FLORAL DEVELOPMENT AND GENETICS IN NYMPHAEALES

By

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To my parents

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Nymphaeales (water lilies) one of the basal lineages of angiosperms, and this clade is sister to all extant flowering plants except *Amborella.* Nymphaeales also exhibit floral morphology that differs from other basal angiosperms, for example, in having whorled phyllotaxis. Thus, studying floral evolution in Nymphaeales will help elucidate floral development in early-diverging angiosperms and contribute to understanding floral evolution and diversification of angiosperms as a whole. The goals of this study were to estimate the divergence times of the major clades of Nymphaeales, to obtain floral developmental sequences for three study species (*Cabomba caroliniana*, *Nuphar advena*, and *Nymphaea odorata*), to examine the expression patterns of putative floral organ identity genes in floral organs of these three species (and especially in the transition series from perianth to stamens in *Nymphaea*), and to compare expression profiles of the floral transcriptome across floral organs of *Nuphar* using microarrays. Using a phylogeny based on the multiple genes, I found that extant Nymphaeales diversified into two major clades during the Eocene (44.6±7.9 mya), indicating that extant Nymphaeales diversified relatively recently, in contrast to their ancient fossil record. The three study species follow the same 10 developmental stages in the very same order. *Nuphar*, which is sister to other members of Nymphaeaceae, exhibits *Nymphaea*-like or *Cabomba*-like developmental features, and its

phylogenetic position suggests that sharing features between *Nuphar* and *Cabomba* might be ancestral characteristics to core Nymphaeales. Expression patterns of floral genes in these three species of Nymphaeales partially follow the developmental model of well-studied eudicot systems, such as *Arabidopsis*. However, analyses of the floral transcriptome in *Nuphar* and comparisons with other plants show that the genes involved downstream of floral organ identity genes are distinctive in their expression patterns in this species. Furthermore, there is greater overlap in floral transcriptional programs between floral parts in *Nuphar* than in the eudicot *Arabidopsis*, paralleling the less specialized floral organization of basal angiosperms. The results for *Nuphar*, coupled with similar data for the basal angiosperms, *Persea*, indicate that the first flowers deployed developmental programs more broadly than the vast majority of modern species.

CHAPTER 1
INTRODUCTION

Nymphaeales comprise nine genera and approximately 80 species of aquatic plants, with a worldwide distribution in tropical to temperate regions (Saarela et al. 2007; Schneider and Williamson 1993; Williamson and Schneider 1993). Nymphaeales are clearly monophyletic, appearing as a well-supported clade in a series of molecular phylogenetic analyses (Borsch et al. 2007; Chase et al. 1993; Hilu et al. 2003; Löhne et al. 2007; Soltis et al. 2000).

Phylogenetic relationships among genera of Nymphaeales were well resolved and strongly supported in phylogenetic analyses of molecular data (Borsch et al. 2007; Löhne et al. 2007; Les et al. 1999). This clade is composed of three subclades: Cabombaceae, Nymphaeaceae, and Hydatellaceae. The former two subclades are traditionally recognized as two families, Cabombaceae and Nymphaeaceae (Richard 1828; Williamson and Schneider 1993), which share many features, including vegetative, reproductive, and molecular characters. The remaining subclade, Hydatellaceae, is sister to Cabombaceae + Nymphaeaceae and was recently placed in Nymphaeales based on molecular phylogenetics (Saarela et al. 2007). Furthermore, its placement in Nymphaeales is confirmed by the presence of a 4-celled embryo sac like that of other Nymphaeales (Friedman 2008). However, floral development differs among the three families. For example, *Cabomba* and *Brasenia* (Cabombaceae) are characterized by oligomerous flowers and simultaneous initiation of calyx and corolla, whereas Nymphaeaceae (*Barclaya*, *Euryale*, *Nuphar*, *Nymphaea*, *Ondinea*, and *Victoria*) have polymerous flowers and exhibit a unidirectional order of perianth initiation (Endress 2001; Ito 1987; Les et al. 1999; Schneider et al. 2003). Hydatellaceae (*Hydatella* and *Trithuria*) show unique floral development: the presence of involucral bracts, no perianth, and separate staminate and carpellate flowers (Rudall et al.

2007). In this study, I only focused on three genera, *Nuphar*, the phylogenetically derived *Nymphaea* (Nymphaeaceae), and *Cabomba* (Cabombaceae).

Cabomba and *Brasenia* (Cabombaceae) (Cronquist 1981; Takhtajan 1980; Takhtajan 1997) are united by the presence of free-floating stems, in addition to rhizomes. They also have distinct carpels, nutlike fruits, and six-tepaled flowers without petal-like staminodes (Judd et al. 2002). There are numerous non-DNA synapomorphies for Nymphaeaceae, including the presence of star-shaped sclerids, spirally inserted stamens with laminar filaments, and laminar placentation (Les et al. 1999; Moseley et al. 1993). *Nuphar* is sister to all other genera of Nymphaeaceae (Borsch et al. 2007, 2008; Les et al. 1999; Löhne et al. 2007), and it has a superior ovary and monosulcate pollen. In contrast to *Nuphar*, *Barclaya, Ondinea, Nymphaea, Euryale*, and *Victoria* have perigynous/epigynous flowers and zonasulculate pollen (Ito 1987; Les et al. 1999). *Barclaya* is then sister to *Euryale*, *Victoria*, *Nymphaea,* and *Ondinea*; relationships among the latter are not clear. In recent phylogenetic studies, the monotypic *Ondinea* was embedded in the *Nymphaea* clade (Borsch et al. 2007, 2008; Löhne et al. 2007). A clade of *Euryale* and *Victoria* is sister to all species of *Nymphaea* (Borsch et al. 2007), or is included within the *Nymphaea* clade (Borsch et al. 2008; Löhne et al. 2007). In *Nymphaea*, *Euryale*, and *Victoria*, the gynoecial vascular strand originated from the receptacular plexus and is the source of the petal trace (Ito 1987; Les et al. 1999). The clade of *Victoria* and *Euryale* is united morphologically by prickles on the petiole and on the abaxial leaf surface.

The flowers of Nymphaeales range from small, ca. 0.5-1.5 cm (*Cabomba*) to very large, up to 50 cm (*Victoria*) (Schneider and Williamson 1993). Organ number varies widely, with three or four "sepals", zero to four (*Ondinea*) up to six to 51 (*Nymphaea*) and 50-70 (*Victoria*) "petals", six (*Cabomba*) up to 15-750 (*Nymphaea*) stamens, and three to 14 (*Ondinea*) up to five to 47

(*Nymphaea*) carpels. The highest number of floral parts occurs in *Victoria* and *Nymphaea*, but *Nuphar*, the sister to all remaining Nymphaeaceae, has also numerous parts. Thus, the proliferation of floral parts in Nymphaeales might be the result of several instances of secondary increase (Borsch et al. 2008; Les et al. 1999).

Several labs are trying to understand the genetic architecture of floral development, including the origin and subsequent diversification of the flower (D. Soltis et al. 2002). The basal angiosperm lineages are of particular interest in studies of floral evolution due to their diversity in the arrangement and number of floral parts. Importantly, members of Nymphaeales exhibit floral morphology that differs from the other basalmost angiosperms (i.e., *Amborella*, Austrobaileyales) in several features, including the presence of large flowers in some members (e.g., *Nuphar*, *Nymphaea*, *Euryale*, and *Victoria*) rather than small or moderate-sized flowers, whorled rather than spiral phyllotaxis, eudicot-like perianth differentiation (as in *Cabomba* and *Nuphar*), and the occurrence of blue perianth organs containing anthocyanins, which are absent in other basal angiosperms (Endress 2001; Schneider et al. 2003). Nymphaeales are sister to all extant angiosperms except *Amborella* (Borsch et al. 2005; Hilu et al. 2003; Jansen et al. 2007; Löhne and Borsch 2005; Leebens-Mack et al. 2005; Mathews and Donoghue 1999; Moore et al. 2007; Qiu et al. 1999; Qiu et al. 2005; Soltis et al. 1999; Soltis et al. 2000; Soltis et al. 2005; Zanis et al. 2002; Zanis et al. 2003). Thus, studying floral evolution in Nymphaeales will help elucidate floral evolution in early-diverging angiosperms, and thus provide insight into the early diversification of the flower.

Therefore, the main goals of this study were to: (1) estimate the divergence times of the major genera of Nymphaeales using a tree based on multiple genes, (2) obtain floral developmental sequences of *Cabomba caroliniana*, *Nuphar advena*, and *Nymphaea odorata*, (3)

examine the expression patterns of floral organ identity genes in the transitional series of stamens of *Nymphaea*, which has a complete series from petaloid staminodes to functional stamens, (4) examine the expression patterns of floral organ identity gene homologues in three genera (*Cabomba*, *Nuphar*, and *Nymphaea*), and (5) compare expression profiles of *Nuphar* floral-derived genes in different floral and vegetative tissues using microarrays.

These goals are addressed in the following five chapters. In chapter 2, I estimated the divergence times of the major genera of Nymphaeales using a tree based on multiple genes and several methods (NPRS (Sanderson 1997), penalized likelihood (Sanderson 2002), and Bayesian approaches (Thorne and Kishino 2002)). The estimated divergence times may help clarify the timing of evolutionary events suggested in my molecular studies below.

In chapter 3, I present floral developmental sequences for *Nuphar advena*, *Nymphaea odorata*, and *Cabomba caroliniana*. Although developmental studies of Nymphaeales have been reported previously (Endress 2001; Moseley 1958, 1961, 1965, 1972; Moseley et al. 1993; Schneider et al. 2003), the complete developmental series from initiation of the floral meristem to anthesis provides the framework for understanding the developmental characteristics across a diverse array of Nymphaeales.

In chapter 4, I investigated the expression patterns of floral organ identity gene homologues in floral organs of *Cabomba*, *Nuphar*, and *Nymphaea*, and so called "water lily" *Nelumbo*, which is previously considered to be related to Nymphaeales, now recognized as eudicots, and compared their gene expression profiles. In particular, I focused on gene expression patterns associated with perianth differentiation among these three genera, and the transitional series of petaloid staminodes to functional stamens of *Nymphaea*. Also, I

investigated whether morphological similarities between *Nymphaea* and *Nelumbo* are related to similar floral developmental genetics.

In chapter 5, I compared of expression profiles of *Nuphar* floral-derived genes in different floral and vegetative tissues using microarrays. I used a cluster analysis and comparative approach to elucidate the floral transcriptome of *Nuphar* and compare it using data from other plants, such as *Persea* and *Arabidopsis*.

Chapter 6 is a general conclusion in which I summarize what has been done and what I learned about floral developmental genetics and evolution in Nymphaeales. Furthermore, I suggest future directions that will help clarify flower development in Nymphaeales as well as basal angiosperm lineages.

CHAPTER 2
DIVERGENCE TIMES AND HISTORICAL BIOGEOGRAPHY OF NYMPHAEALES

Introduction

[*]Nymphaeales comprise eight genera and approximately 70 species of aquatic plants, with a worldwide distribution in tropical to temperate regions (Schneider and Williamson 1993; Williamson and Schneider 1993). Nymphaeales are clearly monophyletic, appearing as a well-supported clade in a series of molecular phylogenetic analyses (Chase et al. 1993; Hilu et al. 2003; Savolainen et al. 2000; Soltis et al. 2000; Zanis et al. 2002).

Phylogenetic relationships among extant genera of Nymphaeales were well resolved and strongly supported in analyses of molecular and morphological data (Les et al. 1999). The order comprises two clades, traditionally recognized as two families (Richard 1828; Williamson and Schneider 1993), Cabombaceae and Nymphaeaceae, but sometimes recognized as a single family (Nymphaeaceae), generally with two subfamilies (Cabomboideae and Nymphaeoideae) (e.g., (APG II 2003; Caspary 1888; Henkel et al. 1907). We will recognize these two clades at the familial rank for consistency with most previous treatments.

Cabomba and *Brasenia* (Cabombaceae) (Cronquist 1981; Takhtajan 1980; Takhtajan 1997) are united by the presence of free-floating stems, in addition to rhizomes. They also have distinct carpels, nutlike fruits, and six-tepaled flowers without petal-like staminodes (Judd et al. 2002). Numerous morphological synapomorphies unite Nymphaeaceae, including the presence of star-shaped sclereids, spirally inserted stamens with laminar filaments, and laminar placentation (Les et al. 1999; Moseley et al. 1993). Within Nymphaeaceae, relationships can be summarized as follows: (*Nuphar* (*Barclaya* (*Ondinea* (*Nymphaea* (*Euryale, Victoria*))))) (Fig. 2-1). Species of *Nuphar*, the second largest genus, share several unique morphological characters

[*]Reprinted with permission from the American Society of Plant Taxonomists. Original publication: Yoo, M.-J., C. D. Bell, P. S. Soltis, and D. E. Soltis. 2005. Divergence Times and Historical Biogeography of Nymphaeales

that provide support for the monophyly of the genus (Padgett et al. 1999), including pollen with ektexine spines (Takahashi 1992). *Nuphar* has hypogynous flowers and monosulcate pollen, while *Barclaya, Ondinea, Nymphaea, Euryale*, and *Victoria* have perigynous/epigynous flowers and zonasulculate pollen (Ito 1987; Les et al. 1999). *Nymphaea* is sister to the *Victoria-Euryale* clade, a relationship supported by anatomical characters (Ito 1987; Les et al. 1999); the monophyly of *Nymphaea* is weakly supported by sequence data (Borsch 2000). In these three genera, the gynoecial vascular strand originates from the receptacular plexus and is the source of the petal trace (Ito 1987; Les et al. 1999). *Victoria* and *Euryale* are united morphologically by prickles on the petiole and on the abaxial leaf surface.

The robust phylogeny provided by Les et al. (1999) is also useful for estimating divergence times of major clades and genera within Nymphaeales. In this study we estimated the divergence times of genera of Nymphaeales using DNA sequence data from nuclear 18S rDNA and plastid *rbcL* and *matK*, and the maximum parsimony tree of Les et al. (1999). We applied four methods—a strict molecular clock (Langley and Fitch 1974), nonparametric rate smoothing (Sanderson 1997, 1998), penalized likelihood (Sanderson 2002), and a Bayesian method (Kishino et al. 2001; Thorne et al. 1998; Thorne and Kishino 2002)—to estimate divergence times. The use of molecular data to date divergences is laden with potential problems, and these, along with potential inadequacies of the fossil record, have been reviewed (Magallón 2004; Sanderson and Doyle 2001; Sanderson et al. 2004; Soltis et al. 2002). Despite potential drawbacks, these approaches have provided estimates that have converged and are in general agreement with the fossil record, especially regarding the origin of the angiosperms (Bell et al. 2005; Magallón 2004; Sanderson et al. 2004). In addition, as the estimated divergence times are

important for interpretation of historical biogeography, we inferred the historical biogeographic pattern of major clades in Nymphaeales in light of these divergence times.

Materials and Methods

Taxon Sampling and Topology

Our molecular sampling employed the sequence data from Les et al. (1999) and includes eight species of Nymphaeales, representing the eight genera of the order. To estimate the divergence time of Nymphaeaceae and Cabombaceae, sequences of other basal angiosperm lineages, *Amborella* and three representatives of Austrobaileyales (*Austrobaileya*, *Illicium*, and *Schisandra*), were included. Four sequences of extant gymnosperms (*Larix*, *Taxus*, *Gnetum*, and *Ginkgo*) were included to permit estimation of the age of the angiosperms. For each species, nuclear 18S DNA sequences and plastid *rbcL* and *matK* sequences were retrieved from GenBank (Table 2-1). The sequences were aligned using CLUSTAL X (Thompson et al. 1997) with the default options, and the alignment was then adjusted manually. Data matrices used in this study are deposited in TreeBase (www.Treebase.org).

The topology for Nymphaeales used in our study is the single maximum parsimony tree obtained by Les et al. (1999) inferred from analysis of morphological characters, plus *rbcL, matK*, and 18S rDNA sequences (Fig. 2-1). In general, the individual and combined data sets (for all genes but 18S rDNA) gave very similar topologies (reviewed in Les et al. 1999). The *matK* topology (see Hilu et al. 2003) is also highly similar to the Les et al. total evidence topology. Phylogenetic relationships among Nymphaeales, *Amborella trichopoda*, and Austrobaileyales were constrained to match those reported in a series of recent studies (Borsch et al. 2003; Graham and Olmstead 2000; Hilu et al. 2003; Mathews and Donoghue 1999; Parkinson

et al. 1999; Qiu et al. 1999; Soltis et al. 1999; Soltis et al. 2000; Zanis et al. 2002) (Fig. 2-1). This topology was constrained during estimation of divergence times.

