GAI Homologues in the Hawaiian Silversword Alliance (Asteraceae-Madiinae): Molecular Evolution of Growth Regulators in a Rapidly Diversifying Plant Lineage

David L. Remington[1] *and Michael D. Purugganan*

Department of Genetics, North Carolina State University

Accelerated evolution of regulatory genes has been proposed as an explanation for decoupled rates of morphological and molecular evolution. The Hawaiian silversword alliance (Asteraceae-Madiinae) has evolved drastic differences in growth form, including rosette plants, cushion plants, shrubs, and trees, since its origin ~6 MYA. We have isolated genes in the DELLA subfamily of putative growth regulators from 13 taxa of Hawaiian and North American Madiinae. The Hawaiian taxa contain two copies of *DaGAI* that form separate clades within the Madiinae, consistent with an allotetraploid origin for the silversword alliance. *DaGAI* retains conserved features that have previously been identified in DELLA genes. Selective constraint in the Hawaiian *DaGAI* copies remains strong in spite of rapid growth form divergence in the silversword alliance, although the constraint was somewhat relaxed in the Hawaiian copies relative to the North American lineages. We failed to detect evidence for positive selection on individual codons. Notably, selective constraint remained especially strong in the gibberellin-responsive DELLA region for which the gene subfamily is named, which is truncated or deleted in all identified dwarf mutants in *GAI* homologues in different angiosperm species. In contrast with the coding region, however, ~900 bp of the upstream flanking region shows variable rates and patterns of evolution, which might reflect positive selection on regulatory regions.

Introduction

A quarter century ago, Wilson and colleagues (King and Wilson 1975; Cherry, Case, and Wilson 1978) noted a lack of correlation between rates of morphological and molecular evolution and proposed variation in regulatory genes as an explanation. Under this hypothesis, a small number of regulatory genes may have been responsible for the evolution of major differences in plant growth form and thus central to the generation of terrestrial biodiversity. Mutations in these genes might occasionally result in morphological changes that allow organisms to colonize new ecological niches, leading to the isolation and eventually the formation of new species (Orr 2001; Schluter 2001). Differences in growth habit among plant taxa contribute to the spatial and temporal diversity of plant communities and thus to the characteristic features of various ecosystems (Oliver and Larson 1990, p. 355). Colonization of island environments in particular often involves adaptive radiation with rapid evolution of novel growth forms adapted to unoccupied niches (Robichaux et al. 1990; Schluter 2000, p. 163). Evaluating whether major ''biodiversity genes'' are responsible for rapid morphological evolution will require studying plant lineages that have undergone adaptive radiation in growth form and identifying the genetic basis of these differences.

The Hawaiian silversword alliance (Asteraceae-Madiinae) is an outstanding example of plant adaptive radiation (Raven, Evert, and Eichhorn 1999, pp. 250–251; Schluter 2000) and serves as a key system for understanding the origin of plant morphological diversity (Robichaux et al. 1990). The 30 extant species in the Hawaiian Madiinae putatively arose from a herbaceous allopolyploid North American tarweed ancestor ~5 MYA (Baldwin et al. 1991; Baldwin and Sanderson 1998; Barrier et al. 1999) and have subsequently evolved spectacular differences in vegetative and reproductive morphology, as well as in physiology and habitat (Carr 1985; Robichaux et al. 1990). Shoot elongation in the basal rosette plants of the genus *Argyroxiphium* is largely limited to reproductive stems, whereas the other two genera (*Wilkesia* and *Dubautia*) have elongated woody vegetative stems. The *Dubautia* spp., moreover, show tremendous variation in the rate and extent of stem elongation and vary from diminutive shrubs and cushion plants (e.g., *D. scabra*, *D. ciliolata*, *D. waialealeae*) to trees up to 6 m tall (*D. reticulata*, *D. knudsenii*, and *D. arborea*) (Carr 1985; Robichaux et al. 1990). Some of the most spectacular morphological differences occur between closely related taxa. For example, *D. arborea* and *D. ciliolata* are essentially indistinguishable by microsatellite and AFLP polymorphisms (unpublished data; E. Friar, personal communication) but exhibit striking differences in height, leaf size and morphology, and floral and capitulescence characteristics (Carr 1985; Robichaux et al. 1990).

Genes involved in transcriptional regulation could be especially important in the evolution of major phenotypic differences because they tend to control specific developmental pathways (Doebley and Lukens 1998; Purugganan 2000). Consistent with this prediction, Barrier, Robichaux, and Purugganan (2001) found accelerated rates of evolution in *ASAP1* and *ASAP3/TM6*, orthologues of the floral homeotic genes *APETALA1* and

[1] Present address: Department of Biology, University of North Carolina at Greensboro.

Abbreviations: *GAI*, *GA INSENSITIVE;* MITE, miniature inverted-repeat transposable element; MRCA, most recent common ancestor.

Key words: Madiinae, silversword alliance, *GAI*, adaptive radiation, selection, regulatory genes.

Address for correspondence and reprints: David L. Remington, Department of Biology, University of North Carolina at Greensboro, P.O. Box 26174, Greensboro, NC 27402. E-mail: dlreming@uncg.edu.

Mol. Biol. Evol. 19(9):1563–1574. 2002
 ISSN: 0737-4038

APETALA3, in Hawaiian Madiinae lineages relative to the North American lineages. Significantly less rate acceleration was found in orthologues of the structural gene *CAB9*. These results suggest that positive selection, and not merely relaxation of selection because of gene duplication, has contributed to the accelerated evolutionary rates in *ASAP1* and *ASAP3*. It is not clear, however, whether these results apply to transcriptional regulators in general or only to a small subset of regulatory loci.

Regulatory genes known to play key roles in plant growth regulation would seem especially likely to be under selection in the silversword alliance. The Arabidopsis genes *GA INSENSITIVE* (*GAI*) and *RGA* (Peng et al. 1997; Silverstone, Ciampaglio, and Sun 1998) and related genes of the DELLA subfamily encode growth regulators (Peng et al. 1999) and have been implicated in quantitative variation in developmental traits (Thornsberry et al. 2001). The DELLA genes, which also include the three *RGL* genes in Arabidopsis (Dill and Sun 2001; Wen and Chang 2002), *Rht-1* in wheat, *d8* in maize (Peng et al. 1999), and *SLR1* in rice (Ogawa et al. 2000; Ikeda et al. 2001), are a subset of the GRAS family of plant transcriptional regulatory genes (Pysh et al. 1999) whose products modulate gibberellin (GA) responses (Peng et al. 1999). Semidominant mutations in *GAI*, *RGA*, *RGL1*, *Rht-1*, *d8*, and *SLR1*, all of which involve deletions in or truncations of the N-terminal DELLA region for which the gene subfamily is named, have been shown to confer a GA-unresponsive dwarf phenotype (Peng et al. 1997; Peng et al. 1999; Dill and Sun 2001; Ikeda et al. 2001; Wen and Chang 2002). Mutants are viable and have become important constituents of green revolution grain varieties (Peng et al. 1999). Different *d8* and *Rht-1* mutants show varying degrees of dwarfism, suggesting that alterations within the DELLA region may have broad importance for the modulation of gibberellin responses (Peng et al. 1999). A broader spectrum of developmental variation in maize, including time to flowering, has been shown to be strongly associated with polymorphisms in *d8* outside the DELLA region and with sites in the promoter (Thornsberry et al. 2001). DELLA genes differ somewhat from one another in expression patterns and effects of GA on expression levels, suggesting some degree of variation in gene regulation (Silverstone, Ciampaglio, and Sun 1998; Ogawa et al. 2000; Wen and Chang 2002). The finding that the wild-type DELLA proteins quantitatively affect GA-induced growth responses in a dose-dependent manner (Dill and Sun 2001; Fu et al. 2001; King, Moritz, and Harberd 2001; Wen and Chang 2002) and the association of sites in the *d8* promoter with flowering time (Thornsberry et al. 2001) also suggest that transcript levels might be involved in phenotypic variation. Posttranslational regulation is also important, at least in RGA and SLR1 proteins, which undergo rapid degradation in response to the GA treatment (Silverstone et al. 2001; Itoh et al. 2002).

