptome of Arabidopsis. Genome Biol. 4:R20.

Martin-Cuadrado AB, Duenas E, Sipiczki M, Vazquez de Aldana CR, del Rey F. 2003. The endo-beta-1,3-glucanase eng1p is required for dissolution of the primary septum during cell separation in *Schizosaccharomyces pombe*. J Cell Sci. 116:1689–1698.

Memelink J, Linthorst HJ, Schilperoort RA, Hoge JH. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol Biol. 14:119–126.

Nicol F, Hofte H. 1998. Plant cell expansion: scaling the wall. Curr Opin. Plant Biol. 1:12–17.

Rinne PL, Kaikuranta PM, van der Schoot C. 2001. The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. Plant J. 26:249–264.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 19:1572–1574.

Roudier F, Schindelman G, DeSalle R, Benfey PN. 2002. The COBRA family of putative GPI-anchored proteins in Arabidopsis. A new fellowship in expansion. Plant Physiol. 130:538–548.

Samuels AL, Giddings TH Jr, Staehelin LA. 1995. Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. J. Cell Biol. 130:1345–1357.

Schmid M, Davidson TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU. 2005. A gene expression map of *Arabidopsis thaliana* development. Nat Genet. 37:501–506.

Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, Van Den Elzen P, Cornelissen B. 1993. Only specific tobacco (Nicotiana tabacum) chitinases and [beta]-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101:857–863.

Stone BA, Clarke AE. 1992. Chemistry and biology of (1-3)-beta-D-glucans. Victoria (Australia): La Trobe University Press.

Sun L, Gurnon JR, Adams BJ, Graves MV, Van Etten LJ. 2000. Characterization of a beta-1,3-glucanase encoded by chlorella virus PBCV-1. Virology. 276:27–36.

Takeda H, Yoshikawa R, Liu XZ, Nakagawa N, Li YQ, Sakurai N. 2004. Molecular cloning of two exo-beta-glucanases and their in vivo substrates in the cell walls of lily pollen tubes. Plant Cell Physiol. 45:436–444.

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ. 2005. The botany array resource: e-northerns, expression angling, and promoter analyses. Plant J. 43:153–163.

Varghese JN, Garrett TP, Colman PM, Chen L, Hoj PB, Fincher GB. 1994. Three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities. Proc Natl Acad Sci USA. 91:2785–2789.

Villanueva JM, Broadhvest J, Hauser BA, Meister RJ, Schneitz K, Gasser CS. 1999. INNER NO OUTER regulates abaxial-adaxial patterning in Arabidopsis ovules. Genes Dev. 13: 3160–3169.

Ward E, Payne G, Moyer M, et al. (11 co-authors). 1991. Differential regulation of ß-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiol. 96:390–397.

Yaish MWF, Doxey AC, McConkey BJ, Moffatt BA, Griffith M. 2006. Cold active winter rye glucanases with ice-binding capacity. Plant Physiol. 141:1459–1472.

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 136:2621–2632.

Zuo J, Niu QW, Nishizawa N, Wu Y, Kost B, Chua NH. 2000. KORRIGAN, an Arabidopsis endo-1,4-beta-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. Plant Cell. 12:1137–1152.

Charles Delwiche, Associate Editor

Accepted January 29, 2007