Tests of Rate Heterogeneity

For each gene taken separately and all genes combined, rate heterogeneity across lineages was tested using a likelihood ratio (LR) test (Felsenstein 1988). Significance was determined by comparing the difference between -ln likelihood (-ln$L$) of the tree, with and without enforcing a molecular clock, based on a χ^2 distribution with n - 2 degrees of freedom where n is the number of taxa. In addition, rate heterogeneity between pairs of genes was also tested using a LR test. Significance was assessed by comparing Λ = -2logLR with a χ^2 distribution, in which LR=[ln$L$-(lnL_1 + lnL_2)], where L_1 is the likelihood of the tree with one data set, L_2 is the likelihood of the tree with the second data set, and L is the likelihood of the tree with both data sets combined (Sanderson and Doyle 2001). Degrees of freedom were calculated according to Sanderson and Doyle (2001).

Estimation of Ages

Because all tests of rate heterogeneity among lineages were highly significant, we used three approaches that have been proposed for use with heterogeneous rates, NPRS (Sanderson 1997, 1998), PL (Sanderson 2002), and a Bayesian method (Kishino and Hasegawa 1989; Thorne et al. 1998; Thorne and Kishino 2002). Although a molecular clock was rejected for our data (which is typically the case in such analyses; reviewed in Sanderson et al. 2004), we calculated divergence times under a strict molecular clock, using the LF (Langley and Fitch 1974) method as implemented in the computer program r8s v. 1.6 (Sanderson 2003), for comparison with methods that attempt to accommodate rate heterogeneity. The LF method uses maximum likelihood to estimate divergence times under the assumption of rate constancy among

lineages (i.e., a molecular clock). All LF, NPRS, and PL analyses were performed on a fixed topology (Fig. 2-1) with branch lengths (for single genes and the combined gene data set) optimized under both maximum parsimony (MP) and maximum likelihood (ML) criteria using a GTR + Γ model of nucleotide substitution in PAUP* 4.0 (Swofford 2002). We used a hierarchical likelihood ratio test (hLRT) to choose the best-fitting model for our data (Felsenstein 1981; Goldman 1993).

Using NPRS, the trees with branch lengths were transformed into ultrametric trees as implemented in r8s (Sanderson 2003). To transform relative time to absolute ages, we calibrated the trees by using the minimum age of the angiosperm crown group constrained to 131.8 mya which is based on the oldest unambiguous fossils (see Soltis et al. 2002b and references therein). To compute confidence intervals for the divergence times estimated, we used the bootstrap resampling method (Baldwin and Sanderson 1996).

PL is a semi-parametric smoothing method that allows a range of modes for rate differences among lineages, from nearly clock-like behavior to a condition in which each branch is allowed its optimal substitution rate (Sanderson 2002). The PL method attempts to combine the statistical power of parametric methods with the robustness of non-parametric methods, by assigning penalties that limit rate changes between adjacent branches on a phylogeny. All PL analyses were conducted using the computer program r8s. The program r8s implements a data-driven, cross-validation procedure that systematically prunes terminals from the tree and then estimates parameters from the sub-matrix and a given smoothing value. It then predicts the data for pruned taxa using the estimated parameters. Finally, it calculates a chi-squared error associated with the difference between the predicted and observed data. The optimal smoothing level is chosen as the one that minimizes the chi-squared error (Sanderson 2002).

Bayesian methods (Kishino and Hasegawa 1989; Thorne et al. 1998; Thorne and Kishino 2002) that relax a strict molecular clock were also used to estimate divergence times using MULTIDIVTIME (available from J. Thorne, North Carolina State University). This parametric approach relaxes the assumption of a strict molecular clock with a continuous autocorrelation of substitution rates across the phylogeny and allows the use of several calibrations/time constraints.

Divergence date estimation with MULTIDIVTIME involved two steps. First, ESTBRANCHES was run to estimate branch lengths from the data and a fixed tree topology (Fig. 2-1) using the F84 (Felsenstein 1984; Kishino and Hasegawa 1989) model of sequence evolution, with rates allowed to vary among sites following a discrete gamma (Γ) distribution with four rate categories (Yang 1994) along with their variance-covariance matrix. The F84 substitution model is the most 'complicated' model currently available in this program, and it is therefore the most appropriate for comparison with the GTR model used with the other three methods. Parameter values for the F84 + Γ model were estimated using the BASEML program in PAML (Yang 2000).

Next, the outgroups (in our case, the extant gymnosperm species) were pruned from the tree, and MULTIDIVTIME was used to estimate the prior and posterior ages of branching events, their standard deviations, and the 95% credibility intervals via Markov chain Monte Carlo (MCMC). The Markov chain was run for 1,000,000 generations and sampled every 100 generations after an initial burn-in period of 10,000 cycles. To check for convergence of the MCMC, analyses were run from at least two different starting points.

Fossils assigned to Nymphaeales were reported recently by (Friis et al. 2001) and (Gandolfo et al. 2004). We attempted to reconcile our estimated divergence times with the reported ages and putative phylogenetic positions of these fossils. For this part of our study, we used PL to estimate the age of the angiosperms using each fossil as a single calibration point.

These estimated ages were then compared with the fossil record and with other recent molecular-based estimates to assess the correspondence between the reported placements of these fossils and molecular-based dates. Only PL was used for these analyses because it is favored over NPRS in simulations, and it provides similar estimates to those of the Bayesian analysis.

Dispersal-Vicariance Analysis

We analyzed the distributions of major clades and genera in Nymphaeales using DIVA (Ronquist 1996; Ronquist 1997). The program optimizes distributions for each node of the tree by favoring vicariance events and minimizing the number of assumed dispersals and extinctions (Ronquist 1997). DIVA assigns a cost to changes in distribution interpreted as extinctions or dispersals but no cost to changes interpreted as vicariance. Therefore, optimizing vicariance minimizes the cost, and those reconstructions are favored. The distributions retrieved by DIVA were compared with the estimated divergence times. Seven main areas of distribution were used: (1) North America, (2) Central America, (3) South America, (4) Europe, (5) Africa, (6) Asia, and (7) Australia. The dates estimated by the Bayesian method were used to construct a chronogram with biogeographic events indicated; although the estimated dates vary somewhat among methods, estimates obtained via both NPRS and PL fall within the 95% credibility intervals of the Bayesian estimates, indicating that all estimates are essentially equivalent.

Results

Tests of Rate Heterogeneity among Genes and Lineages

All genes, separate and combined, show significant rate heterogeneity among lineages based on likelihood ratio tests. In addition, all pairs of genes evolve at significantly different rates across this tree. Therefore, a molecular clock is rejected.

Comparison of Estimates from Different DNA Data Sets

The estimated divergence times varied considerably among genes (Table 2-2). For example, with NPRS, the estimates for node 3 (Nymphaeales) ranged from 43.9 mya (combined data set) to 61.1 mya (18S rDNA) (Table 2-2). Plastid genes generally yielded the oldest age estimates for node 2, 18S rDNA typically provided the oldest ages for all other nodes (Table 2-2). Divergence time estimates based on 18S rDNA sequences have also been older than those obtained with other genes (e.g., *rbcL, atpB*) in other studies (e.g., Soltis et al. 2002b).

Comparison of Estimates from Different Methods

Although the molecular clock was rejected for our data set, we calculated divergence times under a strict molecular clock, using the Langley-Fitch method, for comparison with the other three methods. The estimated divergence times using Langley-Fitch were generally younger than those inferred from NPRS, PL, and the Bayesian method (Table 2-2).

We used NPRS to accommodate the significant rate heterogeneity among lineages, using both MP and ML branch lengths. The ML estimates are older than the MP estimates inferred from *matK* and the combined data set (Table 2-2). In general, however, the divergence times obtained with the two branch-length optimizations were similar, mostly dating to the Eocene and Miocene, so the effects of the optimization method seem to be small.

Although none of the standard deviations are large, for the more recent divergences (e.g., nodes 8 and 9; *Nymphaea*, *Euryale*+*Victoria*) the confidence intervals inferred using MP branch lengths were considerably narrower than those computed with ML branch lengths (Table 2-2). Furthermore, in many cases the confidence intervals using MP branch lengths did not cover the estimate from the original data (Table 2-2). Considering that the estimates based on the original data using both MP and ML branch lengths were very similar across genes and that the bootstrap

confidence intervals using ML branch lengths contained the original estimates, the bootstrap analyses using MP branch lengths seem to be systematically underestimating node ages and associated variances. To our knowledge, this problem has not been reported before; in fact, previous estimates inferred from bootstrapping and the original data differ by only 2-4 million years across a time span of 50 to over 400 mya (Soltis et al. 2002). However, it may be best not to rely on standard deviations calculated with MP branch lengths and NPRS.

The estimated ages obtained from PL are usually slightly younger than those obtained from NPRS (Table 2-2). Sanderson (2002) has shown that PL outperforms NPRS in simulations. In our analyses, use of the Bayesian method provides estimated ages that are similar to those derived from PL, except for the age of crown-group Cabombaceae (node 4) as estimated using 18S rDNA data (34.6-32.6 mya (PL) vs. 17.2 mya (Bayesian); Table 2-2). The methods give similar values despite the use of different nucleotide substitution models for the NPRS and PL analyses vs. the Bayesian analysis.

Despite some differences among the three methods that attempt to account for rate heterogeneity among lineages, the estimated divergence times are very similar for most of the nodes. For example, the 95% credibility interval of the Bayesian method includes the ages estimated from both NPRS and PL. Further discussion is based on ages obtained with the Bayesian method. We also think it is reasonable that more data will give better branch length estimates, so we favor the estimated dates calculated from the combined data set.

Biogeography

The results of the DIVA analysis are shown in Figure 2-2. The ancestors of Nymphaeales were found to be distributed in the American and Eurasian continents, and the present distributional patterns require several dispersal and extinction events (Fig. 2-2).

Discussion

Inferences from Estimates of Divergence Times

The estimated divergence times indicate that crown-group Nymphaeales (node 3, Figs. 2-1, 2-2) date to the Eocene (44.6±7.9 mya); Nymphaeaceae and Cabombaceae split at that point. Extant genera of Nymphaeaceae began to diversify in the late Eocene to early Oligocene (41.1±7.7 mya; node 5, Figs. 2-1, 2-2), and the two extant genera of Cabombaceae diverged during the Miocene (19.9±5.6 mya; node 4, Figs. 2-1, 2-2). These dates for crown-group Nymphaeales are slightly younger than fossil seeds described from the Middle Eocene Allenby Formation of Princeton, British Columbia, and attributed to Nymphaeaceae (Cevallos-Ferriz and Stockey 1989). However, on the basis of the fossil seed record, Cevallos-Ferriz and Stockey (1989) inferred that Cabombaceae and Nymphaeaceae probably diverged as early as the Middle Eocene, consistent with our older estimates for the age of this divergence.

Our divergence time estimates indicate that extant Nymphaeales diversified relatively recently, whereas the stem lineage to Nymphaeales is old, based on a fossil attributed to Nymphaeales from the Early Cretaceous (125-115 mya; Friis et al. 2001) and a fossil attributed to Nymphaeaceae from the middle Cretaceous (~90 mya; Gandolfo et al. 2004). These results for Nymphaeales indicating recent diversification in an ancient lineage agree with similar findings for the basal angiosperms Chloranthaceae (Zhang and Renner 2003) and *Illicium* (Illiciaceae; (Morris et al. 2007). The fossil record indicates clearly that Chloranthaceae represent one of the oldest angiosperm lineages, with unequivocal reproductive structures resembling those of *Hedyosmum* from the Barremian-Aptian boundary, approximately 125 mya (Doyle et al. 2003; Eklund et al. 2004; Friis et al. 1994; Friis 1997; Friis et al. 1999) for recent interpretations and lists of earlier references). However, divergence time estimates based on molecular data indicate

that the extant genera of Chloranthaceae are relatively young (i.e., 60-29 mya for *Hedyosmum*, 22-11 mya for *Chloranthus*, and 18-9 mya for *Ascarina*; Zhang and Renner 2003).

Reconciling Molecular-based Age Estimates with the Fossil Record

Two putative, ancient water lily fossils are extremely important in discussions of the diversification of Nymphaeales: the water lily fossil reported by Friis et al. (2001) and *Microvictoria*, described by Gandolfo et al. (2004). To reconcile molecular-based estimates of divergence times with the fossil dates, we placed the Friis et al. (2001) and the Gandolfo et al. (2004) fossils on several different nodes of the phylogenetic tree for extant Nymphaeales (Fig. 2-1) as calibration points, and then used PL to estimate the age of the angiosperms. Friis et al. (2001) reported a water lily flower fossil that they estimated to be 125 to 115 million years old, indicating the antiquity of the Nymphaeales lineage. Although the arrangement of the carpels around a central protrusion of the floral apex is found in both some Nymphaeales and *Illicium* and despite abundant fossil seeds from the same locality attributed to "Illiciales", Friis et al. (2001) argued that the fossil belongs to Nymphaeales because of its perigynous/epigynous perianth, syncarpous gynoecium, and the presence of numerous ovules per carpel. In contrast, *Illicium* flowers are hypogynous with an apocarpous gynoecium and a single ovule per carpel. A phylogenetic analysis, based on the morphological data set of Les et al. (1999) for extant Nymphaeales, placed the fossil as sister to Nymphaeaceae; synapomorphies with extant Nymphaeaceae included a syncarpous gynoecium, a perigynous or epigynous perianth, and a central protrusion of the floral apex between the carpels (although neither of the latter two characters is found consistently throughout Nymphaeaceae) (Friis et al. 2001). Based on this analysis, plesiomorphies for Nymphaeales (shared by the fossil and Cabombaceae) seem to include a trimerous perianth, monocolpate pollen, floral parts in apparent whorls, linear stamen

filaments, and separate radiating stigmatic areas; some of these characters (monocolpate pollen, trimerous perianth, discontinuous stigmatic areas) are also shared by at least some species of *Nuphar* (Padgett et al. 1999; Schneider et al. 2003). Gandolfo et al. (2004) proposed that the Friis et al. "water lily" fossil is equally compatible with *Illicium*, as well as other angiosperm families. The homoplasy in character of hypogyny vs. perigyny/epigyny and the large number of symplesiomorphies between the fossil and Cabombaceae suggest that a reanalysis that includes taxa outside Nymphaeales may be informative regarding the placement of the fossil and the evolution of these characters.

The placement of the water lily fossil as sister to all extant Nymphaeaceae (Friis et al. 2001) makes its point of attachment to the tree older than node 5, but younger than node 3 (Fig. 2-1). Therefore, the appropriate node for calibration using this fossil is node 3. Using this calibration point, the estimated age of the angiosperms using PL is 414 mya (Table 2-3), an age that would place the origin of flowering plants shortly after the first appearance of land plants, approximately 450 mya based on the fossil record, and close to the initial radiation of tracheophytes (Kenrick and Crane 1997). However, given the homoplasy in the character that place the fossil and given its similarity in some respects to *Illicium* (Friis et al. 2001; Gandolfo et al. 2004), it is possible that the fossil should be placed on the stem lineage leading to Nymphaeales, between nodes 2 and 3. Using the Friis et al. fossil as a calibration point at node 2, which comprises all angiosperms except *Amborella*, results in a reasonable estimate for the age of the angiosperms (138.8 mya; Table 2-3). Hence, moving the fossil calibration point just one node deeper in the tree reconciles the ages inferred from the fossil record and from molecular data. This placement, on the stem lineage to Nymphaeales, suggests that the fossil may have been part of an ancient assemblage that included Nymphaeales and Austrobaileyales.

A fossil flower from the Raritan Formation of New Jersey was recently placed within Nymphaeaceae (Gandolfo et al. 2004). Based on a phylogenetic analysis of a combined morphological and molecular data set, the new fossil taxon, *Microvictoria*, was placed within the crown Nymphaeaceae, in a clade with the modern genera *Victoria, Euryale*, and *Nymphaea.* The fossil *Microvictoria* dates to the Turonian (~90 mya), and the paleoclimate of the collection site is described as subtropical to tropical, which corresponds to the climate of extant *Victoria* and *Euryale*.