Our objective in this study was to test whether the acceleration in protein evolution observed in *ASAP1* and *ASAP3/TM6* (Barrier, Robichaux, and Purugganan 2001) can be generalized to other regulatory genes in the Hawaiian silversword alliance. We tested whether the rates of evolution were accelerated in Hawaiian relative to North American lineages of *DaGAI-A* and *DaGAI-B*, two DELLA genes in the silversword alliance, and whether some codons showed evidence of positive selection. Furthermore, to test the prediction that *cis*-regulatory regions of transcriptional regulators are the most likely locations for important variation (Doebley and Lukens 1998), we evaluated portions of the *DaGAI* upstream flanking regions as well as the coding regions. Finally, the results would also provide some evidence of whether *DaGAI* might have played a major role in at least some of the episodes of morphological evolution that gave rise to the 30 existing Hawaiian Madiinae. The molecular signature of such a role could include mutations in key conserved residues and domains, such as the DELLA region important in GA responsiveness, or accelerated evolutionary rates in putative promoter sequences.

Materials and Methods

Plant Materials, DNA and RNA Isolation

Plant materials were obtained from the field from natural populations in 1998 and 2000. DNA was extracted from a leaf tissue using a modified cetyl-trimethylammonium bromide protocol (Doyle J. J. and Doyle J. L. 1990). DNA from *A. sandwicense*, *Madia sativa*, and *Anisocarpus madioides* was prepared from flower heads using DNeasy Plant Mini Kits (Qiagen). Bruce Baldwin and Marianne Barrier kindly provided DNA from *Carlquistia muirii* and *Calycadenia multiglandulosa*, respectively. Total RNA from *D. arborea* tissue was obtained using an RNeasy Plant Kit (Qiagen).

Isolation and Sequencing of *GAI* Homologues

Short genomic fragments (~130–140 bp) of a *GAI* homologue (designated *DaGAI*) were isolated by PCR from *D. arborea*, using degenerate primers designed from conserved domains in the N-terminal regions of the published sequences. An initial round of amplification was performed using primers gaiU103deg (5′-TACAAGGT NMGNTCNTCNGANATG-3′) and gaiL278deg (5′-GG AGGRTTNARNTCNGWNARCAT-3′), followed by seminested amplifications using gaiU103deg and gaiL234deg (5′-CTCCGCNGGRTTRTARTGNACNGT-3′) or gaiU136deg (5′-GCTCAGAARYTNGARCARYT NGA-3′) and gaiL278deg.

Nested forward primers for 3′ rapid amplification of cDNA ends (3′ RACE) (Frohman, Dush, and Martin 1988) were designed from degenerate PCR products. First-strand cDNA synthesis and nested amplification of *DaGAI* transcripts were done with a 3′ RACE kit (Life Technologies), using total RNA from *D. arborea* shoot tips and leaves as the template. Nested reverse primers designed from 3′ RACE products were used to obtain genomic sequences from *D. arborea* corresponding to the 5′-end of the *DaGAI*-coding region and approximately 900 bp of the upstream flanking sequences, using the Universal GenomeWalker kit (Clontech) to perform

anchored PCR (Siebert et al. 1995). Further verification of *DaGAI* expression in elongating shoot tips was obtained with RT-PCR using the Access RT-PCR kit (Promega) and gene-specific primers. Reactions without reverse transcriptase were used as negative controls for genomic DNA contamination.

Specific PCR primers designed from the *D. arborea* sequences or subsequently obtained sequence data were used to amplify *DaGAI* from nine Hawaiian Madiinae (*D. arborea*, *D. ciliolata*, *D. menziesii*, *D. knudsenii*, *D. raillardioides*, *D. microcephala*, *A. sandwicense*, *A. kauense*, and *W. gymnoxiphium*) and four North American Madiinae (*A. madioides*, *C. muirii*, *M. sativa*, and *C. multiglandulosa*). PCR amplification was performed using the proofreading *Pwo* polymerase (Roche) to minimize the amount of sequence error introduced during PCR. The *DaGAI* region was amplified as two overlapping fragments; one fragment covered virtually the entire putative coding region, and the second fragment encompassed ~600–1,000 bp of the upstream flanking sequence plus ~400 bp of the 5′-end of the coding region. Overlapping regions were used to determine which sequences from the two sets of products were allelic. Further GenomeWalker library construction and amplification were required to isolate the upstream region from *C. multiglandulosa* and the second *DaGAI* copy in *D. arborea*.

All PCR products were cloned into TOPO TA or Zero Blunt vectors (Invitrogen) for sequencing. Cycle sequencing reactions were performed using BigDye terminator-polymerase mixes (Applied Biosystems), and sequences were resolved on ABI 377 automated sequencers or ABI 3700 DNA analyzers. PHRED (Ewing and Green 1998; Ewing et al. 1998) was used to call bases and assign quality scores. Most, but not all, regions were covered by overlapping sequence reads. Discrepancies and unique sequence changes at sites with single coverage and low-quality scores were visually rechecked against chromatograms. All sequences are deposited in GenBank (accession numbers AF492562–AF492590).

Sequence Alignment and Phylogeny Reconstruction

The two *DaGAI* sequences from *D. arborea* were visually aligned with *d8* from maize, *SLR1* from rice, and the five known *GAI* family members in *Arabidopsis thaliana*: *GAI*, *RGA*, and the three ''*RGA*-like'' sequences that have been designated as *RGL1*, *RGL2*, and *RGL3* (Dill and Sun 2001). Phylogenies were evaluated with PAUP* Version 4.0b6 (Swofford 1998). Only sites that could be aligned confidently in the in-group taxa were included, and third codon positions were excluded. The 3′ portion of the GRAS family *SCARECROW* gene from *A. thaliana*, which can be aligned with the DELLA subfamily sequences, was included as an out-group. Neighbor-joining tree construction used the HKY85 substitution model with gamma rate variation, and shape parameter $\alpha = 0.5$. Maximum parsimony trees were constructed using a branch-and-bound search to find the single minimum-length tree. Gaps were treated as missing data. Branch support for both neighbor-joining and maximum parsimony trees was evaluated using 500 bootstrap replicates. A maximum likelihood tree was constructed using the HKY85 substitution model with gamma rate variation, with the shape parameter estimated from the data. A heuristic search was performed using four random addition replicates followed by tree bisection/reconnection (TBR) branch swapping.

The alignment of sequences from the thirteen Hawaiian and North American Madiinae taxa included both copies from *D. arborea*, *D. menziesii*, *D. raillardioides*, *A. sandwicense*, and *A. kauense* for a total of 18 sequences. *Calycadenia multiglandulosa* was chosen as an out-group for phylogenetic analyses, on the basis of previous phylogenetic investigations in the Madiinae (Baldwin and Wessa 2000; Barrier, Robichaux, and Purugganan 2001). Maximum parsimony trees were generated in PAUP* using a branch-and-bound search. Branch support was evaluated using 500 bootstrap replicates. Maximum likelihood analysis used the HKY85 substitution model with the gamma shape parameter estimated from the data. A maximum likelihood tree was generated heuristically using five random addition replicates followed by TBR branch swapping.

Molecular Evolution Analyses

Maximum likelihood analyses of molecular evolution in *DaGAI* were carried out in HY-PHY version 0.901 beta (peppercat.stat.ncsu.edu/~hyphy) using the maximum likelihood tree topology generated in PAUP*. Nucleotide-based relative rate tests used the HKY85 substitution model with global or local transition-transversion ratios. Codon-based analyses of replacement-synonymous substitution ratios (K_A/K_S, or ω) and relative rates used the Muse-Gaut 3×4 codon substitution model (Muse and Gaut 1994), in which the separate nucleotide frequencies at each of the three codon positions are used to calculate the codon substitution matrix. Nested models with different numbers of constrained parameters were used to conduct likelihood ratio hypothesis tests. Models included (1) a global model in which a single ω ratio was applied to the entire tree; (2) a 3-ω model in which three separate ω ratios were applied to branches descending from the most recent common ancestor (MRCA) of the Hawaiian *A* copy, those descending from the MRCA of the Hawaiian *B* copy, and to North American branches, respectively; (3) 2-ω models in which two of the three categories in the 3-ω model were combined; and (4) an unrestricted local model in which separate K_A and K_S values were determined for each branch of the tree. In the 2-ω and 3-ω models, the branches leading to the common ancestors of the Hawaiian *A* and *B* copies were considered to be North American, even though some of the evolution along these branches may have occurred after Hawaiian introduction. The null distribution of the likelihood ratio test statistic was assumed to follow a χ^2 distribution with degrees of freedom equal to the difference in the number of unconstrained parameters between the two hypotheses. Analyses were performed for the *DaGAI*-coding re-

gion as a whole and for sliding windows of 300 bp with 150-bp offsets between successive windows. To test the sensitivity of the results to the tree topology, all analyses were also run using an alternate topology, with slight differences in both Hawaiian gene lineages, generated in HY-PHY by stepwise addition without branch swapping.