We used the age of *Microvictoria* as reported by Gandolfo et al. (2004) as a calibration point and repeated the divergence time exercise described above to evaluate the correspondence of molecular- and fossil-derived ages. Based on Gandolfo et al.'s analysis, *Euryale*, *Victoria*, *Nymphaea*, and *Microvictoria* form a polytomy with 68% bootstrap support. This placement most likely corresponds to node 8. However, calibration using node 8 results in an angiosperm age estimate of 773 mya (Table 2-3). This placement of *Microvictoria* is not strongly supported. Alternative placements deeper in the tree, based on higher bootstrap values, correspond to calibration at nodes 6 and 7, but calibration at these nodes results in age estimates for the angiosperms that are also too old, 410 and 687 mya, respectively. In fact, all estimates using this fossil are too old, until the fossil is placed outside Nymphaeales. For example, the age estimate for angiosperms is 311 mya when node 3, which subtends all crown-group Nymphaeales, is the calibration point; this age corresponds more closely to the age of extant seed plants (Mapes and Rothwell 1984; Mapes and Rothwell 1991). However, if node 2, which subtends all crown-group angiosperms except *Amborella*, is the calibration point, the age estimate for the angiosperms is 104 mya, which is too young, perhaps suggesting that *Microvictoria* might be best placed along the stem lineage leading to extant Nymphaeales (as found for the Friis et al. fossil), although

such a placement is at odds with the derived placement of *Microvictoria* within Nymphaeaceae favored by Gandolfo et al. (2004). However, Gandolfo et al.'s topology disagrees with the morphological analysis of Les et al. (1999), who found strong support for *Victoria* + *Euryale*, consistent with many previous inferences of relationship in Nymphaeaceae; this sister-group relationship is not evident in Gandolfo et al.'s tree, which places *Victoria*, *Euryale*, *Nymphaea*, and *Microvictoria* in a polytomy supported by number of petals greater than five and number of stamens greater than 50. In the two shortest trees of Gandolfo et al. (2004), *Microvictoria* is alternatively placed as either sister to *Victoria* or as sister to *Victoria* + *Euryale*. The collapse of the *Victoria* + *Euryale* sister group in the strict consensus tree of Gandolfo et al. (2004) when *Microvictoria* is included must be due to the "one-to-one structural and positional correspondence" of floral organs of *Victoria* and *Microvictoria* reported by Gandolfo et al. (2004). Conflict between characters that unite *Victoria* and *Euryale* and those that unite *Victoria* and *Microvictoria* is apparently responsible for the lack of resolution in this clade, although it is not possible to discern the pattern of character support from their study.

There seem to be two possible explanations for the disparity between the fossil record for angiosperms and the age inferred here using PL and *Microvictoria*, as placed within Nymphaeaceae by Gandolfo et al. (2004) as the calibration point. Either methods of estimating divergence times from molecular data are highly dubious or the phylogenetic placement of *Microvictoria* in Gandolfo et al. (2004) may need to be reconsidered. Regarding the first possibility, several authors have reviewed the limitations of these methods (e.g., (Magallón 2004; Sanderson and Doyle 2001; Sanderson 2002; Sanderson et al. 2004; Soltis et al. 2002). However, despite these limitations, when combined data sets of multiple genes have been used, a series of recent studies has converged on similar, reasonable age estimates for the angiosperms (e.g.,

approximately130-190 mya; Magallón and Sanderson 2001; Sanderson and Doyle 2001; Soltis et al. 2002b; reviewed in Sanderson et al. 2004; Bell et al. 2005). Current evidence therefore indicates that, despite errors inherent in the process of divergence time estimation, methods that account for rate heterogeneity among lineages typically provide estimates consistent with the fossil record, at least when all relevant lineages have been sampled and multiple genes have been included. Of course, lineage-specific rate deceleration, as observed in both angiosperms (Sanderson and Doyle 2001) and across tracheophytes (Soltis et al. 2002), may also account for the anomalously old ages inferred using both the Friis et al. (2001) and Gandolfo et al. (2004) fossils as calibration points. However, no such rate deceleration was observed for Nymphaeales in our study or in broader studies of angiosperms (e.g., Zanis et al. 2002), although the apparent non-clocklike "delay" in diversification of most extant genera of Nymphaeales relative to the age of the clade may contribute to the old ages estimated using these fossils.

Alternatively, *Microvictoria* may be misplaced in the phylogenetic analysis of Gandolfo et al. (2004), perhaps due to homoplasy in the crucial morphological characters scored and included in that study. That is, a now-extinct assemblage of early angiosperms may have possessed suites of traits not found in any extant groups. Friis et al. (2000) stressed that many early angiosperm fossils exhibit character combinations unknown in extant angiosperms. *Microvictoria* may belong instead on the stem lineage to Nymphaeales, a position that could not be evaluated by Gandolfo et al. (2004) because only Nymphaeales were included in their study. The placement of early angiosperm fossils in phylogenetic trees may be extremely challenging due to the mixture of characters present in early angiosperms. However, it would be worthwhile to reassess the features of both *Microvictoria* and the Friis et al. (2001) fossil and conduct additional phylogenetic analyses that include taxa from outside Nymphaeales.

Biogeography

Nymphaeales have a worldwide distribution in tropical to temperate regions (Schneider and Williamson 1993; Williamson and Schneider 1993). The oldest putative fossil of Nymphaeales is from western Portugal (Friis et al. 2001), although caution regarding the phylogenetic placement of this fossil may be warranted (see above). The fossil records from the Northern Hemisphere reveal that *Brasenia* and *Nymphaea* exhibited great species diversity in these areas during the Miocene (Cevallos-Ferriz and Stockey 1989; Collinson 1980; Dorofeev 1973; Dorofeev 1974; Mai 1988). However, three genera (*Barclaya, Ondinea*, and *Victoria*) of Nymphaeales are not known from the fossil record, and their present distributions are restricted to small geographic areas (see below).

The results of the DIVA analysis explain the present distributional pattern of Nymphaeales with seven inferred dispersal and two extinction events (Fig. 2-2). Based on our estimated divergence times, Nymphaeales may have been widely distributed in the American continents and Eurasia during the Eocene (44.59±7.9 mya) (Fig. 2-2). This inference is consistent with the fossil record, with fossils of Nymphaeales found in the American continents and Eurasia (Anzotegui 2004; Cevallos-Ferriz and Stockey 1989; Collinson 1980; Dorofeev 1974; Knobloch and Mai 1984).

Cabombaceae were ancestrally distributed in the American continents and Eurasia. After the two genera diverged, *Cabomba* either diversified only on the American continents (Fig. 2-2) or, if it did occur in Eurasia, it subsequently became extinct there. Unfortunately, however, there is no fossil record of *Cabomba* to help infer the past distributional pattern of the genus. Two separate dispersals and an extinction event are needed to explain the present distributional pattern of *Brasenia*. The distribution of *Brasenia* in Australia can be explained by dispersal from

southeastern Asia, but the presence of the genus in Africa is interpreted as ambiguous. *Brasenia* could have dispersed from either South America or Europe to Africa. In the latter case, southward migration from Europe would have been followed by the subsequent extinction of the genus in Europe, probably during glaciation. The fossil record lends support to this hypothesis; *Brasenia* was distributed in Europe during the Eocene to Oligocene (Collinson 1980). Based on our estimated divergence times, Cabombaceae diversified during the Miocene (19.91 ± 5.6 mya), and at that time the connection between South America and Africa had already disappeared (Leys et al. 2002; Smith et al. 1994; Storey 1995), making this migration route unlikely (unless via long-distance dispersal). Davis et al. (Davis et al. 2002a; Davis et al. 2002b) provided evidence for a similar biogeographical scenario for members of Malpighiaceae with lineages occurring ancestrally in the Northern Hemisphere, but with subsequent migration to the tropics. Thus, the presence of *Brasenia* in Africa can be explained more plausibly by dispersal from Europe to Africa than by dispersal from South America to Africa.

The present distribution of Nymphaeaceae provides a more complicated pattern than that of Cabombaceae. The oldest *Nuphar* fossil is from the Paleocene of North America (Chen et al. 2004), and a fossil seed from the Early Eocene was recently reported from China (Chen et al. 2004). The fossil record therefore indicates that *Nuphar* was already widespread in North America by the Early Eocene. The present distribution of Nuphar can be explained by retention of its ancestral distribution in the Northern Hemisphere. *Nuphar* is sister to the remaining extant Nymphaeaceae. Following this divergence, four of the remaining five genera (*Barclaya*, *Ondinea*, *Euryale*, and *Victoria*) seem to have experienced range contraction or separate dispersal events, based on their current distributional patterns. At present, these four genera are distributed in small geographic areas; *Barclaya* and *Euryale* are found only in southeastern Asia, *Ondinea*

occurs only in western Australia, and *Victoria* is native to South America, especially the Amazon River basin and Paraguay.

Fossils of *Euryale* are known from Eurasia (Cevallos-Ferriz and Stockey 1989; Dorofeev 1974; Miki 1960). Thus, we can infer that the present distribution of *Euryale* might be the result of a more widespread distribution in the Northern Hemisphere followed by extinction in Europe, leaving the genus only in southeastern Asia. However, there are no fossils for *Barclaya*, *Ondinea*, or *Victoria*, so it is difficult to infer their past distributional patterns.

Nymphaea has a cosmopolitan distribution, and its present occurrence is wider than the inferred ancestral distribution of Nymphaeales. The wide distribution of *Nymphaea* can be explained by three separate dispersal events (Fig. 2-2). The ancestral area of distribution for *Nymphaea* may have been North America, Europe, and Asia, because fossils of *Nymphaea* have been reported from North America and Eurasia (Cevallos-Ferriz and Stockey 1989; Dorofeev 1974). Based on our estimated divergence times, *Nymphaea* may have experienced range expansion from North America to South America via Central America. A fossil of *Nymphaea* from the Late Miocene of Argentina (Anzotegui 2004) supports our inference of range expansion from North America to South America during the Miocene. In addition, *Nymphaea* may have also dispersed from Eurasia into Africa and Australia during the Miocene. The modern worldwide distribution of *Nymphaea* may be the result of an ability of this lineage to adapt to a wider range of temperatures than other genera of Nymphaeales.

Future Directions

Estimates of divergence times and analyses of historical biogeography rely heavily on the fossil record. In this study, we have relied on published interpretations of the placement of fossils of Nymphaeales. However, based on our attempts to reconcile fossil-based and molecular

dates, it appears that additional effort to place Nymphaealean fossils in the context of other lineages of early angiosperms is warranted. Likewise, the placement of fossils attributed to extant genera of Nymphaeales should be re-evaluated using explicit analyses of characters and taxa. The morphological matrix of Les et al. (1999) provides an outstanding starting point for synthetic analyses of fossil and extant Nymphaeales.

Table 2-1. List of taxa used in this study with GenBank accession numbers and references

Ingroup: Nymphaeales

Cabombaceae. *Brasenia schreberi* J. F. Gmelin: 18S rDNA AF206874 (Soltis et al. 1999); *rbcL* M77031 (Les et al. 1991); *matK* AF092973 (Les et al. 1999). *Cabomba caroliniana* A. Gray: 18S rDNA AF206878 (Soltis et al. 1999); *rbcL* M77027 (Les et al. 1991); *matK* AF108719 (Les et al. 1999).

Nymphaeaceae. *Barclaya longifolia* Wall.: 18S rDNA AF096692 (Les et al. 1999); *rbcL* M77028 (Les et al. 1991); *matK* AF092982 (Les et al. 1999). *Euryale ferox* Salisb.: 18S rDNA AF096694 (Les et al. 1999); *rbcL* M77035 (Les et al. 1991); *matK* AF092994 (Les et al. 1999). *Nuphar variegata* Durand: 18S rDNA AF096695 (Les et al. 1999); *rbcL* M77029 (Les et al. 1991); *matK* AF092979 (Les et al. 1999). *Nymphaea odorata* Aiton: 18S rDNA AF206973 (Soltis et al. 1999); *rbcL* M77034 (Les et al. 1991); *matK* AF092988 (Les et al. 1999). *Ondinea purpurea* den Hartog: 18S rDNA AF096697 (Les et al. 1999); *rbcL* AF102549 (Les et al. 1999); *matK* AF108722 (Les et al. 1999). *Victoria cruziana* Orb.: 18S rDNA AF096698 (Les et al. 1999); *rbcL* M77036 (Les et al. 1991). *Victoria amazonica* (Poepp.) Sowerby: *matK* AF092991 (Les et al. 1999).

Amborellales.

Amborellaceae. *Amborella trichopoda* Baill.: 18S rDNA U42497 (Soltis et al. 1997); *rbcL* L12628 (Qiu et al. 1993); *matK* AJ506156 (Goremykin et al. 2003).

Austrobaileyales.

Austrobaileyaceae. *Austrobaileya scandens* C. T. White: 18S rDNA U42503 (Soltis et al. 1997); *rbcL* L12632 (Qiu et al. 1993); *matK* AF543726 (Hilu et al. 2003).

Schisandraceae. *Illicium parviflorum* Michx. ex Vent.: 18S rDNA L75832 (Soltis et al. 1997); *rbcL* L12652 (Qiu et al. 1993). *Illicium floridanum* J. Ellis: *matK* AF543738 (Hilu et al. 2003). *Schisandra chinensis* (Turcz.) Baill.: 18S rDNA L75842 (Soltis et al. 1997). *Schisandra sphenanthera* Rehder & E.H. Wilson: *rbcL* L12665 (Qiu et al. 1993). *Schisandra rubriflora* Rehder & E.H. Wilson: *matK* AF543750 (Hilu et al. 2003).

Outgroup: Gymnosperms

Taxaceae. *Taxus mairei* (Lemee et H.Lev.) S.Y. Hu ex T.S. Liu: 18S rDNA D16445 (Chaw et al. 1993); *rbcL* AB027316 (Chaw et al. 2000); *matK* AB024001(Cheng et al. 2000). Pinaceae. *Larix leptolepis* (Siebold & Zucc.) Gordon: 18S rDNA D85294 (Chaw et al. 1997). *Larix decidua* Mill.: *rbcL* AB019826 (Wang et al. 1999). *Larix gmelini* (Rupr.) Rupr.: *matK* AF143433 (Wang et al. 2000).

Gnetaceae. *Gnetum gnemon* L.: 18S rDNA U42416 (Soltis et al. 1997); *rbcL* U72819 (Price 1996); *matK* AF542561 (Hilu et al. 2003).

Ginkgoaceae. *Ginkgo biloba* L.: 18S rDNA D16448 (Chaw et al. 1993); *rbcL* AJ235804 (Chase et al. 1993); *matK* AF456370 (Quinn et al. 2002).

Table 2-2. Divergence time estimation: Age estimates for nodes 2-9 for different age estimation methods (LF=Langley-Fitch; NPRS=nonparametric rate smoothing; PL=penalized likelihood). Bootstrap= standard deviations from bootstrap re-sampling, λ=smoothing factor. CI=Bayesian credibility intervals. All estimates based on minimum age of the angiosperm clade (node 1, Fig. 2-1) constrained to 131.8 mya (Soltis et al. 2002). All dates estimated on fixed tree (Les et al. 1999) with branch lengths for LF, NPRS, and PL optimized under maximum likelihood (ML) and maximum parsimony (MP) using a GTR+Γmodel of sequence evolution; optimization of branch lengths in Bayesian analysis under F84+Γ model

Genes	LF_{ML}	LF_{MP}	$NPRS_{ML}$	Bootstrap for $NPRS_{ML}$	$NPRS_{MP}$	Bootstrap for $NPRS_{MP}$	PL (λ)	PL_{ML}	PL (λ)	PL_{MP}	Bayesian	95% CI
18S rDNA												
2	109.2	94.6	114.6	112.4±8.9	100.7	88.1±4.9	0.001	114.5	0.010	96.1	115.5±24.3	68.9-164.2
3	52.0	54.4	60.6	61.5±8.9	61.1	71.4±5.0	0.001	58.8	0.010	52.6	62.4±18.6	32.8-105.5
4	31.2	36.0	33.0	39.4±7.6	39.2	26.1±5.0	0.001	32.5	0.010	34.6	17.2±9.2	4.5-40.4
5	36.6	44.8	46.2	48.3±11.7	53.3	41.2±7.0	0.001	45.0	0.010	43.1	54.8±18.6	28.0-94.0
6	32.6	31.9	40.7	46.2±10.4	42.5	34.6±5.6	0.001	45.0	0.010	33.1	43.3±14.9	20.1-78.9
7	22.5	22.4	36.0	38.9±7.5	33.1	22.0±4.7	0.001	34.2	0.010	23.5	35.9±13.6	15.1-67.8
8	19.5	17.0	32.0	32.2±7.8	25.8	14.5±3.3	0.001	30.8	0.010	17.8	27.6±11.8	11.0-56.8
9	15.11	12.5	27.2	28.9±9.1	17.7	10.2±2.9	0.001	25.8	0.010	13.0	19.5±10.0	5.6-44.8
rbcL												
2	111.3	104.9	115.4	115.2±7.5	107.7	83.7±4.5	0.001	112.4	0.0001	105.2	116.7±23.5	75.2-167.2
3	34.4	35.3	49.7	48.1±8.9	50.0	69.7±4.9	0.001	34.4	0.0001	35.6	42.3±15.5	20.5-80.9
4	20.7	20.7	28.7	27.5±8.0	27.7	14.4±3.3	0.001	20.6	0.0001	20.8	26.2±11.2	9.7-54.3
5	19.4	22.7	40.8	37.8±10.1	43.8	27.5±4.9	0.001	25.0	0.0001	23.1	27.1±12.2	12.1-59.4
6	15.2	17.8	33.8	34.8±8.9	36.6	21.2±4.7	0.001	19.3	0.0001	18.1	22.2±10.5	9.3-50.4
7	11.8	13.9	26.6	28.6±8.5	29.4	13.8±4.0	0.001	14.5	0.0001	14.1	17.4±8.8	6.7-41.1
8	8.9	9.0	19.9	24.5±8.1	19.5	10.2±2.7	0.001	10.6	0.0001	9.1	12.1±6.9	3.5- 30.6
9	2.8	2.9	7.2	8.8±5.4	7.1	3.8±1.4	0.001	3.1	0.0001	2.9	4.7 ±1.3	0.2-15.4
matK												
2	109.6	95.5	118.3	122.4±5.0	102.4	71.8±3.0	0.0001	116.9	0.0001	99.9	116.4±23.3	75.9-167.8
3	28.9	29.1	56.2	49.6±11.1	44.3	53.0±2.9	0.0001	50.5	0.0001	37.9	34.4±9.8	19.1-57.4
4	13.4	9.8	26.5	24.2±11.4	16.2	6.2±1.2	0.0001	24.0	0.0001	12.4	16.5±6.4	3.7-20.1
5	19.5	22.3	48.3	32.2±20.2	39.1	18.0±2.2	0.0001	43.0	0.0001	32.5	33.2±9.8	18.2-55.7
6	15.7	17.5	34.3	42.4±11.4	31.8	11.9±1.8	0.0001	32.2	0.0001	26.2	27.4±8.9	14.9-48.3
7	13.4	7.7	26.5	23.6±9.1	15.1	4.7±1.2	0.0001	26.5	0.0001	12.1	14.8±6.5	6.1-31.1

Table 2-2. Continued.