Tests of variable selection among codons used two different null models. In the first (neutral) model, all codons were assumed to belong either to an invariant ($\omega = 0$) or a neutral ($\omega = 1$) class. This model was tested against an alternative (selection) model containing a third class of sites with an ω ratio estimated from the data. In the second null model (beta model), ω was assumed to follow a beta distribution with $0 \leq \omega \leq 1$, which was discretized into three classes of equal frequency for the purposes of estimation. This model was tested against an alternative (beta+ω) model containing an additional class of positively selected sites, in which ω was constrained to be greater than 1.0 and was estimated from the data. The sensitivity of this test to model assumptions was tested by reducing the number of discretized β distribution categories to two and by changing the codon substitution model to the Goldman-Yang 3×4 model (Goldman and Yang 1994).

Analyses of the upstream flanking sequences were done in HY-PHY using the HKY85 nucleotide substitution model. The upstream sequence was divided into three regions (proximal, intermediate, and distal) on the basis of the changes in nucleotide composition and the frequency of indels. Constraint and branch length heterogeneity were evaluated by comparing each region separately with the third codon position sites in the coding region. Three nested models were used for each comparison: (1) a model in which both the upstream region and third position sites were constrained to have identical branch lengths; (2) a model in which branch length ratios were constrained to be identical but could vary proportionately between the two regions; and (3) a model in which all branch lengths were estimated independently for the two sections. The likelihood ratio test of model 3 against model 2 (the relative ratio test) evaluates branch ratio heterogeneity between the two sections, and the test of model 2 against model 1 evaluates differences in overall substitution rates between the sections. A sliding window analysis was also performed, using windows of 200 bp and offsets of 50 bp between successive windows.

Results and Discussion

Duplicated Copies of *DaGAI* in the Hawaiian Madiinae

We used PCR techniques to isolate *DaGAI* from *D. arborea* genomic DNA and total RNA from shoot tips and leaves. The isolated sequences showed the highest similarities to other DELLA genes in BLASTX searches of the GenBank NR database. As in other DELLA genes, the predicted *DaGAI*-coding region consisted of a single exon of ~1.6 kb. The name *DaGAI* was chosen to reflect its strong similarity to *GAI* and not necessarily to imply orthology to *GAI*, which is part of a five-member gene subfamily in Arabidopsis. We then amplified the coding region and 5′ flanking regions of *DaGAI* from nine Hawaiian and four North American Madiinae. Two distinct classes of coding region sequences were apparent in the Hawaiian taxa. Phylogenetic analysis of the sequences showed that the Hawaiian sequences comprised two monophyletic groups with separate origins within the Madiinae, which confirmed the presence of two duplicated loci. We designated the two Hawaiian copies as *DaGAI-A* and *DaGAI-B* based on their apparent phylogenetic placement within the Madiinae, so as to maintain consistency with the previous designation of the *A* and *B* gene copies in the Hawaiian Madiinae (Barrier et al. 1999; Barrier, Robichaux, and Purugganan 2001). We obtained clones for the *DaGAI*-coding region from the four North American taxa, and for *DaGAI-A* and *DaGAI-B* each from seven of the nine Hawaiian species. We also obtained upstream flanking sequence of *DaGAI-B* from the same seven Hawaiian taxa; of *DaGAI-A* from *A. sandwicense*, *A. kauense*, and *D. arborea* and from all of the North American taxa except *M. sativa*. RT-PCR experiments showed that both *DaGAI-A* and *DaGAI-B* are expressed in *D. arborea* elongating shoots, consistent with a function in growth regulation.

A comprehensive alignment of the *DaGAI* sequences from the Madiinae encompassed ~0.6–1.0 kb of the upstream flanking sequence plus the entire coding region, except for 65 bases at the 3′-end. Pairwise nucleotide distances for the *DaGAI* coding region sequences show divergences of 1.9% or less within both *DaGAI-A* and *DaGAI-B* in the Hawaiian taxa. Distances between *A* and *B* copies range from 5.6% to 6.5%. The *C. multiglandulosa* sequence was by far the most divergent, with distances of 16.7%–17.4% from the *DaGAI-A* sequences, 17.6%–18.1% from the *DaGAI-B* sequences, and 17.7%–18.3% from the other North American *DaGAI* sequences. *Calycadenia multiglandulosa* was included as an out-group on the basis of previous studies that have shown it to belong to a separate tarweed lineage, whereas the other three North American species were included as close relatives of the silversword alliance ancestors (Baldwin and Wessa 2000; Barrier, Robichaux, and Purugganan 2001). The distances are thus consistent with a common origin for all the *DaGAI* sequences within a single lineage of the Madiinae.

Phylogenetic trees were constructed from the entire aligned coding regions. Two minimum-length trees were found using maximum parsimony. Two likelihood peak ''islands'' were found when maximum likelihood methods were used, and the tree corresponding to the higher peak was identical in topology to one of the two minimum-length parsimony trees (fig. 1). The Hawaiian *A* and *B* copy clades were strongly supported in maximum parsimony bootstrap analyses. *Anisocarpus madioides* was strongly supported as sister to the Hawaiian *A* copy, consistent with the findings for the *A* copy of *ASAP3/TM6* relative to *A. scabridus*, the sister species to *A. madioides* (Barrier et al. 1999; Barrier, Robichaux, and Purugganan 2001). The *B* copy clade was strongly sup-

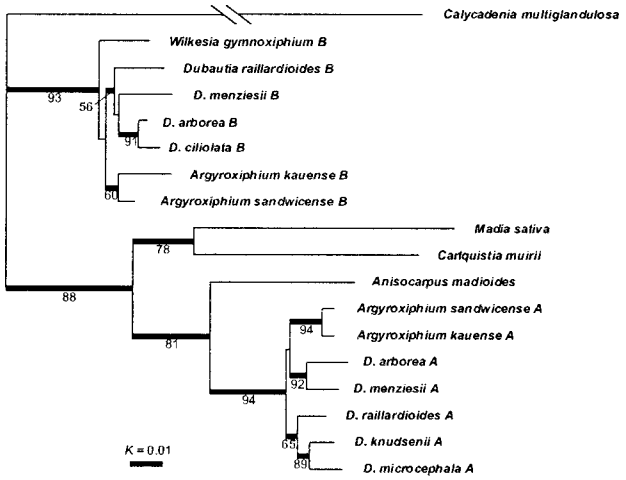

FIG. 1.—Maximum likelihood tree of *DaGAI* sequences, using *C. multiglandulosa* as an out-group. Branches with bootstrap support >50% from 500 maximum parsimony replicates are printed in bold, and bootstrap percentages are printed below the branches.

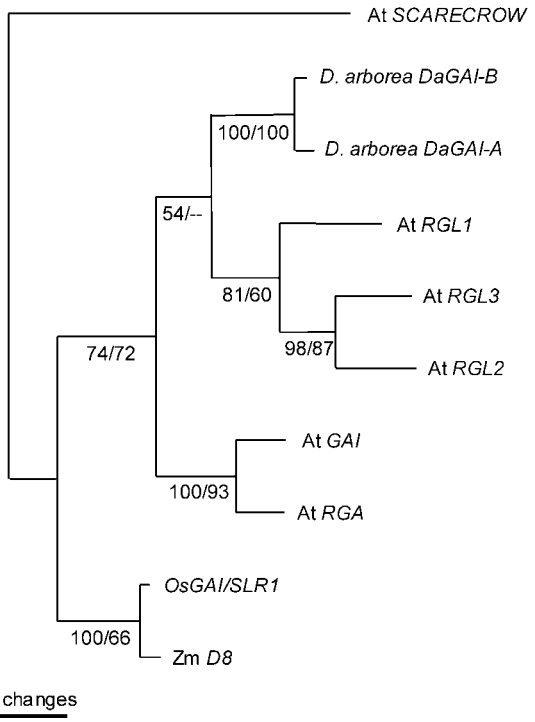

FIG. 2.—Single minimum-length maximum parsimony tree (817 steps) of the DELLA subfamily sequences from *A. thaliana* (At), rice (Os), maize (Zm), and *D. arborea*. The *A. thaliana SCARECROW* sequence was used as an out-group. Bootstrap percentages from 500 maximum parsimony and 500 neighbor-joining replicates, respectively, are shown for each node.

ported as sister to all the other ingroup taxa, in contrast with the sister relationship to *C. muirii* shown by *ASAP3/TM6* (Barrier et al. 1999; Barrier, Robichaux, and Purugganan 2001). Support was much weaker for clades within the Hawaiian species for both the *A* and *B* copies, consistent with a rapid adaptive radiation of the silversword alliance after introduction to Hawaii. All within-copy clades with at least moderate support were consistent with chloroplast DNA and rDNA ITS phylogenies (Baldwin et al. 1991; Baldwin and Sanderson 1998). Phylogenetic trees constructed from upstream flanking sequences produced similar topologies with respect to the major clades (data not shown).