Genes	LF_{ML}	LF_{MP}	$NPRS_{ML}$	Bootstrap for $NPRS_{ML}$	$NPRS_{MP}$	Bootstrap for $NPRS_{MP}$	PL (λ)	PL_{ML}	PL (λ)	PL_{MP}	Bayesian	95% CI
8	7.5	7.2	25.7	23.6±9.1	13.6	4.1±1.0	0.0001	25.7	0.0001	11.0	11.7±5.6	4.3-25.6
9	6.2	5.9	20.5	19.1±9.0	10.4	3.1±0.8	0.0001	20.5	0.0001	8.7	8.5±4.7	2.2-20.2
Combined												
2	110.9	97.4	116.3	115.1±3.9	100.8	73.4±2.7	0.0001	116.1	0.0001	97.9	118.4±5.2	107.5-127.9
3	33.7	34.7	46.9	45.3±4.6	43.9	55.8±2.7	0.0001	45.8	0.0001	37.6	44.6±7.9	29.5-60.7
4	18.6	16.3	24.8	23.9±3.1	19.9	9.5±1.2	0.0001	24.0	0.0001	16.4	19.9±5.6	11.0-32.9
5	27.8	26.5	44.6	37.2±9.7	37.9	20.4±2.8	0.0001	43.4	0.0001	31.3	41.1±7.7	26.8-56.9
6	21.3	20.3	37.3	36.1±4.3	30.1	14.5±2.3	0.0001	36.2	0.0001	24.6	34.7±7.3	21.5-50.0
7	11.8	11.8	23.0	23.0±4.4	18.4	6.8±1.6	0.0001	22.3	0.0001	14.7	23.3±6.2	13.0-37.1
8	10.5	9.4	20.2	20.7±4.6	14.0	5.1±1.1	0.0001	19.7	0.0001	11.5	20.4±5.8	11.0-33.2
9	7.6	6.7	13.8	14.8±4.3	8.9	3.2±0.8	0.0001	13.4	0.0001	7.8	14.5±4.8	7.2-25.5

Table 2-3. Effect of fossil placement. Inferred age (in millions of years) of the angiosperms if each of two described fossils is used as a calibration point at that node. All age estimates were computed using the penalized likelihood method (Sanderson 2002). Node numbers are given in Figure 2-1

Constrained node	Inferred age of angiosperms (Gandolfo et al. ~90 mya)	Inferred age of angiosperms (Friis et al. ~ 120 mya)
2	104.1	138.9
3	310.9	414.5
4	603.2	804.2
5	334.9	446.5
6	410.3	547.1
7	686.8	915.8
8	772.7	1030.3
9	1093.0	1457.3

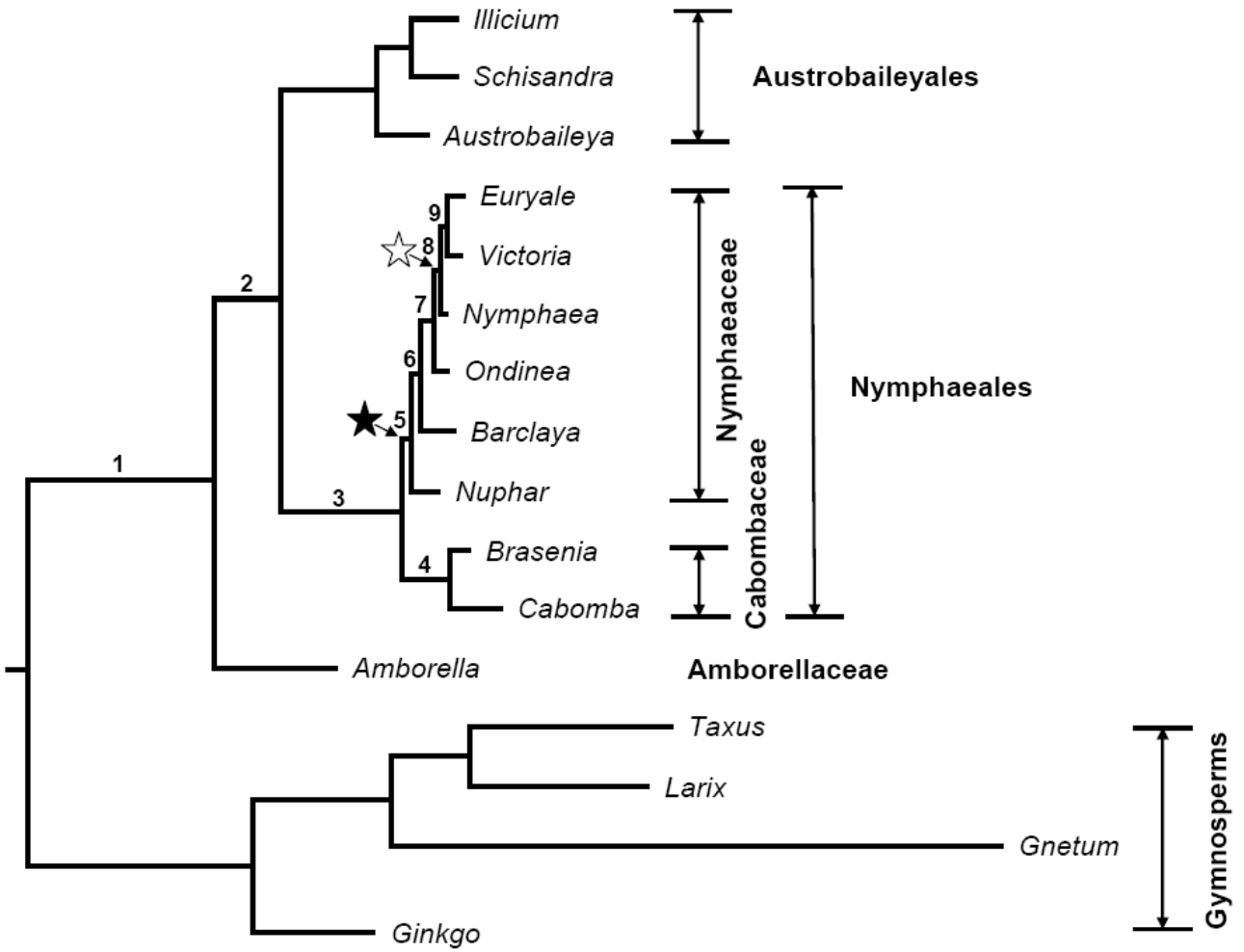


Figure 2-1. Phylogenetic tree of Nymphaeales from Les et al. (1999), with *Amborella*, Austrobaileyales, and gymnosperm outgroups added, based on many recent analyses (see text). Phylogram depicts branch lengths based on the combined data set of 18S rDNA, *rbcL*, and *matK* optimized via maximum parsimony. Numbers signify nodes for which divergence times were estimated. Solid star on branch leading to node 5 indicates the preferred placement of the Friis et al. (2001) fossil by those authors, and open star on branch leading to node 8 represents the preferred position of *Microvictoria* by Gandolfo et al. (2004).

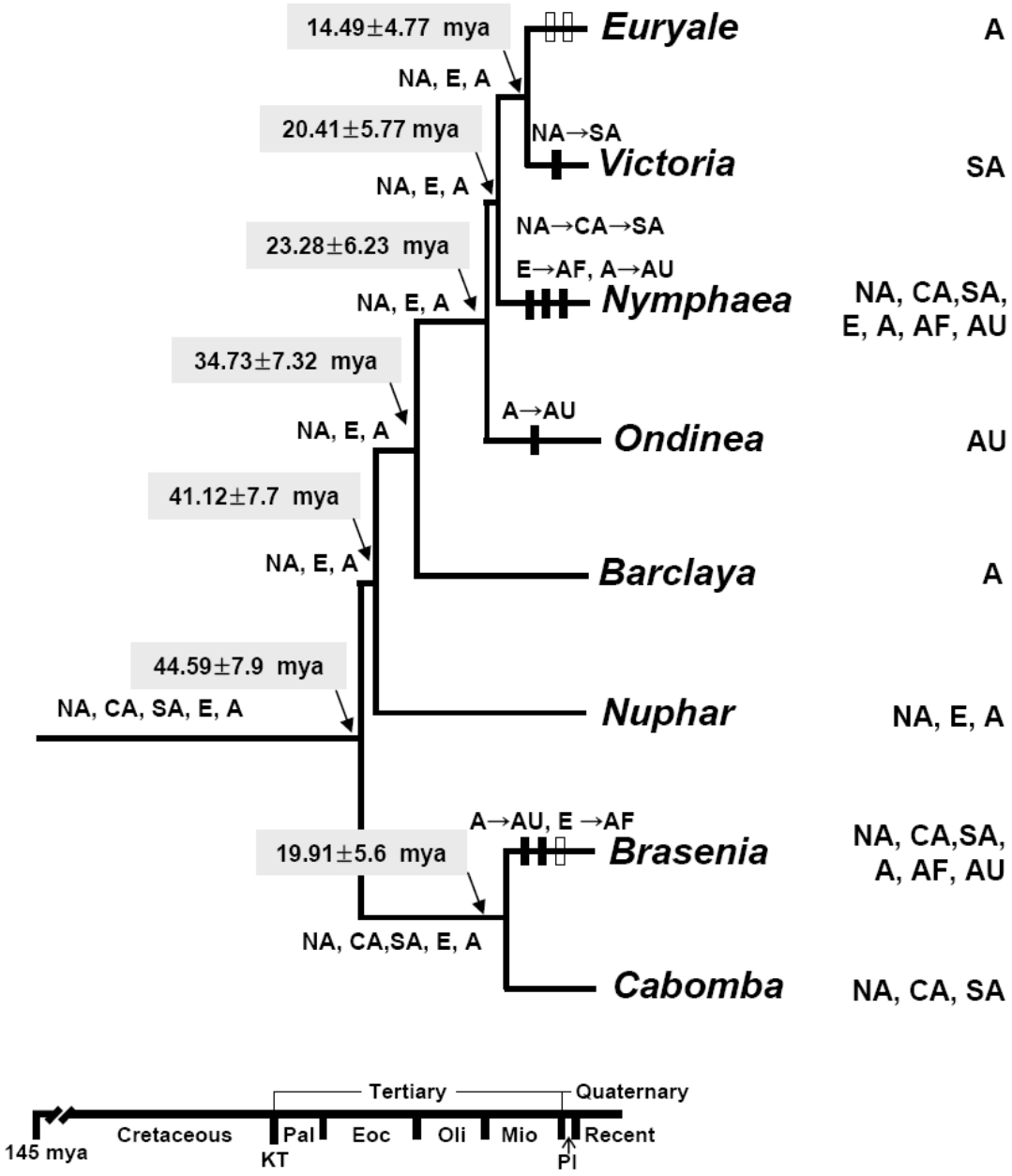


Figure 2-2. Chronogram for Nymphaeales showing timing of inferred divergences and biogeographic events. Closed bars represent dispersal events, and open bars indicate extinction events. Present distributions are given as NA=North America, SA=South America, CA=Central America, E=Europe, A=Asia, AF=Africa, AU=Australia. Divergence time estimates for each node are from the Bayesian analysis, based on the combined data set of 18S rDNA, *rbcL*, and *matK* (Table 2-2). Scale at bottom indicates geological time scale: Pal = Paleocene, Eoc = Eocene, Oli = Oligocene, Mio = Miocene, Pl = Pliocene, KT = the Cretaceous-Tertiary boundary.

CHAPTER 3
COMPARATIVE STUDIES OF FLORAL DEVELOPMENT IN NYMPHAEALES

Introduction

The molecular genetics of floral development is well established for model eudicot plants such as *Arabidopsis* (Coen and Meyerowitz 1991; Ma and dePamphilis 2000; Smyth et al. 1990; Theissen et al. 2000; Weigel 1995; Zhao et al. 2001). The well-known ABCDE model, based on mutant studies in *Arabidopsis*, explains floral organ formation by combinatorial action of spatially specific transcription factors. According to this model, A and E class genes control sepal identity; A, B, and E class genes control petal identity; B, C, and E class genes control stamen identity; C and E class genes control carpel identity; D and E class genes control ovule identity (Coen and Meyerowitz 1991; Colombo et al. 1995; Pelaz et al. 2000; Theissen 2001a). Many features of the ABCDE model are conserved across angiosperms (i.e., *Petunia hybrida* (Angenent et al. 1993); *Silene latifolia* (Hardenack et al. 1994); *Gerbera hybrida* (Yu et al. 1999); *Oryza sativa* (Fornara et al. 2003; Kater et al. 2006; Kyozuka et al. 2000); *Pisum sativum* (Taylor et al. 2002); *Zea mays* (Whipple et al. 2004); *Magnolia grandiflora* (Kim et al. 2005); *Persea americana* (Chanderbali et al. 2006), *Akebia trifoliata* (Shan et al. 2006); *Elaeis guineensis* (Adam et al. 2007); *Taihangia rupestris* (Lü et al. 2007); *Vitis vinifera* (Poupin et al. 2007).

Buzgo et al. (2004a) suggested that comparative studies of developmental morphology and genetics will be required to build up a new set of model plants for floral genetics outside of those organisms studied to date, most of which are eudicots. However, so far these have been few studies involving basal angiosperms. Noteworthy studies include investigation of a basal eudicot, *Eschscholzia californica* (Becker et al. 2005), basal angiosperms, *Amborella trichopoda* (Buzgo et al. 2004), and a magnoliid, *Persea americana* (Buzgo et al. 2007). Additional comparative studies throughout angiosperms are needed for a comprehensive comparative floral developmental paradigm.

Nymphaeales are the sister to all extant angiosperms except *Amborella* (Borsch et al. 2005; Hilu et al. 2003; Jansen et al. 2007; Löhne and Borsch 2005; Leebens-Mack et al. 2005; Mathews and Donoghue 1999; Moore et al. 2007; Qiu et al. 1999; Qiu et al. 2005; Soltis et al. 1999; Soltis et al. 2000; Soltis et al. 2005; Zanis et al. 2002; Zanis et al. 2003). They exhibit distinct floral morphologies from those of the other basalmost angiosperms (i.e., *Amborella*, Austrobaileyales) in several features, including the presence of large flowers in some members (i.e., *Nuphar*, *Nymphaea*, *Euryale*, and *Victoria*) rather than small or moderate-sized flowers, whorled rather than spiral phyllotaxis, eudicot-like perianth differentiation (as in *Cabomba* and *Nuphar*), and the occurrence of blue perianth organs containing anthocyanins, which are absent in other basal angiosperms (Endress 2001; Schneider et al. 2003). Thus, studying floral development in Nymphaeales will help elucidate floral evolution in early-diverging angiosperms, and thus provide insight into the early diversification of the flower.

There have been several previous floral developmental studies in Nymphaeales (Cutter 1957a, b, 1961; Endress 2001; Moseley 1958, 1961, 1965, 1972; Moseley et al. 1993; Schneider et al. 2003; Tucker and Douglas 1996). For example, Cutter (1957a, b, 1959, 1961) mainly focused on early floral development in *Nuphar*, with an emphasis on the scale-appendage. She considered that organ to be a sepal because it is formed by the young primordium in the same position as a sepal of *Nymphaea*, although Moseley (1972) argued for its interpretation as a bract. However, most of these earlier works focused only on specific genera, such as *Nymphaea* and *Nuphar* (Cutter 1957a, b, 1959, 1961; Moseley 1961, 1965, 1972), on specific developmental stages or organs, or on morphological features (Ito 1983, 1984, 1986; Khanna 1964, 1967; Moseley 1958; Moseley et al. 1984; Moseley et al. 1993; Osborn and Schneider 1988; Prance and Arias 1975; Schneider 1976; Schneider and Moore 1977; Schneider and Jeter 1982; Schneider 1983; Williamson and Moseley 1989; Williamson

and Schneider 1994). Recently, Endress (2001) examined floral development of extant basal angiosperms, and he included three species from Nymphaeales: *Cabomba furcata*, *Nuphar advena*, and *Victoria cruziana*. However, the most extensive study of flower development in Nymphaeales is that of Schneider et al. (2003). They investigated developmental stages from flower initiation or sepal initiation to carpel formation across all genera of water lilies except *Barclaya* and Hydatellaceae, the latter only recently placed in Nymphaeales based on molecular phylogenetics (Saarela et al. 2007). However, Schneider et al. (2003) did not examine developmental stages after carpel formation. Furthermore, they did not align the developmental sequences among genera; recommendations for standardizing descriptions of developmental sequences to allow for alignment among species were presented by Buzgo et al. (2004a), after Schneider et al.'s (2003) study.

In this study, I describe the floral developmental series of three species of Nymphaeales, *Cabomba caroliniana* A. Gray, *Nuphar advena* Aiton, and *Nymphaea odorata* Aiton. First, I completed examination of the floral developmental series of all three species. Early developmental stages are well-documented for these three species (Endress 2001; Schneider et al. 2003; Tucker and Douglas 1996), so I focused on later developmental stages from carpel formation to anthesis, and I then aligned the floral developmental series according to Buzgo et al. (2004a). I then compared the aligned stages for these three species to each other and to other angiosperms.

In addition, I examined the third whorl of *Nuphar advena* in detail. Organs in the third whorl were traditionally considered "petals" based on the presence of nectaries (e.g., Crow and Hellquist 2000; Endress 2001; Moseley 1965, 1972; Padgett et al. 1999; Schneider and Williamson 1993; Schneider et al. 2003; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994), while some authors interpreted these organs as "staminodes" based on their stamen-like appearance (Judd et al. 2002; Kim et al. 2002; Warner et al. 2008). To try to

elucidate whether these organs are “petals” or “staminodes”, I observed their development in detail and compared it with that of stamens. In this chapter, I used the term “petals” to refer to the third-whorl organs.

The results of this study were used in several subsequent studies, for example, floral gene expression profiles at the level of specific genes (i.e., floral organ identity genes in chapter 4) and of the entire transcriptome (chapter 5).

Materials and Methods

Floral buds at various developmental stages ranging from flower initiation to prior to anthesis were collected from the following taxa: *Cabomba caroliniana*, plants purchased from a local aquarium store, Gainesville, FL, USA (Yoo 10020, FLAS); *Nuphar advena*, in Waccassasa River, near Waccassasa Preserve Area, Levy Co., FL, USA (Yoo & Koh 1000, FLAS); *Nymphaea odorata*, in Waccassasa Preserve Area, Levy Co., FL, USA (Yoo & Koh 1001, FLAS); and *Nymphaea capensis* Thunb., plants purchased from a local aquarium store, Gainesville, FL, USA (Yoo 10021, FLAS). Samples were fixed in FAA (formalin, acetic acid, alcohol) and then transferred and stored in 70% ethanol.

Studies were performed by scanning electron microscopy (SEM) and serial microtome sectioning. For SEM, samples were dehydrated, critical-point dried, gold sputtered, and examined with a Hitachi S-4000 FE-SEM with an acceleration of 4.0 kV at the University of Florida’s ICBR Electron Microscopy Laboratory. For the serial sections, samples were dehydrated in a series of alcohol, and then transferred to xylene and embedded in paraplast. Samples were sectioned to a thickness of 5 μm to 20 μm using a rotary microtome. Sectioned samples were stained with Sass’s safranin-fast green or toluidine blue O (pH < 5) (Ruzin 1999).

To facilitate comparison among the taxa surveyed, I employed the developmental landmarks for alignment of floral developmental stages proposed by Buzgo et al. (2004a).

Stages 1 to 5 were well described from previous studies for all three species investigated here (Cutter 1957b; Endress 2001; Moseley 1961, 1972; Schneider et al. 2003; Tucker and Douglas 1996), so I mainly focused on later developmental stages although I included some early developmental stages to ensure overlap.

Results

Developmental Stages

Flower development in the three species investigated here were aligned according to the developmental landmarks proposed by Buzgo et al. (2004a) and generally proceeds through 10 developmental stages in the same order (Table 3-1). Description of floral developmental stages of each species is followed below.

***Cabomba caroliniana*:** The flower of *Cabomba caroliniana* consists of four trimerous whorls: three sepals, three petals, six stamens, and three carpels. Early developmental features of *Cabomba* were well studied by Moseley et al. (1984), Tucker and Douglas (1996), Endress (2001), and Schneider et al. (2003), so I only focused on developmental features from carpel initiation to anthesis. At stage 5 (carpel initiation), carpel primordia arise from a flat apical meristem (Fig. 3-1*A*) and become cylinder-shaped (Fig. 3-1*B*). Later, carpels become ascidiate at stage 6, at which time microsporangia initiate at the abaxial side of the stamen (Fig. 3-1*C*). At this stage, the anther locule is filled with sporogenous tissue (Fig. 3-1*D*). Just after this, ovules initiate along the dorsal wall of the locule (stage 7: Fig. 3-1*E*), and their development continues until megaspores have formed (Fig. 3-1 *H*, *J*). After ovule initiation, male meiosis occurs within the anthers (stage 8): the microspore mother cell and tetrads were observed in Fig. 3-1 *F* and *G*, respectively. At stage 9 (female meiosis), megaspores are formed (Fig. 3-1*H*, *I*), and nuclei were observed within the ovule (Fig. 3-1*J*). Anthesis occurs over two consecutive days: during the first day the flower is pistillate, and

the stigma is receptive (Fig. 3-1*L*), and in the second day the flower is staminate, and the stamens are dehiscent (Fig. 3-1*M*).

***Nuphar advena*:** The flower of *Nuphar advena* has a single bract, six sepals in two whorls, 16 to 18 petals in two whorls, numerous stamens, and many carpels. Although many researchers had studied floral development in *Nuphar*, most focused on various features, such as bracts and flower initiation (Cutter 1957a, b), and early floral development (Endress 2001; Moseley 1965, 1972; Schneider et al. 2003). I included several early developmental stages. At stage 2 (initiation of sepals), six sepal primordia are initiated in two whorls; three of them occurred in the first whorl, and the other three sepals in the second whorl. There is a long plastochron between initiation of the third and fourth sepals, so at this stage a big size difference between sepals was observed (Fig. 3-2*A*). At stage 3, petal primordia are initiated in the third whorl (Fig. 3-2*B*), and two trimerous whorls of petals appear at stage 5 (carpel initiation; Fig. 3-2*H* and Fig. 3-4*D*). Stamen primordia are initiated in the fourth whorls, and later they showed orthostichies (Fig. 3-2*B*-*E*). The carpel primordia broaden at stage 5 (Fig. 3-2F), and later carpellary ridges are formed and deepen (Fig. 3-2*G*, *H*). Next, microsporangia are formed on the adaxial surface of stamens (Fig. 3-2*I*, *J*), and ovules are initiated along the lateral and dorsal walls of carpel locules (Fig. 3-2*K*). At stage 8, male meiosis initiates; the pollen sac is filled with sporogenous tissues (Fig. 3-2*L*). Female meiosis occurs at stage 9, at which point nuclei are observed within ovules (Fig. 3-2*N*, *O*). As in *Cabomba caroliniana*, anthesis occurs over two consecutive days: pistillate flower in the first day, and staminate flower in the second day.

***Nymphaea odorata*:** The flower of *Nymphaea odorata* consists of several tetramerous whorls; four sepals, 28 petals, numerous stamens, and many carpels. The general developmental features of *Nymphaea* were studies by Moseley (1961) and Schneider et al. (2003). After sepal initiation, petal primordia are initiated alternating with the first four

perianth members, which are sepals (Fig. 3-3*A*). At stage 4, stamen primorida are initiated along the dome-shaped apical meristem, but the central region is flat (Fig. 3-3*B*). Later, the apical meristem becomes depressed, and along the peripheral region of this central region more stamens are formed (Fig. 3-3*C*). At a later stage, the central area bulges, and it becomes a floral apex (Fig. 3-3*D*). When a visible floral apex has formed, carpel primorida are initiated at the peripheral region (Fig. 3-3*E*). At stage 6, microsporangia are formed at the adaxial surface of the stamens, and a floral apex becomes a ball-shaped structure (Fig. 3-3*F-H*). Also, at this time, carpellary locules are formed (Fig. 3-3*G*), and later ovules initiate along the lateral and dorsal walls of those locules (Fig. 3-3*I*). After ovule initiation, male meiosis occurs; the pollen sac is filled with sporogenous tissue (Fig. 3-3*J*). At stage 9, female meiosis initiates; ovules are anatropous, and at this time the megaspore is formed in each ovule (Fig. 3-3*K*, *L*). Anthesis also occurs over two consecutive days, and it shows the same patterns as those observed in *Cabomba* and *Nuphar*.

Developmental Features of the Third-Whorled Organs of *Nuphar advena*

At anthesis, there are 16 to 18 stamen-like organs or "petals" in the third whorl between two perianth whorls and stamens of *Nuphar advena*. In fact, they are developed from the third, fourth, and/or fifth whorls of a floral bud in early developmental stages (see below). However, later in development, they are densely packed in a limited space, so they appear to be present in the third whorl at anthsis. In this chapter, I refer thses third-whorl organs to "petals".

"Petal" primordia arise at the third whorl (Fig. 3-4*A*), and they are much larger than stamen primordia at the stage of stamen initiation. At stage 4, the petal primordia broaden and become flat (Fig. 3-4*B*), and at the stage of carpel initiations (stage 5) a tip of the stamen primordia is projected and petal primordia become perianth-like structures (Fig. 3-4*C*). There seem to be two whorls of petal primordia (Fig. 3-4*D*); however, at maturity, petals appear to

occurr in the third whorl (Fig. 3-4*E*). 16 to 18 petals are observed at anthesis (Fig. 3-4*F*). At stage 9 (female meiosis), stamens have four microsporangia, and petals have nectaries on the abaxial side (Fig. 3-4*E*). Stamens have a ridge in the middle of the abaxial side (Fig. 3-4*G*, *H*), but petals do not have such a structure (Fig. 3-4*I*-*O*). There are also intermediate forms of petal-like structures which have characteristics of petals as well as stamens (Fig. 3-4*I*-*N*). They have sterile or fertile microsporangia on the adaxial surface, but no ridges on the abaxial side, and generally they do not have nectaries on the abaxial surface like normal petals (Fig. 3-4*I*-*L*). However, there are some petals with sterile microsporangia on the adaxial surface and nectaries on the abaxial side (Fig. 3-4*M*). At stage 9, stamens have four fertile microsporangia, and the stamen tip is projected (Fig. 3-4*N*). However, a ridge on the abaxial side of stamens is very weakly developed (Fig. 3-4*O*). At anthesis, mature petals have two weak ridges on the adaxial side (Fig. 3-4*P*), and the stamens are now dehiscent (Fig. 3-4*Q*).

Discussion

Comparison of Developmental Stages in Three Species of Nymphaeales

Flower development in the three species investigated here, *Cabomba caroliniana*, *Nuphar advena*, and *Nymphaea odorata*, proceed through 10 developmental stages in the same order (Table 3-1). However, variation occurs among species in several stages. At stage 1 (flower initiation) in *Nuphar* and *Nymphaea*, floral buds arise as lateral shoots on the rhizome, so they replace a leaf in the ontogenetic spiral (Cutter 1957a, b; Schneider et al. 2003). However, in *Cabomba*, flowers are axillary to floating leaves, which have decussate phyllotaxy if two floating leaves are present. Generally, the next flower occurs proximally and distantly against the previous flower.

All three species exhibit acropetal organogeny. Two or more whorls of perianth initiate in a similar way in all three species. In *Cabomba*, the trimerous whorl of sepals emerges

simultaneously, and subsequently a trimerous whorl of petals initiates in alternate position to the sepals. *Nuphar* displays similar developmental features to *Cabomba*; a trimerous whorl of sepals initiates, although there is a time lapse among the first three sepals (they are not simultaneous), and then after a long plastochron, another trimerous whorl of sepals emerges in alternate position to the previous sepal primordia. In contrast, *Nymphaea* exhibits a different sequence of sepal initiation although it also has whorled phyllotaxy. The four sepals are initiated in a unidirectional sequence; first, the abaxial sepal, next the two lateral sepals, and last, the adaxial sepal. Next, the first four petal primordia occur alternating with the first four sepals, and two additional petal whorls form. Finally, in the fifth and sixth whorl of *N. odorata*, eight petal primordia initiate in alternating position to the previous petal primordia, resulting in many petals in *Nymphaea*.

The difference among the three species studied is clear at stage 4 (initiation of stamens). In *Cabomba*, six stamen primordia form in alternate position with petal primordia. *Nuphar* and *Nymphaea* have numerous stamens, so they have a bigger apical meristem. In *Nuphar*, the central region becomes dome-shaped, so many stamen primordia are formed along the peripheral region. *Nymphaea* also has a bulging apical meristem at stage 4, but it becomes depressed as stamen primordia are formed. After stamen primordia formation, the central area gets swollen again and becomes the floral apex. Around this floral apex, carpel primordia initiate. The main developmental difference at this point between *Nuphar* and *Nymphaea* is that the floral apex (central area) becomes the carpels in *Nuphar*, while only the peripheral region gives rise to carpel primordia in *Nymphaea*. The central area of *Nymphaea* becomes the apical residuum, and this is considered a derived character for members of Nymphaeaceae, such as *Ondinea*, *Victoria*, and *Euryale* (Schneider et al. 2003). In *Cabomba*, a trimerous whorl of carpel primordia initiates in alternate position to three of the paired stamens.

At stage 6, microsporangia initiate on the abaxial (*Cabomba*) or adaxial (*Nuphar* and *Nymphaea*) side of the stamens. All three species show a similarity in ovule initiation; ovules initiate along the dorsal (*Cabomba*) or dorsal/lateral (*Nuphar* and *Nymphaea*) walls of the locules. After ovule initiation, male meiosis initiates. At stage 9, ovules are anatropous in all three species, and the megaspore is observed. At anthesis, they all have protogynous flowers, so the flower is pistillate on the first day and is staminate on the second day.

From comparison of developmental stages in all three species, sometimes *Nuphar* exhibits *Nymphaea*-like or *Cabomba*-like features. For example, *Nuphar* exhibits *Nymphaea*-like characteristics; in flower initiation of both species, the floral primordia replace a leaf in a phyllotactic spiral. Also, the stamen primordia of *Nuphar* are initiated along the peripheral region of the central area as those of *Nymphaea* are formed around the periphery of the central area. *Nuphar* and *Nymphaea* also exhibit similar microsporangia initiation; four microsporangia initiate on the adaxial surface of the stamens. Finally, ovules of *Nuphar* and *Nymphaea* initiate along the lateral and dorsal walls of locules, which is due to their syncarpous carpels. However, *Nuphar* also exhibits *Cabomba*-like developmental features. First of all, initiation of sepals and petals occurs in the same way in *Nuphar* and *Cabomba*; a trimerous whorl of sepals emerges simultaneously (*Cabomba*) or subsequently (*Nuphar*), and another trimerous whorl of petals (or sepals in *Nuphar*) initiates in an alternate position with the first three sepals. Next, six stamen or petal primordia arise in the third whorl of *Cabomba* and *Nuphar*, respectively, in a double position against the previous petal or sepal primodia. These developmental features of *Nuphar* compared to *Cabomba* and *Nymphaea* may be explainable by the phylogenetic position of *Nuphar*. Nymphaeaceae and Cabombaceae are sister families that share many features, including vegetative, reproductive, and molecular characters (Borsch et al. 2007, 2008; Löhne et al. 2007; Les et al. 1999). Within Nymphaeaceae, *Nuphar* is sister to all remaining genera within Nymphaeaceae (Borsch et al.