Duplicate copies of *DaGAI* in the Hawaiian Madiinae are consistent with an allotetraploid origin of the silversword alliance (Barrier et al. 1999). The basal position of the Hawaiian *B* copy, however, suggests that this copy may have a different origin than does the *B* copy of *ASAP3/TM6* (Barrier et al. 1999; Barrier, Robichaux, and Purugganan 2001). Such strong support for conflicting topologies between *DaGAI* and *ASAP3/TM6* seems unlikely to be caused by lineage sorting because intralocus recombination would tend to produce ambiguous rather than strong support for alternate topologies. A more likely explanation for topological inconsistencies would be the hybridization among tarweed lineages before the origin of the Hawaiian ancestor. Hybridization has been suggested previously as an explanation for discordance between cpDNA phylogenies and cytogenetic data in the Hawaiian Madiinae (Baldwin, Kyhos, and Dvorak 1990). It is also possible that we have not found the ''true'' *B* copy of *DaGAI* and that our *DaGAI-B* is actually the product of an earlier duplication within the same lineage of the Madiinae. We did not do genomic Southern blots to evaluate the possible presence of other closely related *DaGAI* copies. We have not isolated sufficient quantities of high-purity DNA for genomic Southern hybridizations to date because of low DNA yields resulting from high tissue pectin concentrations and limited field collections of leaf and flower tissue. However, neither sequencing of 50 clones from four degenerate PCR experiments in *D. arborea* nor analysis of 22 clones from coding region amplifications of 10 taxa (which amplified the *A* and *B* copies with equal affinity) revealed any evidence of additional loci.

Common Ancestry of Monocot and Dicot DELLA Genes

We aligned the sequences of *DaGAI-A* and *DaGAI-B* from *D. arborea* with publicly available sequences of *GAI*, *RGA*, and three other *RGA*-like sequences (*RGL1*, *RGL2*, and *RGL3*) from *A. thaliana*, *d8* from maize, and *SLR1* from rice. We constructed phylogenetic trees from the nine genes using conserved regions of the *A. thaliana SCARECROW* gene as an out-group. The neighbor joining, maximum parsimony, and maximum likelihood methods produced identical topologies (fig. 2). The two *DaGAI* copies formed a strongly supported clade, as did the Arabidopsis *GAI-RGA* and *RGL2-RGL3* gene pairs. The *GAI-RGA* and *RGL2-RGL3* sister relationships are also supported by genomic evidence; *GAI* and *RGA* are located in duplicated Arabidopsis chromosomal blocks (10b and 10a) identified by Vision, Brown, and Tanksley (2000), as are *RGL2* and *RGL3* (blocks 71a and 71b). The two *DaGAI* sequences grouped with the three Arabidopsis *RGL* sequences rather than to *GAI* and *RGA*, but bootstrap support for this branch was weak (54% with maximum parsimony and <50% with neighbor-joining). The two monocot sequences form a strongly

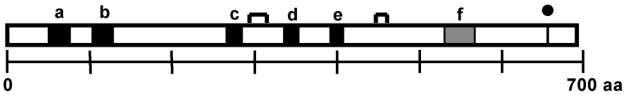

FIG. 3.—Schematic drawing showing the structure of DELLA proteins. Conserved features, per Peng et al. (1999) are: (a) and (b) DELLA region; (c) and (e) valine-rich regions; (d) putative nuclear localization signal; and (f) SH2-like domain. Square brackets denote regions of leucine heptad repeats, and dot indicates the putative tyrosine phosphorylation site. Scale is based on alignment of *DaGAI-A, DaGAI-B, d8, SLR1, GAI, RGA, RGL1, RGL2,* and *RGL3.*

supported sister clade to the dicot sequences, which provides phylogenetic support for previous assumptions of orthology between the monocot sequences and *GAI* (Peng et al. 1999).

The phylogenetic reconstructions suggest that the DELLA genes in monocots and dicots originated from a single ancestor and that the subsequent duplications leading to multiple subfamily members in Arabidopsis occurred within the dicots. This is consistent with evidence that *SLR1* is a single-copy gene in rice (Ikeda et al. 2001). The association of *GAI-RGA* and *RGL2-RGL3* with duplicated chromosomal blocks was also recently noted by Dill and Sun (2001), but no phylogenetic analysis was done to evaluate the relative ages of the gene duplications and taxonomic divergences. Our analysis provides clear evidence that the duplications leading to all five DELLA genes in Arabidopsis occurred after the monocot-dicot divergence and suggests that the monocot and dicot DELLA genes are indeed orthologous to each other.

The ambiguous relationships between *DaGAI* and the Arabidopsis DELLA genes may reflect duplication dates for the *GAI-RGA* and *RGL* gene ancestors close to the time of the rosid-asterid divergence ~112–156 MYA (Yang et al. 1999). In a comparison of a tomato genomic region with the Arabidopsis genome sequence, Ku et al. (2000) found evidence of two genomic duplications in the Arabidopsis lineage, the older of which may have occurred very near the time of the rosid-asterid divergence. If the *GAI-RGA* and *RGL* lineages diverged before the rosid-asterid divergence and *DaGAI* were orthologous to the latter, which is weakly suggested by our data, another, as yet undetected, set of DELLA loci orthologous to *GAI* and *RGA* may be present in the Madiinae. Genes in both the *GAI-RGA* and *RGL* lineages in Arabidopsis, however, have now been shown to function in regulation of gibberellin growth responses (Wen and Chang 2002), so the precise phylogenetic relationships between *DaGAI* and the Arabidopsis genes lack any obvious functional implications.

Patterns of Sequence Conservation and Divergence in *DaGAI*

The *DaGAI* sequences shared conserved features that have been previously reported for the DELLA subfamily (Peng et al. 1999), including (1) a conserved N-terminal DELLA domain, (2) regions of leucine-heptad repeats, (3) two valine-rich regions, (4) key conserved residues in an SH2-like domain, and (5) a conserved tyrosine near the C-terminus that is a putative phosphorylation site in signal transducer and activator of transcription (STAT) proteins (see fig. 3). The putative start of translation is identified by the amino acid sequence MKR a short distance upstream from the start of the DELLA domain, as in the other *GAI* homologues. The only substantial difference is that conserved arginine residues have been changed (to alanine and glutamine, respectively) in both sections of the putative bipartite nuclear localization signal.

Three indels were found near the N-terminus of the *DaGAI*-coding region, and there were 20 indels in a 260-bp region after the DELLA domain. One of the indels involved variable numbers of imperfect single-codon N/D/T repeats, and another consisted of variation in two-codon (G/P/E)N repeats. The region between the indel-rich sections, consisting primarily of the DELLA domain (here defined as spanning regions I and II of Peng et al. 1999), and the entire coding region after the first ~0.5 kb were almost entirely lacking in indels. This pattern corresponds closely to what was seen in the broader DELLA subfamily, except that the regions containing indels were more limited in the Madiinae. Transformation experiments have shown that both wild-type *GAI* and DELLA-truncated mutant *gai* from Arabidopsis maintain their expected function in rice and behave in a very similar manner to rice transformed with wild-type and mutant forms of *SLR1* (Fu et al. 2001; Ikeda et al. 2001). Moreover, all three Arabidopsis DELLA genes that have been studied functionally (*GAI, RGA,* and *RGL1*) show very similar effects on growth, both in wild-type and DELLA-truncated forms (Wen and Chang 2002). This shows that patterns of structural conservation in DELLA genes, which the *DaGAI* loci share, are correlated with strong functional conservation as well.