2007, 2008; Löhne et al. 2007; Les et al. 1999). Although *Nuphar* shares several morphological features with other members of Nymphaeaceae, *Nuphar* also shares some morphological characteristics with *Cabomba*, such as, anasulcate pollen, absence of staminodes (although its absence is doubtable), separate or discontinuous stigmatic surface, and flower maturation above water (Ito 1987; Les et al. 1999). Based on these characteristics, several authors have suggested that Nupharaceae be recognized as a separate family (Kerner von Marilaun 1891; Nakai 1943; Takhtajan 1997). However, despite of these morphological traits, its phylogenetic position as as sister to other Nymphaeaceae is strongly supported by three genome analyses (Borsch et al. 2008). Therefore, sharing developmental features of *Nuphar* with *Cabomba* may reflect ancestral state for core Nymphaeales.

"Outer and Inner Tepals" or "Sepals and Petals"

Although Endress (2008) reviewed the perianth structure of *Cabomba* and *Nuphar*, here I focused on whether perianth members of the three study species are differentiated into sepals and petals. In fact, there has long been a question as to whether the outer sepaloid and inner petaloid tepals of these water lilies are homologous to the sepals and petals of eudicots, respectively. However, most researchers use the term outer and inner tepals instead of sepals and petals for basal angiosperms because of only slight morphological differentiation between them and their spiral arrangement. For Nymphaeales, many authors have used the terms sepals and petals for the outer and inner tepals, respectively, based on their position and color difference. In this study, I used term sepals and petals because the two whorls are somewhat differentiated from each other (see Warner et al. 2008).

In *Cabomba*, following their simultaneous appearance, sepal primordia continue their development, so at stage 8 (male meiosis), they enclose a bud. In contrast, petal primordia remain very small and do not expand until just prior to anthesis. In addition to this developmental retardation in petals, petals are easily distinguished from sepals by having

auricular-shaped nectaries at the base. However, both sepals and petals in *Cabomba* have a single vascular trace (Ito 1986; Moseley et al. 1984), similar sheath-like texture, and similar color. Therefore, the two organs are not easily distinguishable except for developmental features and the presence of nectaries. Such undifferentiated perianth members are common in monocots.

In *Nuphar*, two whorls of sepals initiate, and they are much bigger than the petal primordia. Endress (2001) pointed out that petals of *Nuphar* are retarded in development relative to the sepals and stamens. However, I could not observe any retardation in petal development in *Nuphar* in this study. Petal primordia are initiated after sepal formation, and they continue their development. I can see broad and spatulate petals at stage 4 (stamen initiation), and some petals become thicker and larger than stamens at stage 5 (carpel initiation). As a result, in *Nuphar*, petals are different from sepals with regard to development. Also, the six sepals have three vascular traces, while petals and stamens each have a single trace (Moseley 1958). Therefore, considering that there are no members in Nymphaeales having two whorls of sepals, and the similarity of petals to stamens (see below), the petals of *Nuphar* may not be homologous to petals of other Nymphaeales. Alternatively, the first three perianth parts are sepals, and the next three perianth members may be petals.

In *Nymphaea*, four sepal primordia are initiated in the first whorl and develop fast, so at stage 4 (initiation of stamens), they are much bigger than petal primordia. Petal primordia also develop continuously, so at the later stage of stamen initiation they consist of several whorls (or layers) of petals. Therefore, there is no distinctive difference between sepals and petals in *Nymphaea* except for their position and color. Also, these organs all have three vascular traces (Moseley 1961).

Sepals are generally differentiated from petals in many ways; their position (1st whorl for sepals vs. 2nd whorl for petals), function (protection in sepals vs. attraction of pollinators

in petals), development (spiral initiation of sepals vs. simultaneous initiation of petals), vascular system (three traces in sepals vs. one trace in petals), color (greenish sepals vs. colorful petals), epidermal cell type (flat in sepals vs. conical in petals), and general similarity (foliage leaves vs. stamens) (reviewed in Albert et al. 1998 and Warner et al. in press). However, these characteristics are found in eudicots, which have bipartite perianth members. Based on the features above, perianth members of Nymphaeales are distinguished by a limited number of traits. Thus, sepals and petals may not be appropriate terms for the perianth in Nymphaeales even though perianth members are differentiated to some degree.

Identity of the Third-Whorled Organs of *Nuphar advena*: Petals or Staminodes

Petals of *Nuphar* can be easily distinguished from stamens by their position (occurrence at the third whorl) and size (much wider than stamens). However, the outer appearance of petals of *Nuphar advena* is very similar to those of stamens, although there are several differences. Petals are oblong to spatulate with truncate tip, and they have nectaries on the abaxial surface, while stamens are oblong with projected tip with two pairs of microsporangia on the adaxial surface. They also share developmental features. Petal primordia arise alternately with the first three sepal primordia in double position (Endress 2001). Thus, the first six petals are present in the third whorl at stages 2-5. Next, nine petal primordia formed in the fourth whorl, but these two whorls are relatively indistinctive after stage 5 as their size increases. Subsequently, in the very next whorl, stamen primordia initiate. I observed 16 to 18 petals in flowers from the Waccassasa River population. However, from those two whorls of petal primordia only 15 petals can be formed. Our results from serial sectioning showed that one to three stamens from the outermost stamen primordia convert into petals, perhaps due to their position. These stamens are located next to the petal whorls, and sometimes extra space is present between petal primordia. Therefore, some stamen primordia can be placed at that position, and they can become petals. In this case, they have

sterile or fertile microsporangia on the adaxial surface, but do not have nectaries on the adaxial surface, although there are exceptional cases (see below).

Petals and stamens initiate in different whorls; petals from the third and fourth whorls, and stamens from the fifth whorl to the more inner whorls. Also, the primordia of stamens and petals are different in shape as well as position. Petal primordia become flat and scale-like structures as stages proceed (Fig. 3-4), while stamen primordia are dome-shaped and the apices of primordia become projected (sterile appendages) (Padgett 2007). At stage 8, petals have nectaries on the abaxial surface and two ridges on the adaxial surface. At the same stage, stamens have well-developed microsporangia on the adaxial surface and a conspicuous ridge on the abaxial surface. I observed intermediate forms of petals, which have microsporangia on the adaxial surface, ranging from sterile (or aborted) to fertile (fully developed). Those petals are relatively small and present in the borders between petals and stamens. Also, they do not have a ridge on the abaxial surface nor nectaries. At anthesis, the number of petals ranges from 16 to 18, and sometimes there are intermediate forms between stamens and petals in petal whorls.

In Nymphaeales, perianth members from the first and second whorls (sepals and petals, respectively) are similar in shape and size. For example, *Cabomba* has two petaloid whorls (similar to monocots), and *Nymphaea* has four greenish sepals and many petals. However, only *Nuphar* has been regarded as having perianth members of different size; petals are very small compared to sepals. In addition, sepals occur in the first two whorls, resulting in presence of petals in the third whorls. In contrast to the presence of staminodes in Nymphaeaceae, but not Cabombaceae, Moseley (1958) noted that *Nuphar* does not have staminodes because there is no morphological gradual transition from stamens to petals.

To define the identity of the organs at the third whorl, we have to think of three categories of homology; historical homology, positional homology, and process homology

(Albert et al. 1998). First of all, these organs of *Nuphar* are positionally homologous to stamens of other members of Nymphaeales, particularly in having a small number of perianth organs, as do *Cabomba*, *Brasenia*, and *Ondinea*. Considering the important phylogenetic position of *Barclaya* as subsequent sister to all other Nymphaeaceae after *Nuphar*, developmental data for *Barclaya* is required to elucidate this problem (Borsch et al. 2007; Borsch et al. 2008; Löhne et al. 2007; Les et al. 1999). Also, when we examine the developmental features of the organs in the third whorls, they are much more similar to stamens, not to the organs of the first two whorls. In addition, developmental evidence presented here also suggests that intermediate forms between stamens and petals at the third whorls might have originated from stamens, although there is no gradual transition from stamens to these organs.

There is controversy in the definition of a staminode, but aborted stamens, whatever their structures are, should obviously be referred to as staminodes (Ronse De Craene and Smets 2001). According to this view, the organs in the third whorl of *Nuphar* should be considered staminodes because some of them show reminiscent characteristics of stamens, for example, sterile or fertile microsporangia on the abaxial surface. In addition, their position between two whorls of perianths and stamens, and similar developmental features to stamens further support this idea. However, detailed investigation of staminodes from other members of Nymphaeales is needed to assess whether they are all developmentally and historically homologous. Also, comparison of gene expression patterns of these organs with those of stamens will be helpful to clarify this problem.

Table 3-1. Stage alignment using descriptions of floral developmental stages for three taxa

Stage, developmental landmark	*Cabomba caroliniana*	*Nuphar advena*	*Nymphaea odorata*
1. Flower initiation	[1]Flowers are axillary to floating leaves, which have decussate phyllotaxy (0.17 mm)	[4, 5]Flowers appear as lateral shoots on the rhizome (0.2-0.3 mm)	[3]Flowers appear as lateral shoots on the rhizome (0.09-0.18 mm)
2. Initiation of bract & sepals	[2]A trimerous whorl of sepals emerges simultaneously (0.17 mm in *C. furcata*)	[2, 4, 5]A long plastochron between the initiation of the third and fourth organs (0.28-0.7 mm)	Four calyx members are initiated in unidirectional sequence: first, the abaxial sepal; next the two later sepals; and last the adaxial sepal
3. Initiation of petals	[1, 3]A trimerous whorl of petals initiates in alternate positions to sepals (0.14-0.21 mm)	[2, 5]Petal primordia emerge in double positions (0.7-0.9 mm)	[3]The first four petals are initiated alternating with sepals (0.3-0.65 mm)
4. Initiation of stamens	[1, 3]Six stamen primordia initiate (0.18-0.36 mm)	[2, 5]The floral apex becomes dome-shaped (0.88-1.10 mm)	[2, 6]Stamen primordia are initiated in whorls around periphery of inactive central region, central region becomes depressed, and later floral apex bulges (0.8-1.6 mm)
5. Carpel initiation	[1, 3]A trimerous whorl of carpel primordia initiates in alternate position to three of the stamens (0.32-0.46 mm)	[2, 5]Carpellary primordia broaden laterally slightly (1.5-3.0 mm)	[6]Carpels rise around periphery of floral apex (2.0 mm)
6. Microsporangia initiation	Four microsporangia initiate on abaxial side of stamens (0.6 mm)	Four microsporangia initiate on adaxial side of stamens (3.2-4.0 mm)	Four microsporangia initiate on adaxial side of stamens (2.8-4.0 mm)
7. Ovule initiation	Ovules initiate along the dorsal walls of locules (0.8 mm)	Ovules initiate along the lateral and dorsal walls of locules (4.6-6.4 mm)	Ovules initiate along the lateral and dorsal walls of locules (4.0-4.8 mm)
8. Male meiosis	Formation of sporogenous tissue in anthers, microspore formation (1.2 mm)	Formation of sporogenous tissue in anthers (6.7-9.2 mm)	Formation of sporogenous tissue in anthers, microspore formation (6.4-7.4 mm)

Table 3-1. Continued

Stage, developmental landmark	*Cabomba caroliniana*	*Nuphar advena*	*Nymphaea odorata*
9. Female meiosis	Ovule anatropous, with megaspore (1.6 mm)	Ovule anatropous, with megaspore (11.2 mm)	Ovule anatropous, with megaspore (6.0-9.6 mm)
10. Anthesis 1	Stigma receptive	Stigma receptive	Stigma receptive
10. Anthesis 2	Stamens dehiscent	Stamens dehiscent	Stamens dehiscent

Note- I have observed developmental stages from stamen initiation to anthesis. Some developmental stages were taken from previous studies: [1]Tucker and Douglas (1996); [2]Endress (2001); [3]Schneider et al. (2003); [4]Cutter (1957); [5]Moseley (1972); [6]Moseley (1961).

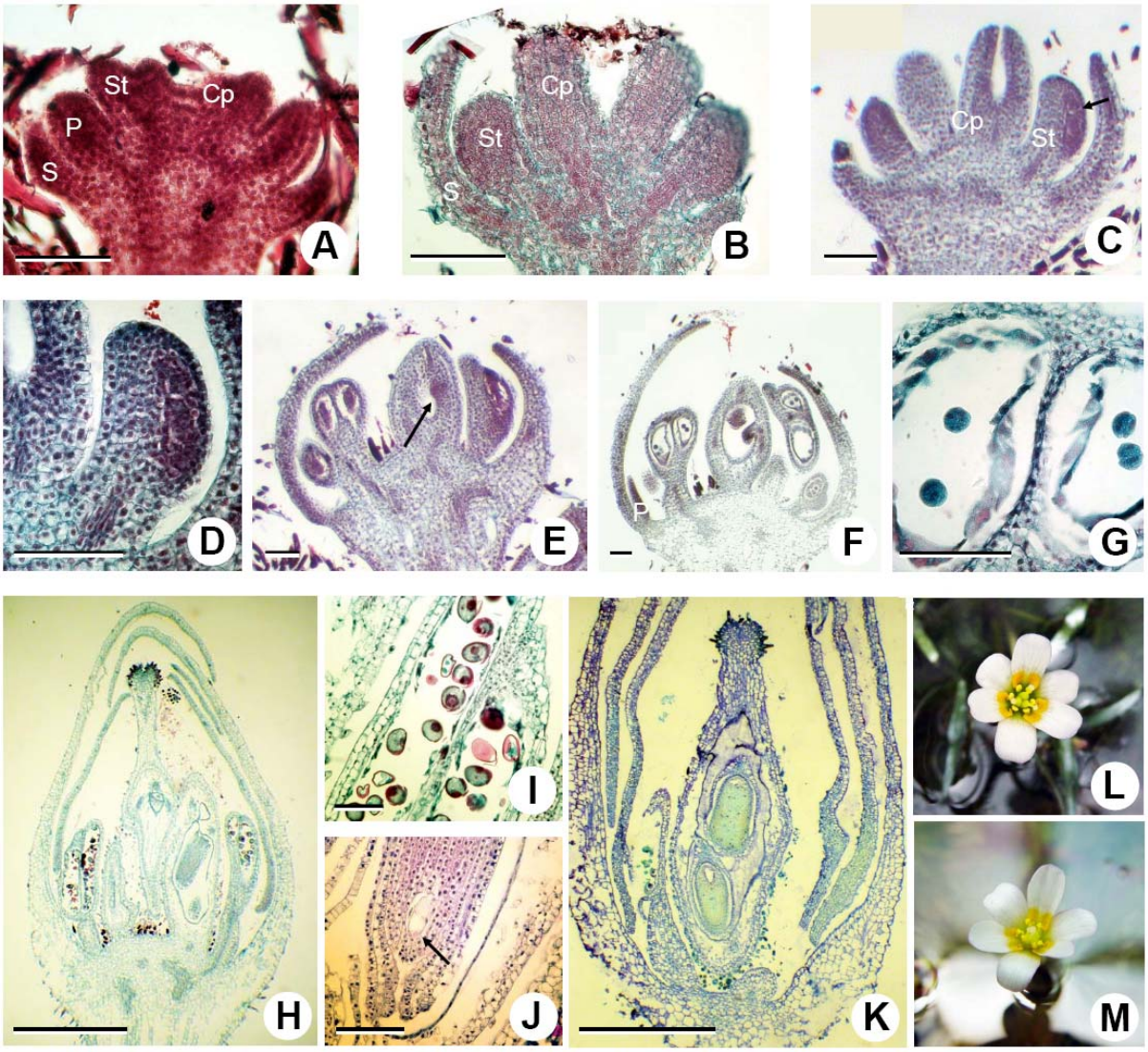


Figure 3-1. Developmental series of *Cabomba caroliniana*. All longitudinal sections. *A*, *B*, Stage 5: carpel initiation. *A*, Carpel primorida emerge. *B*, Carpel formed. *C*, *D*, Stage 6, microsporangia initiation. *C*, Four microsporangia initiate on abaxial side of stamens. *D*, Enlarged image of microsporangia in *C*. *E*, Stage 7, ovule initiation: ovules initiates along the dorsal walls of locules. *F*, *G*, Stage 8, male meiosis. *F*, microspore mother cells formed. *G*, tetrads formed. *H*, *J*, Stage 9, female meiosis. *H*, female meiosis occurred. *I*, Enlarged image of *H*, microspore formed. *J*, nuclei (arrow) formed. *K*, endosperm formed. *L*, *M*, Stage 10, anthesis. *L*, The first day of flowering, stigma receptive. *M*, The second day of flowering, stamens dehiscent. Cp: carpel, P: petal, S: sepal, St: stamen. Scale bars are as follows: A-G, I-J = 0.1 mm, H, K = 1 mm