Rates of Neutral Molecular Evolution are Similar Between *A* and *B* Copies of *DaGAI*

We conducted relative rate tests on the *DaGAI-A* and *DaGAI-B* lineages to evaluate whether rates of evolution differed between copies. We applied maximum likelihood codon and nucleotide substitution models to the *D. arborea A* and *B* copies, using *C. multiglandulosa* as the out-group. Both synonymous and replacement substitution rates were higher for the *A* copy than for the *B* copy in the codon models, but the differences were not significant. Similarly, relative rates did not differ significantly with the nucleotide models applied to codon positions 1–2 only or position 3 only. Elevated substitution rates for the *A* copy were marginally significant only when maximum likelihood nucleotide substitution models were applied to the entire coding region ($P = 0.045$ using a global transition-transversion rate). The branch lengths of the maximum likelihood tree (fig. 1) appear to show that faster rates of evolution in the *A* copy lineage relative to the *B* copy lineage are confined to the more basal, North American branches, or are related to the placement of the root. Thus, there is no suggestion of differences in neutral evolutionary rates between the two *DaGAI* copies in the Hawaiian lineages.

Table 1
Summary of Replacement : Synonymous Substitution Ratios (ω) and Likelihoods for the Overall Coding Region Under Global, Constrained (2-ω and 3-ω), and Local Models of Rate Variation

Hypothesis	Replacement : Synonymous Substitution Ratios (ω)				Ln(*L*)[a]	df[b]
	Global	HI-*A*	HI-*B*	NA		
Global ω	0.177	—	—	—	−4,496.945	33
2-ω_A: HI-*A*, HI-*B* + NA	—	0.367	0.151[c]		−4,491.849	34
2-ω_B: HI-*A* + *B*, NA	—	0.264[d]		0.140	−4,492.734	34
3-ω: HI-*A*, HI-*B*, NA	—	0.368	0.196	0.139	−4,491.087	35
Local ω	—	—	—	—	−4,465.622	62

[a] Natural logarithm of the maximum value of the likelihood function.
[b] Degrees of freedom for the likelihood function.
[c] Combined ω for HI-*B* and NA branches.
[d] Combined ω for HI-*A* and HI-*B* branches.

Selective Constraints Differ Between the Hawaiian and North American *DaGAI* Lineages

To evaluate the heterogeneity in the selective constraint in *DaGAI*, we analyzed the rates of synonymous (K_A) and nonsynonymous (K_S) substitutions along each branch of the maximum likelihood tree using codon-based maximum likelihood models. We excluded *C. multiglandulosa* from these analyses so that the results would not be unduly influenced by evolution along the out-group branch. We imposed different sets of constraints on K_A/K_S (or ω) ratios along various tree branches (table 1), which allowed us to conduct a number of nested hypothesis tests of constraint and selection (table 2). We also tested the robustness of the maximum likelihood models to alterations in the tree topology and obtained very similar results when an alternative topology was used (data not shown).

A test of the global model with a single ω value against the 3-ω model, in which separate ω ratios were estimated for Hawaiian *A* and *B* copy branches and North American branches, indicated significant variation in selective constraint, with the Hawaiian *B* copy showing slightly less constraint (higher ω) than the North American branches and the Hawaiian *A* copy showing a substantial reduction in constraint. In tests of 2-ω models against the global model, the combined Hawaiian *A* and *B* copy branches showed significantly less constraint than did the North American branches. However, the Hawaiian *A* copy showed an even more significant relaxation of constraint relative to the combined Hawaiian *B* copy and North American branches. The 3-ω model did not produce a significant improvement over either of the 2-ω models, indicating that the Hawaiian *B* copy ω ratios by themselves are not significantly different from either of the other two classes.

The test results suggest both a general relaxation of the selective constraint in the Hawaiian copies and a relaxation of constraint specific to the Hawaiian *A* copy. Nevertheless, even the *A* copy branches remained under substantial selective constraint (ω = 0.37). These results stand in sharp contrast to results from other regulatory genes (Barrier, Robichaux, and Purugganan 2001), with average ω ratios of 0.79 and 0.98 for *ASAP3/TM6* and *ASAP1*, respectively. The mild degree of relaxed selective constraint in the two Hawaiian copies of *DaGAI* corresponds more closely to that for the structural gene *ASCAB9* (Barrier, Robichaux, and Purugganan 2001).

The North American and two Hawaiian *DaGAI* branches showed similar patterns of selective constraint and maintained the same relative order of ω ratios across nearly the entire coding region. We used a sliding window analysis to evaluate the patterns of constraint in the three sets of *DaGAI* branches (fig. 4 and table 3). The patterns of constraint in all three sets of branches closely

Table 2
Likelihood Ratio (LR) Test Statistics and *P* Values for the Tests of Hypotheses Shown in Table 1

Hypothesis Tests	LR Statistic	df Difference	*P* Value
2-ω_A:global ω	10.192	1	0.0014
3-ω:global ω	11.716	2	0.0029
3-ω:2-ω_A	1.524	1	0.2170
2-ω_B:global ω	8.422	1	0.0037
3-ω:2-ω_B	3.294	1	0.0695
local ω:3-ω	50.930	27	0.0035

NOTE.—Likelihood ratio (LR) statistic is twice the difference in Ln(*L*) between the alternative and null hypotheses.

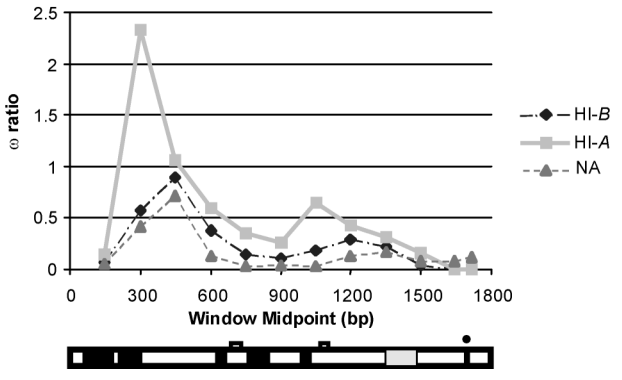

FIG. 4.—Sliding window plot of replacement-synonymous substitution ratios (ω) across the *DaGAI*-coding region for North American (NA), Hawaiian *A* copy (HI-A) and Hawaiian *B* copy (HI-B) branches. Window size is 300 bp of aligned sequence, with a 150-bp offset between windows.

Table 3
Summary of Sliding Windows Tests of Replacement : Synonymous Substitution Ratios (ω) in the *DaGAI*-Coding Region, Under Global ω and Constrained (3-ω) Models

Starting Position (bp)	Ending Position (bp)	Ln(*L*) (3-ω)	Ln(*L*) (global ω)	LR Statistic	*P* Value (2 df)
0 . . .	300	−548.772	−549.194	0.844	0.6557
150 . . .	450	−757.713	−759.435	3.444	0.1787
300 . . .	600	−883.879	−884.068	0.378	0.8278
450 . . .	750	−825.204	−829.058	7.708	0.0212
600 . . .	900	−733.551	−739.636	12.170	0.0023
750 . . .	1050	−753.607	−756.389	5.564	0.0619
900 . . .	1200	−770.969	−781.388	20.838	<0.0001
1050 . . .	1350	−940.035	−942.751	5.432	0.0661
1200 . . .	1500	−894.383	−894.884	1.002	0.6059
1350 . . .	1650	−642.160	−642.705	1.090	0.5798
1500 . . .	1784	−460.922	−462.985	4.126	0.1271
1650 . . .	1784	−151.726	−153.213	2.974	0.2260

NOTE.—Positions are based on consensus alignment of the coding region.

mirrored the degree of conservation observed in the broader DELLA subfamily. Only two nonsynonymous substitutions were observed within either the Hawaiian *A* or *B* copies over the entire DELLA region. The section encompassing the valine-rich regions and the C-terminal region beginning with the SH2-like domain also showed especially strong constraint. The variable region downstream of the DELLA region and, to a lesser extent, the region just upstream of the SH2-like domain showed relaxed levels of constraint.

The relaxation of selection in the Hawaiian lineages was only significant over small portions of the coding region. When the 3-ω model was tested against the single-ω model in each window, only the position 601–900 and 901–1,200 windows had significantly different ω ratios when *P* values were adjusted for multiple tests. In both these regions, especially the latter, the ω ratio was substantially elevated in the Hawaiian *A* copy, whereas the Hawaiian *B* copy and North American branches were highly constrained. The ω ratios in the 151–450 region were not significantly different in spite of the greatly elevated ω of 2.37 for the Hawaiian *A* copy, possibly because the large number of indels in this region substantially reduced the amount of sequence data from which substitutions could be inferred.

It is important to note that the evolutionary rate analyses require only that both *DaGAI-A* and *DaGAI-B* be monophyletic, that their common ancestor would have lived after the divergence from *C. multiglandulosa*, and that the MRCAs of the Hawaiian *A* and *B* copies would have lived near the time of the Hawaiian introduction. The relative percentages of sequence divergence and the gene tree (fig. 1) support these assumptions. Neither hybrid introgression that conjoined the *DaGAI-A* and *DaGAI-B* ancestors before allotetraploidy nor duplication within the *A* or *B* copy lineages after introduction to Hawaii would affect any of the aforementioned analyses. The possible presence of other DELLA genes in the Madiinae, whether the result of recent or older duplications, likewise would not affect the validity of our analyses. The strength and patterns of selective constraint we have found suggest that the *DaGAI* genes have important functions in spite of whatever additional duplications may have occurred.

No Evidence for Coding Region Sites Under Positive Selection

We tested the presence of individual codons in *DaGAI* that may be under positive selection. Even though *DaGAI-A* and *DaGAI-B* are under strong overall selective constraint, individual codons representing key functional sites in the proteins could be experiencing positive selection and contributing to adaptive variation. If this were the case, we would expect to find a class of sites with ω ratios greater than 1. A model incorporating a class of sites under selection was significant when tested against the neutral model (table 4). The estimated ω ratio for the additional rate class, however, was far less than 1.0, indicating purifying rather than positive selection. Likewise, addition of a category of sites under positive selection into a beta model (beta+ω model) did not produce a significant increase in likelihood, thus providing no evidence for positive selection. This test was somewhat sensitive to the particular assumptions used and became significant when the number of discrete categories used to approximate the beta distribution was reduced from three to two (data not shown). In all model variants, however, the estimated ω value was not much greater than 1.0 and never exceeded 1.40. The likelihood improvements with the beta+ω model thus appear to be because of a better fit of the constraint distribution and not the presence of sites under positive selection. These results suggest that a subset of codons in *DaGAI* is evolving much faster than the gene as a whole but under approximate neutrality rather than under positive selection.

Table 4
Summary of Tests for Positively Selected Codons

	Codon frequencies		Beta Parameters						
Model	Invariant	Selected	P	Q	ω[a]	Ln(*L*)	LR Statistic	df Difference	*P* Value
Neutral	0.736	—	—	—	—	−4,485.348			
Positive	0.083	0.806	—	—	0.091	−4,472.464	25.768	2	<0.0001
Beta	—	—	0.142	0.626	—	−4,475.115	—	—	—
Beta + ω . . .	—	0.090	2.029	19.756	1.136	−4,472.421	5.388	2	0.0676

[a] ω is the estimated replacement : synonymous substitution ratio for the selected site category.

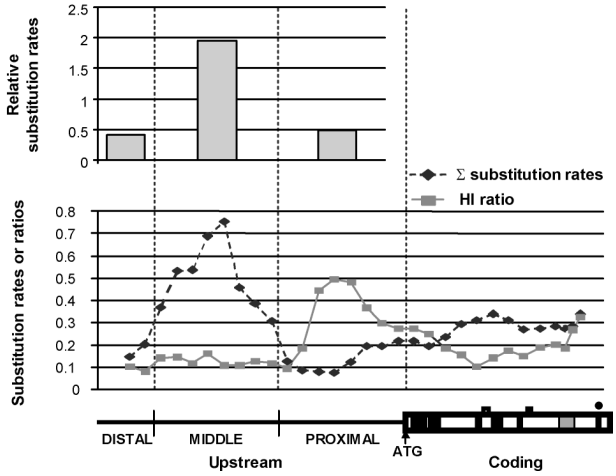

FIG. 5.—Nucleotide substitution rates in three upstream regions, relative to that of the third codon positions in the coding region (top); and sliding window analysis of summed substitution rates per base pairs and proportion of total branch length on Hawaiian branches (bottom).

Variable Patterns of Evolution in *DaGAI* Upstream Flanking Region

We investigated ~1 kb of the upstream flanking region in *DaGAI* for evolutionary patterns that may result from selection. The upstream flanking region can be divided into three regions with contrasting evolutionary dynamics (fig. 5): (1) a proximal region consisting of positions −1 to −260 (relative to the start of the coding region based on the *D. arborea DaGAI-B* sequence) with many indels but moderately constrained substitution rates compared with third codon position sites in the coding region; (2) a middle region (−261 to −630) that is also indel-rich and has greatly elevated mutation rates; and (3) a distal region (−631 to −804) that is completely devoid of indels and has highly constrained substitution rates. The remaining aligned sequence from −805 to −862 again becomes more variable, and the anchored PCR sequences of *C. multiglandulosa* and the *D. arborea A* copy that extend another several hundred base pairs upstream show only weak similarities. Virtually, the entire middle region is deleted in *C. multiglandulosa*. The proximal region is highly pyrimidine-rich (~66% in the *B* copy) on the plus strand. The *B* copy in the *Dubautia* spp. has a 70–90 bp deletion relative to the other sequences at −240. Upstream from position −260, the purine-pyrimidine imbalance disappears and the sequence abruptly becomes more AT-rich, averaging <30% GC content compared with ~40% GC for the proximal region. We checked for the possibility that the conserved distal region is an exon of an adjacent gene. BLAST searches using the distal region as a query sequence turned up no significant similarities to known or predicted genes, however, and all six possible reading frames contain multiple stop codons.

Positions −401 to −479 are an insertion unique to the Hawaiian *B* copy with characteristics of a miniature inverted-repeat transposable element, or MITE (Bureau, Ronald, and Wessler 1996), including a 5′-AGT-3′ target site duplication and an imperfect 20-bp terminal inverted repeat (fig. 6). Four additional base pairs just inside the target site duplication at the 3′-end of the insertion do not have a match at the 5′-end and may have been deleted from the 5′-end shortly after insertion of the element. This insertion shows no homology to the existing elements (Le et al. 2000) and thus appears to belong to a new transposable element family, which we have named *'I'iwi* after a Hawaiian honeycreeper. *Carlquistia muirii* contains a unique 125-bp insertion at −351 and several smaller insertions just downstream;

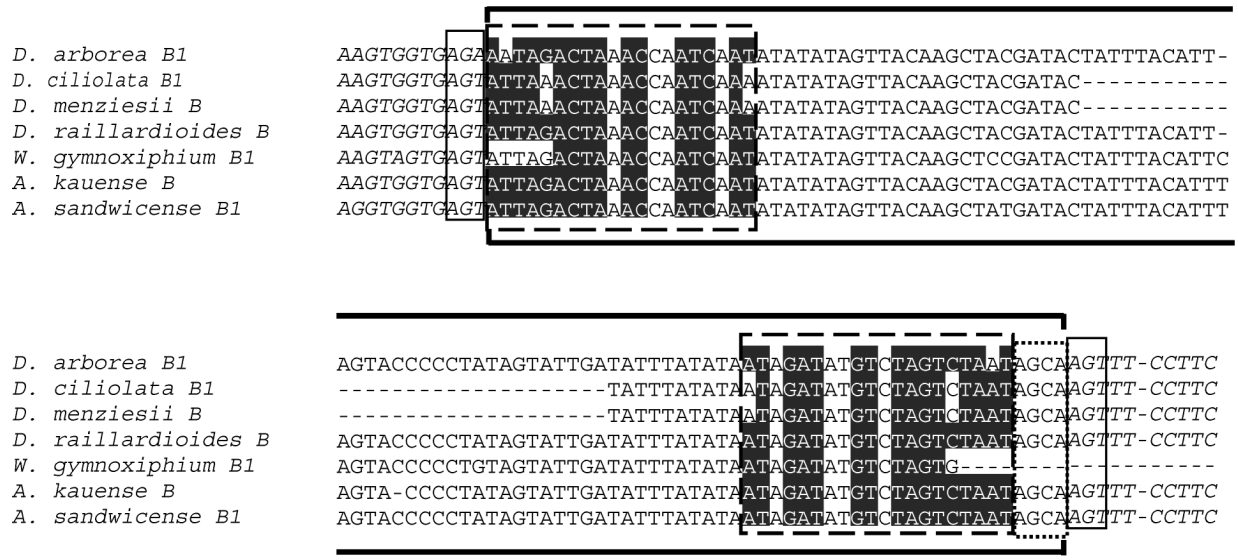

FIG. 6.—Alignment of putative *'I'iwi* element insertion (bracketed) and flanking sequence (in italics) in the upstream flanking region of *DaGAI-B,* for sequences from seven species. Target site AGT repeat is enclosed in a solid box; terminal inverted repeats are enclosed in a dashed box, with complementary residues shown in black shading. Four additional bases at the 3′-end of the element that lack a 5′ complement are enclosed in a dotted box.

this region shows no homology to existing elements and lacks a terminal inverted-repeat structure.