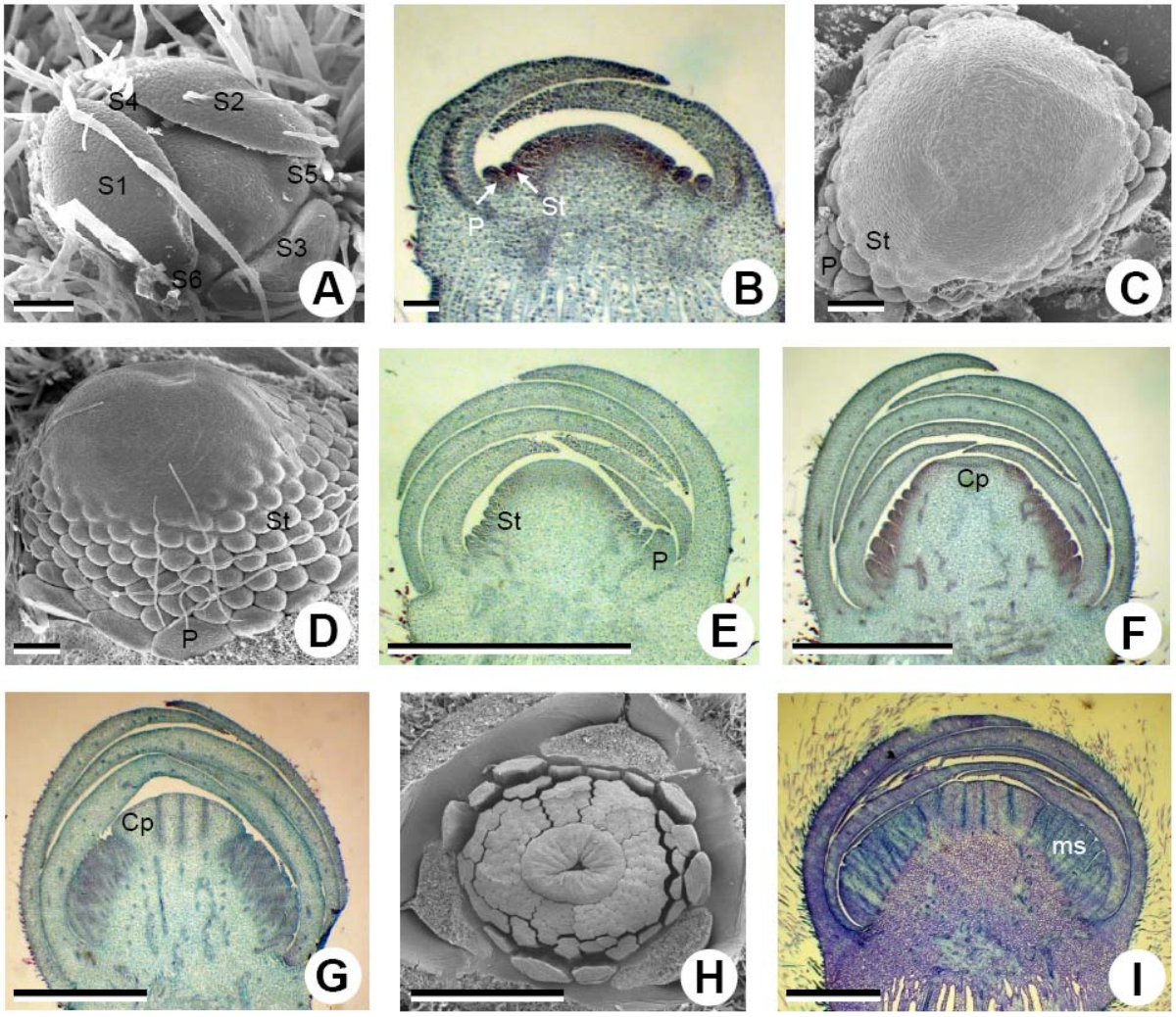


Figure 3-2. Developmental series of *Nuphar advena*. *A*, Stage 2, initiation sepal: first three sepals formed, and other three sepals initiated (SEM, top view). *B*-*E*, Stage 4, initiation of stamens. *B*, Petal whorl formed, and stamen primordia initiated, longitudinal section. *C*, Slightly older stage, central region becomes dome-shaped (SEM, top view). *D*, Older stage of stamen initiation: many stamen primordia formed (SEM, top view). *E*, same stage as D, longitudinal section. *F*-*H*, Stage 5, carpel initiation. *F*, Flat carpel primordia formed, longitudinal section. *G*, Carpellary ridge formed, longitudinal section. *H*, Carpellary ridge deepen (SEM, top view). *I*, *J*, Stage 6, microsporangia initiation. *I*, Four microsporangia initiate on adaxial side of stamens, longitudinal section. *J*, Enlarged image of *I*. *K*, Stage 7, ovule initiation: ovules initiates along the lateral and dorsal walls of locules, longitudinal section. *L*, Stage 8, male meiosis: sporogenous tissue, longitudinal section. *M*-*O*, Stage 9, female meiosis. *M*, female meiosis initiated, longitudinal section. *N*, *O*, nuclei observed, longitudinal section. Cp=carpel, ms=microsporangia, P=petal, S=sepal, St=stamen. Scale bars are as follows: A-D J, N, O = 0.1 mm, E-I, K-M = 1 mm.

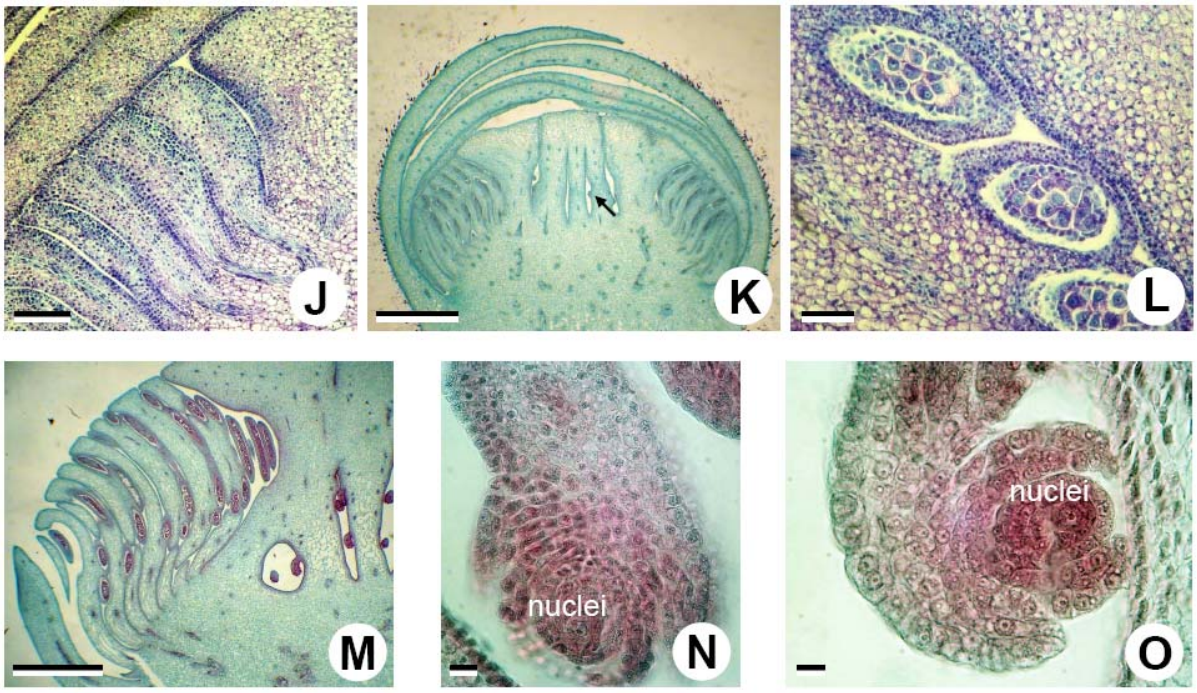


Figure 3-2. Continued.

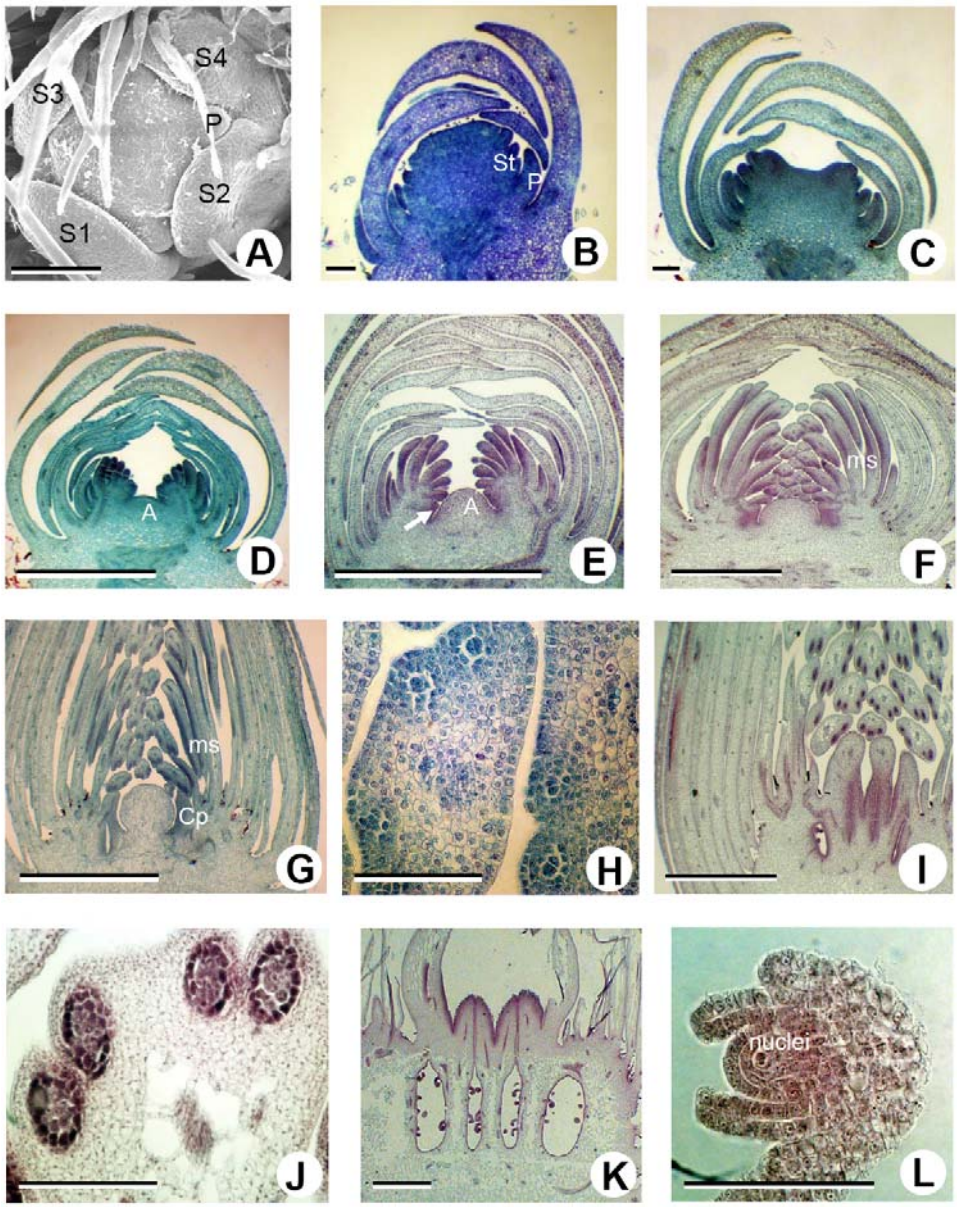


Figure 3-3. Developmental series of *Nymphaea odorata.* All longitudinal sections except *A* (SEM, top view). *A*, Stage 3, initiation of petals: the first four petals are initiated alternating with sepals. *B-D*, Stage 4, stamen initiation. *B*, Stamen primordia initiated, central region is flat. *C*, initiation of later stamens, central region is depressed. *D*, floral apex bulges. *E*, Stage 5, carpel initiation: carpel primodria (arrow) formed. *F*, *G*, Stage 6, microsporangia initiation. *F*, four microsporangia formed on adaxial sides of stamens. *G*, Slightly older stage, carpel locule formation. *H*, cross-sectioned image of microsporangia from stage 6. *I*, Stage 7, ovule initiation: ovules initiates along the lateral and dorsal walls of locules. *J*, Stage 8, male meiosis: pollen sac is filled with sporogenous tissues. K, Stage 9, female meiosis: ovule anatropous, with megaspore. A=floral apex, Cp=carpel, ms=microsporangia, P=petal, S=sepal, St=stamen. Scale bars are as follows: A-C, H, L = 0.1 mm, D-G, I-K = 1 mm.

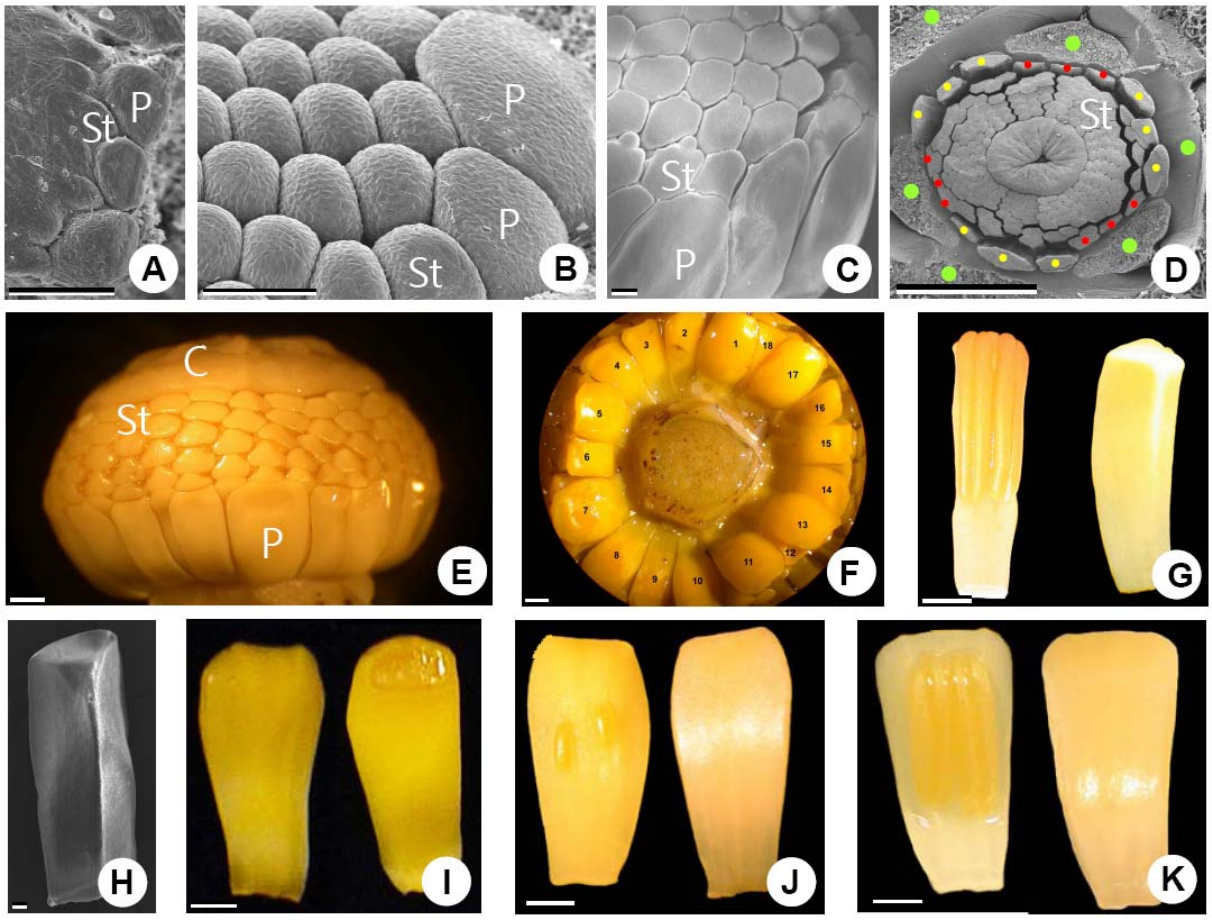


Figure 3-4. Developmental features of the third-whorled organs of *Nuphar advena*. *A*, *B*, Stage 4, stamen initiation. *A*, stamen primordia are alternative with petal primordia (SEM, abaxial view). *B*, Slightly older stage (SEM, abaxial view). *C*, *D*, Stage 5, carpel initiation. *C*, a tip of stamens is projected (SEM, abaxial view). *D*, two whorls of sepals (green dots) and two whorls of petals (red and yellow dots) were formed (SEM, abaxial view). *E*-*O*, Stage 9, female meiosis. *E*, The most outer whorl is composed of 16 petals and nectaries are on the abaxial side of petals. *F*, At antheis, view from the bottom, six sepals are removed. 18 petals formed at the third whorl, and they have nectaries on the abaxial surface. *G*, Adaxial (right) and abaxial (left) view of stamens: four microsporagia formed in adaxial side. *H*, Abaxial view of stamens: there is a ridge in the middle of stamen (SEM). *I*, Adaxial (right) and abaxial (left) view of petals: there is a nectary on the top area in abaxial side. *J*, Adaxial (right) and abaxial (left) view of petals with two microsporangia. *K*, Adaxial (right) and abaxial (left) view of petals with four microsporangia. *L*, Adaxial (right) and abaxial (left) view of petals with four fertile microsporangia. The tip is projected. *M*, Abnormal petal at anthesis with adaxial (right) and abaxial (left) view. It has aborted microsporangia on the adaxial surface as well as nectaries on the abaxial surface. *N*, Abnormal petal with aborted microsporangia (SEM, adaxial view). *O*, Adaxial (right) and abaxial (left) view of petals at anthesis: two ridges are present. *P*, Adaxial (two right) and abaxail (two left) views of stamens at anthesis: stamens are dehiscent and one ridge is present in the middle area. Scale bars are as follows: A-C, H = 0.1 mm D-G, I-P = 1 mm.