Relative ratio tests comparing the three upstream regions with the third codon positions indicated that selective constraint in the proximal region was significantly relaxed ($P = 0.03$) in the Hawaiian lineages relative to the North American branches. Approximately 33% of the estimated substitutions in the proximal region occurred on Hawaiian branches compared with 18% in the third codon positions and 12%–14% in the middle and distal regions. Even greater levels of variation in substitution rates and branch length ratios were apparent in a sliding windows analysis (fig. 5, bottom). Fivefold variation in the proportion of branch lengths on Hawaiian lineages and an order of magnitude difference in overall substitution ratios are apparent within the upstream region.

The Hawaiian *B* copy had an elevated number of indels but not substitutions, relative to the *A* copy for the upstream flanking sequence as a whole. The differences in numbers of substitutions versus indels between the *D. arborea* and *A. sandwicense A* and *B* copies were significant in a contingency test ($P = 0.041$), indicating heterogeneity between the indel and the substitution rates.

Relatively few published studies have examined upstream flanking sequences in a phylogenetic context, so there is limited data that can be used to evaluate whether the patterns we observed are typical. Some recent studies (although involving enzyme-encoding rather than regulatory genes) have found higher rates of sequence polymorphism and divergence in functionally important promoter segments than in adjacent regions, and adaptive explanations have been proposed (Crawford, Segal, and Barnett 1999; Miyashita 2001). Such patterns, however, could also be caused by coevolved sequence differences that preserve existing functions rather than selection for novel functions (Ludwig et al. 2000). The lack of correlation between indel and substitution rates in the upstream regions is not readily explained. One possibility is that the substitutions that modify the transcription factor–binding sites are more deleterious than the indels that change the spacing between sites. Testing this hypothesis, however, would require detailed functional studies.

The elevated substitution rates in the middle upstream region, the branch length heterogeneity between the proximal upstream region and the coding region, and the decoupled rates of indels and substitutions are all possible indicators of positive selection in the *DaGAI* upstream flanking region. None of these observations, however, demonstrate positive selection to the exclusion of other possibilities. Further studies that include within-species sampling of alleles would allow the use of more sensitive tests to distinguish between relaxed selective constraint and positive selection (e.g., the HKA test, Hudson, Kreitman, and Aguade 1987). Ambiguous alignments in parts of the middle region could have influenced some of the results, but the high variability in this region cannot be explained as an artifact of alignment. Elevated rates of substitution and numerous indels are apparent even within the *A* and *B* lineages, where the alignments are straightforward.

Summary

If accelerated evolution of regulatory genes is an important factor in morphological evolution, our results suggest that not all regulatory genes with relevant functions are involved. We failed to find evidence of accelerated evolution in the *DaGAI* coding region in the Hawaiian Madiinae. Selective constraint was only slightly relaxed and to a degree comparable with that previously found in a structural gene (Barrier, Robichaux, and Purugganan 2001). Evidence that selection may be operating on *DaGAI* is limited to the upstream flanking region. If the discordant evolutionary patterns we observed in the upstream region were the result of selection, this would support the hypothesis of Doebley and Lukens (1998) that mutations with important effects on plant form are most likely to be in the *cis*-regulatory regions of the transcriptional regulators rather than in the coding regions. Changes in the promoters of the regulatory genes have been implicated in the changes in the levels or spatial and temporal patterns of gene expression in maize, leading to novel phenotypes (Hanson et al. 1996; Wang et al. 1999).

Lukens and Doebley (2001) also found a lack of evidence for positive selection among *tb1*-like sequences in grasses of the tribe Andropogoneae. Both the *tb1*-like and DELLA genes are putative transcription factors with effects on plant morphology and have had important roles in domesticated crops. One possible explanation is that plant growth and morphology may be affected by large numbers of genes. Individual genes may thus be under very weak selection or else beneficial mutations with large effects may have occurred by chance in other genes. It is also possible that other DELLA genes in the Madiinae, especially those that may have arisen from earlier genomic duplications, might show very different evolutionary patterns.

We were also interested in the more specific question of whether *DaGAI* itself might have had a major role in the adaptive radiation of the silversword alliance, similar to that of DELLA genes in domesticated crops. In this regard, the absence of relaxation in the selective constraint in the DELLA region is especially noteworthy. The dwarf phenotypes of the ''green revolution'' wheat varieties, mediated by DELLA truncations in *Rht-1* genes with consequent reductions in gibberellin responsiveness, are of great advantage because of reduced wind and rain damage and less expenditure on vegetative biomass production (Peng et al. 1999). We had predicted that similar, but possibly less drastic, DELLA mutations might have been important in adaptive evolution in the silversword alliance as well. The region around the SH2-like domain, in which maize indels are strongly associated with flowering time (Thornsberry et al. 2001), likewise shows no evidence of relaxed selection. One possible explanation for our failure to obtain such results is that maintenance of normal gibberellin responsiveness may be important for long-term fitness in natural envi-

ronments, even those that promote strong adaptive divergence in growth habit, in contrast with some crop environments. This explanation assumes that *DaGAI* has a function similar to that of other DELLA genes, which seems reasonable, given their functional conservation in taxa as diverse as *A. thaliana* and various grasses (Peng et al. 1997, 1999; Fu et al. 2001; Ikeda et al. 2001; Wen and Chang 2002).

The possibility of divergent selection on the upstream *DaGAI* regions may warrant further investigation. In particular, some of the larger indels in the upstream region co-occur with major changes in the growth form within the Hawaiian Madiinae. Indels in the upstream region of *d8* show strong association with the developmental phenotypes in maize, suggesting a phenotypic role for regulatory variation in DELLA genes (Thornsberry et al. 2001). The recently demonstrated importance of dosage level in growth response modulation by DELLA genes in Arabidopsis and transgenic rice (Dill and Sun 2001; Fu et al. 2001; King, Moritz, and Harberd 2001; Wen and Chang 2002) suggests that changes in gene copy number could also have profound phenotypic effects. Study of *DaGAI* expression levels and molecular population genetics would be warranted in pairs of closely related taxa with large differences in growth form.

Acknowledgments

We thank Rob Robichaux for his indispensable guidance with field collections, Amy Lawton-Rauh and Sola Halldorsdottir for their assistance with the fieldwork, Marianne Barrier and Bruce Baldwin for providing tissue and DNA samples from North American Madiinae, Elizabeth Friar for her assistance with the DNA preparation protocols, and Sergei Kosakovsky Pond for assistance with customizing HY-PHY batch files. This work was supported in part by an NSF grant to M.D.P. and Robert Robichaux, an Alfred P. Sloan Young Investigator award to M.D.P., and an NIH Individual Postdoctoral Fellowship to D.L.R.

LITERATURE CITED

BALDWIN, B. G., D. W. KYHOS, and J. DVORAK. 1990. Chloroplast DNA evolution and adaptive radiation in the Hawaiian silversword alliance (Asteraceae-Madiinae). Ann. Mo. Bot. Gard. **77**:96–109.

BALDWIN, B. G., D. W. KYHOS, J. DVORAK, and G. D. CARR. 1991. Chloroplast DNA evidence for a North American origin of the Hawaiian silversword alliance (Asteraceae). Proc. Natl. Acad. Sci. USA **88**:1840–1843.

BALDWIN, B. G., and M. J. SANDERSON. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc. Natl. Acad. Sci. USA **95**:9402–9406.

BALDWIN, B. G., and B. L. WESSA. 2000. Origin and relationships of the tarweed-silversword lineage (Compositae-Madiinae). Am. J. Bot. **87**:1890–1908.

BARRIER, M., B. G. BALDWIN, R. H. ROBICHAUX, and M. D. PURUGGANAN. 1999. Interspecific hybrid ancestry of a plant adaptive radiation: allopolyploidy of the Hawaiian silversword alliance (Asteraceae) inferred from floral homeotic gene duplications. Mol. Biol. Evol. **16**:1105–1113.

BARRIER, M., R. H. ROBICHAUX, and M. D. PURUGGANAN. 2001. Accelerated regulatory gene evolution in an adaptive radiation. Proc. Natl. Acad. Sci. USA **98**:10208–10213.

BUREAU, T. E., P. C. RONALD, and S. R. WESSLER. 1996. A computer-based systematic survey reveals the predominance of small inverted-repeat elements in wild-type rice genomes. Proc. Natl. Acad. Sci. USA **93**:8524–8529.

CARR, G. D. 1985. Monograph of the Hawaiian Madiinae (Asteraceae): *Argyroxiphium*, *Dubautia*, and *Wilkesia*. Allertonia **4**:1–123.

CHERRY, L. M., S. M. CASE, and A. C. WILSON. 1978. Frog perspective on the morphological difference between humans and chimpanzees. Science **200**:209–211.

CRAWFORD, D. L., J. A. SEGAL, and J. L. BARNETT. 1999. Evolutionary analysis of TATA-less proximal promoter variation. Mol. Biol. Evol. **16**:194–207.

DILL, A., and T.-P. SUN. 2001. Synergistic derepression of gibberellin signaling by removing RGA and GAI function in *Arabidopsis thaliana*. Genetics **159**:777–785.

DOEBLEY, J., and L. LUKENS. 1998. Transcriptional regulators and the evolution of plant form. Plant Cell **10**:1075–1082.

DOYLE, J. J., and J. L. DOYLE. 1990. Isolation of plant DNA from fresh tissue. Focus **12**:13–15.

EWING, B., and P. GREEN. 1998. Base-calling of automated sequencer traces using *Phred*. II. Error probabilities. Genome Res. **8**:186–194.

EWING, B., L. HILLIER, M. C. WENDL, and P. GREEN. 1998. Base-calling of automated sequencer traces using *Phred*. I. Accuracy assessment. Genome Res. **8**:175–185.

FROHMAN, M. A., M. K. DUSH, and G. R. MARTIN. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**:8998–9002.

FU, X., D. SUDHAKAR, J. PENG, D. RICHARDS, P. CHRISTOU, and N. P. HARBERD. 2001. Expression of Arabidopsis GAI in transgenic rice represses multiple gibberellin responses. Plant Cell **13**:1791.

GOLDMAN, N., and Z. YANG. 1994. A codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol. Biol. Evol. **11**:725–736.

HANSON, M. A., B. S. GAUT, A. O. STEC, S. I. FUERSTENBERG, M. M. GOODMAN, E. H. COE, and J. F. DOEBLEY. 1996. Evolution of anthocyanin biosynthesis in maize kernels: the role of regulatory and enzymatic loci. Genetics **143**:1395–1407.

HUDSON, R. R., M. KREITMAN, and M. AGUADE. 1987. A test of neutral molecular evolution based on nucleotide data. Genetics **116**:153–159.

IKEDA, A., M. UEGUCHI-TANAKA, Y. SONODA, H. KITANO, M. KOSHIOKA, Y. FUTSUHARA, M. MATSUOKA, and J. YAMAGUCHI. 2001. *slender* Rice, a constitutive gibberellin response mutant, is caused by a null mutation of the *SLR1* gene, an ortholog of the height-regulating gene *GAI*/*RGA*/*RHT*/*D8*. Plant Cell **13**:999–1010.

ITOH, H., M. UEGUCHI-TANAKA, Y. SATO, M. ASHIKARI, and M. MATSUOKA. 2002. The gibberellin signaling pathway is regulated by the appearance and disappearance of SLENDER RICE1 in nuclei. Plant Cell **14**:57–70.

KING, K. E., T. MORITZ, and N. P. HARBERD. 2001. Gibberellins are not required for normal stem growth in *Arabidopsis thaliana* in the absence of GAI and RGA. Genetics **159**:767–776.

KING, M. C., and A. C. WILSON. 1975. Evolution at two levels in humans and chimpanzees. Science **188**:107–116.

KU, H.-M., T. VISION, J. LIU, and S. D. TANKSLEY. 2000. Comparing sequenced segments of the tomato and *Arabidopsis* genomes: large-scale duplication followed by selective gene

loss creates a network of synteny. Proc. Natl. Acad. Sci. USA **97**:9121–9126.

LE, Q. H., S. WRIGHT, Z. YU, and T. BUREAU. 2000. Transposon diversity in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **97**:7376–7381.

LUDWIG, M. Z., C. BERGMAN, N. H. PATEL, and M. KREITMAN. 2000. Evidence for stabilizing selection in a eukaryotic enhancer element. Nature **403**:564–567.

LUKENS, L., and J. DOEBLEY. 2001. Molecular evolution of the teosinte branched gene among maize and related grasses. Mol. Biol. Evol. **18**:627–638.

MIYASHITA, N. T. 2001. DNA variation in the 5′ upstream region of the *Adh* locus of the wild plants *Arabidopsis thaliana* and *Arabis gemmifera*. Mol. Biol. Evol. **18**:164–171.

MUSE, S. V., and B. S. GAUT. 1994. A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. Mol. Biol. Evol. **11**:715–724.

OGAWA, M., T. KUSANO, M. KATSUMI, and H. SANO. 2000. Rice gibberellin-insensitive gene homolog, *OsGAI*, encodes a nuclear-localized protein capable of gene activation at transcriptional level. Gene **245**:21–29.

OLIVER, C. D., and B. C. LARSON. 1990. Forest stand dynamics. McGraw-Hill, New York.

ORR, A. 2001. The genetics of species differences. Trends Ecol. Evol. **16**:343–350.

PENG, J., D. E. RICHARDS, N. M. HARTLEY et al. (15 co-authors). 1999. 'Green revolution' genes encode mutant gibberellin response modulators. Nature **400**:256–261.

PENG, J. R., P. CAROL, D. E. RICHARDS, K. E. KING, R. J. COWLING, G. P. MURPHY, and N. P. HARBERD. 1997. The Arabidopsis *GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev. **11**: 3194–3205.

PURUGGANAN, M. D. 2000. The molecular population genetics of regulatory genes. Mol. Ecol. **9**:1451–1461.

PYSH, L. D., J. W. WYSOCKA-DILLER, C. CAMILLERI, D. BOUCHEZ, and P. N. BENFEY. 1999. The GRAS gene family in Arabidopsis: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. Plant J. **18**: 111–119.

RAVEN, P. H., R. F. EVERT, and S. E. EICHHORN. 1999. Biology of plants. 6th edition. W. H. Freeman, New York.

ROBICHAUX, R. H., G. D. CARR, M. LIEBMAN, and R. W. PEARCY. 1990. Adaptive radiation of the Hawaiian silversword alliance (Compositae-Madiinae): ecological, morphological, and physiological diversity. Ann. Mo. Bot. Gard. **77**:64–72.

SCHLUTER, D. 2000. The ecology of adaptive radiation. Oxford University Press, Oxford, U.K.

———. 2001. Ecology and the origin of species. Trends Ecol. Evol. **16**:372–380.

SIEBERT, P. D., A. CHENCHIK, D. E. KELLOGG, K. A. LUKYANOV, and S. A. LUKYANOV. 1995. An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res. **23**:1087–1088.

SILVERSTONE, A. L., C. N. CIAMPAGLIO, and T.-P. SUN. 1998. The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell **10**:155–169.

SILVERSTONE, A. L., H.-S. JUNG, A. DILL, H. KAWAIDE, Y. KAMIYA, and T.-P. SUN. 2001. Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. Plant Cell **13**:1555–1565.

SWOFFORD, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Mass.

THORNSBERRY, J. M., M. M. GOODMAN, J. DOEBLEY, S. KRESOVICH, D. NIELSEN, and E. S. BUCKLER IV. 2001. *Dwarf8* polymorphisms associate with variation in flowering time. Nat. Genet. **28**:286–289.

VISION, T. J., D. G. BROWN, and S. D. TANKSLEY. 2000. The origins of genomic duplications in *Arabidopsis*. Science **290**:2114–2117.

WANG, R.-L., A. STEC, J. HEY, L. LUKENS, and J. DOEBLEY. 1999. The limits of selection during maize domestication. Nature **398**:236–239.

WEN, C.-K., and C. CHANG. 2002. Arabidopsis *RGL1* encodes a negative regulator of gibberellin responses. Plant Cell **14**: 87–100.

YANG, Y.-W., K.-N. LAI, P.-Y. TAI, and W.-H. LI. 1999. Rates of nucleotide substitution in angiosperm mitochondrial DNA sequences and dates of divergence between *Brassica* and other angiosperm lineages. J. Mol. Evol. **48**:597–604.

ELIZABETH KELLOGG, reviewing editor

Accepted May 9, 2002