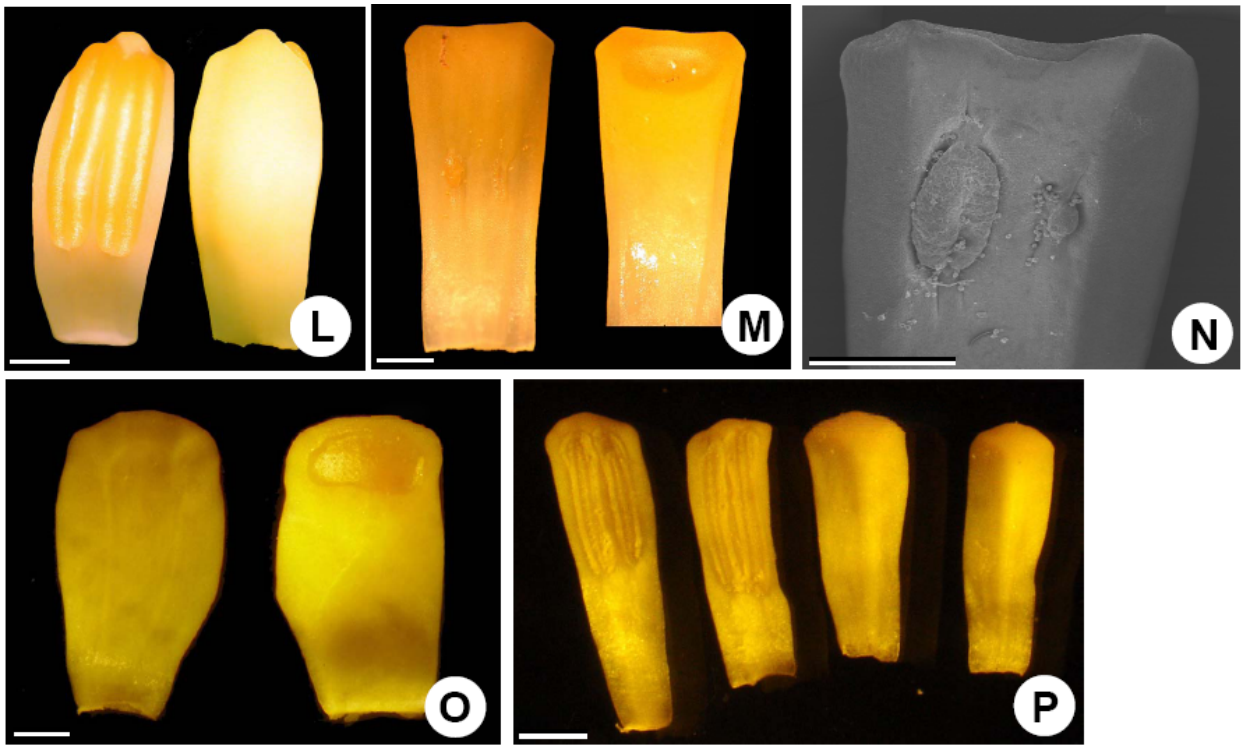


Figure 3-4. Continued.

CHAPTER 4
EXPRESSION OF HOMOLOGUES OF MADS-BOX GENES IN FLOWERS OF TWO DIVERGENT "WATER LILIES": THE BASAL ANGIOSPERM NYMPHAEALES AND THE BASAL EUDICOT *NELUMBO*

Introduction

The basal angiosperm lineages are of particular interest in studies of floral evolution due to their diversity in the arrangement and number of floral parts (e.g., Endress 1994, 2001; Soltis et al. 2002; Zanis et al. 2003). Nymphaeales are the sister to all extant angiosperms except *Amborella* (Borsch et al. 2005; Hilu et al. 2003; Jansen et al. 2007; Löhne and Borsch 2005; Leebens-Mack et al. 2005; Mathews and Donoghue 1999; Moore et al. 2007; Qiu et al. 1999; Qiu et al. 2005; Soltis et al. 1999; Soltis et al. 2000; Soltis et al. 2005; Zanis et al. 2002; Zanis et al. 2003). Members of Nymphaeales exhibit floral morphology that differs from that of the other basal lineages of angiosperms (i.e., *Amborella*, Austrobaileyales) in several features, including the presence of large flowers in some members (i.e., *Nuphar*, *Nymphaea*, *Euryale*, and *Victoria*) rather than small or moderate-sized flowers, whorled rather than spiral phyllotaxis, eudicot-like perianth differentiation (i.e., *Cabomba* and *Nuphar*), and the occurrence of blue perianth organs containing anthocyanins, which are absent in other basal angiosperms (Endress 2001; Schneider et al. 2003). Thus, studying floral development in Nymphaeales will help elucidate floral evolution in early-diverging angiosperms and thus provide insight into the early diversification of the flower.

Nymphaeales comprise nine genera and 80 aquatic species distributed in tropical to temperate regions around the world (Saarela et al. 2007; Schneider and Williamson 1993; Sokoloff et al. 2008; Williamson and Schneider 1993). This clade is composed of three subclades: Cabombaceae, Nymphaeaceae, and Hydatellaceae. The former two subclades have long been recognized as two closely related families (Richard 1828; Williamson and Schneider

1993) that share many features, including vegetative, reproductive, and molecular characters. The remaining subclade, Hydatellaceae, is sister to Cabombaceae + Nymphaeaceae and was only recently placed in Nymphaeales based on molecular phylogenetics (Saarela et al. 2007). The placement of Hydatellaceae in Nymphaeales is further supported by the presence of a 4-celled embryo sac like that of other Nymphaeales (Friedman 2008). However, floral development differs among the three families. For example, *Cabomba* and *Brasenia* (Cabombaceae) are characterized by oligomerous flowers and simultaneous initiation of calyx and corolla, whereas Nymphaeaceae (*Barclaya*, *Euryale*, *Nuphar*, *Nymphaea*, *Ondinea*, and *Victoria*) have polymerous flowers and exhibit a unidirectional order of perianth initiation (Endress 2001; Ito 1987; Les et al. 1999; Schneider et al. 2003). Hydatellaceae show unique floral developmental features: the presence of involucral bracts, no perianth, and separate staminate and carpellate flowers (Rudall et al. 2007). For this study, we focused on three genera of Nymphaeales, *Cabomba*, *Nuphar*, which is sister to all other genera of Nymphaeaceae, and the phylogenetically derived *Nymphaea* (Fig. 1; Borsch et al. 2008).

Cabomba has two petaloid whorls (similar to monocots) that have been variously described as undifferentiated tepals (Crow and Hellquist 2000; Judd et al. 2002; Zomlefer 1994) or a perianth differentiated into sepals (1st whorl) and petals (2nd whorl) (Endress 2001; Fassett 1953; Ito 1986, 1987; Moseley et al. 1993; Ørgaard 1991; Schneider and Jeter 1982; Soltis et al. 2005; Wiersema and Hellquist 1998; Zanis et al. 2003). The inner perianth ("petals") of *Cabomba* is distinguished by the auricular-shaped nectaries at the side of each "petal" (Fig. 1*A*; Endress 2001; Schneider et al. 2003). In addition, the inner perianth ("petals") of *Cabomba* shows retarded development (Endress 2001), as in eudicots. The "petals" remain small until late development in the floral bud when the outer perianth and stamens develop fully, and they

expand just prior to anthesis (Endress 2001; Schneider et al. 2003). This developmental retardation in petals is also observed in *Nuphar* (Endress 2001).

The perianth of *Nuphar* is well differentiated into sepals (1st and 2nd whorls) and petals (the remaining perianth members) (e.g., Crow and Hellquist 2000; Endress 2001; Moseley 1965, 1972; Padgett et al. 1999; Schneider and Williamson 1993; Schneider et al. 2003; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994). In fact, the two whorls of sepals show a difference in color: the outer sepals are green, and the inner sepals are yellow (Fig. 1*B*; Padgett et al. 1999; Warner et al. 2008). Therefore, several authors have regarded the 1st whorl of *Nuphar* flowers as sepals or outer tepals and the 2nd whorl as petals or inner tepals (Judd et al. 2002; Kim et al. 2005; Warner et al. 2008). The remaining perianth members are then regarded as staminodes (Judd et al. 2002; Kim et al. 2005; Warner et al. 2008).

Nymphaea has attracted the attention of many botanists because of its distinctive floral morphology. It is a classic example of a gradual transition among adjacent organ types with a complete range from petaloid staminodes to functional stamens (Crow and Hellquist 2000; Judd et al. 2002; Schneider and Williamson 1993; Wiersema and Hellquist 1998; Zomlefer 1994). Typically, the innermost stamens are functional, but the androecium shows a gradual transition from inner functional stamens toward outer petaloid perianth members; the apical portion of the anther becomes smaller, and the laminar filament area broadens toward the perianth members (Fig.1*C*, *D*). The outermost stamens, also referred to as petaloid staminodes, could be derived from either petals or stamens, but their origins have not yet been studied in detail. Furthermore, the perianth of *Nymphaea* has been variously interpreted as undifferentiated (Doyle and Endress 2000; Llamas 2003; Soltis et al. 2005) or slightly differentiated into sepals (1st whorl) and petals (the remaining perianth members) (Crow and Hellquist 2000; Ito 1987; Judd et al. 2002;

Schneider and Williamson 1993; Schneider et al. 2003; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994).

Recently, Warner et al. (2008) suggested that the perianth organs of Nymphaeales are differentiated, but these plants lack the "typical" sepals and petals of eudicots based on SEM examination. They showed that both sepaloid and petaloid areas are present on individual perianth members, and sepals and petals only differ in the occurrence of trichomes on the abaxial side of the sepals. Thus, Warner et al. (2008) suggest the term tepal is more appropriate for the perianth organs of Nymphaeales because the perianth members of other basal angiosperms, such as *Amborella* and Austrobaileyales, are considered to represent tepals (i.e., Endress 2001; Buzgo et al. 2004b). However, in this study we use the terms sepals and petals for *Cabomba*, *Nymphaea*, and *Nuphar* because the perianth organs are, in fact, differentiated (Crow and Hellquist 2000; Endress 2001; Fassett 1953; Ito 1986, 1987; Moseley 1965, 1972; Ørgaard 1991; Schneider and Jeter 1982; Schneider and Williamson 1993; Schneider et al. 2003; Wiersema 1988; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994).

Nelumbo (Nelumbonaceae) was historically thought to have a close relationship with Nymphaeales due to a superficial similarity in overall floral morphology and habitat (Cronquist 1988; Heywood 1993). However, molecular data have shown that *Nelumbo* is an early-diverging eudicot related to Platanaceae and Proteaceae (APG 1998; APG II 2003; Chase et al. 1993; Soltis et al. 2000; Worberg et al. 2007), and some morphological features also support this placement (Drinnan et al. 1994; Hoot et al. 1999). Although the floral similarity between the distantly related *Nelumbo* and Nymphaeales is an example of convergence rather than homology, this similarity is of interest with respect to floral developmental genetics. *Nelumbo* has a spirally arranged, undifferentiated perianth, and the innermost perianth members have a staminal

appendage (Hayes et al. 2000; Vogel and Hadacek 2004). In *Nelumbo*, the first two perianth parts ("sepals") are greenish and smaller than the other perianth parts ("petals"). The abaxial side of the outermost petals is greenish white, while the inner petals are pinkish white (Hayes et al. 2000; Vogel and Hadacek 2004). Unlike *Nymphaea*, the sepals of *Nelumbo* are smaller than the petals, and have different developmental features. For example, two sepal primordia enclose the floral apex and the sepals become hood-shaped with two basal auricles at the sides at early developmental stages (Hayes et al. 2000). However, at anthesis, the difference between sepals and petals in *Nelumbo* is unclear except for organ size. The innermost petals of *Nelumbo* have a staminal appendage, but no anthers (M.-J. Yoo, personal observation), a feature somewhat similar to the petaloid staminodes of *Nymphaea*.

In flowering plants, many MADS-box genes are involved in the well-known ABCDE model of floral organ identity. In the model organism *Arabidopsis thaliana*, A and E class genes control sepal identity; A, B, and E class genes control petal identity; B, C, and E class genes control stamen identity; C and E class genes control carpel identity; D and E class genes control ovule identity (Coen and Meyerowitz 1991; Colombo et al. 1995; Pelaz et al. 2000; Theissen 2001a). At least some aspects of the ABCDE model are conserved across angiosperms (i.e., *Petunia hybrida* (Angenent et al. 1993); *Silene latifolia* (Hardenack et al. 1994); *Gerbera hybrida* (Yu et al. 1999); *Oryza sativa* (Fornara et al. 2003; Kater et al. 2006; Kyozuka et al. 2000); *Pisum sativum* (Taylor et al. 2002); *Zea mays* (Whipple et al. 2004); *Magnolia grandiflora* (Kim et al. 2005), *Persea americana* (Chanderbali et al. 2006), *Akebia trifoliata* (Shan et al. 2006); *Elaeis guineensis* (Adam et al. 2007); *Taihangia rupestris* (Lü et al. 2007); *Vitis vinifera* (Poupin et al. 2007)). However, other features are not conserved throughout flowering plants, with important variations observed, especially in basal eudicots and basal

angiosperms (i.e., Kim et al. 2005; Kramer et al. 2003; van Tunen et al. 1993; Kanno et al. 2003). For whorled sepaloid or petaloid perianths, the "shifting boundaries model" or the "sliding boundaries model" was introduced to explain sepaloidy or petaloidy in some taxa by shifting the B class gene expression area to the outer whorl (Bowman 1997; Kramer et al. 2003). Recently, Buzgo et al. (2004, 2005) proposed the "fading borders model" to explain morphological intergradation of floral organs in the basal angiosperms. According to this model, each floral organ identity gene is broadly expressed across adjacent floral organs, but only weakly expressed at the edges ("borders") of its zone of activity (Buzgo et al. 2004; Buzgo et al. 2005). The fading borders model is supported by gene expression studies in several basal angiosperms (Kim et al. 2005).

We investigated the molecular determination of floral development in Nymphaeales. Specifically, we identified homologues of well-known floral organ identity genes and investigated their expression patterns across an array of Nymphaeales with varying floral morphologies (*Cabomba*, *Nuphar*, and *Nymphaea*) and in the basal eudicot *Nelumbo*. We focused on the following three issues: (1) patterns of floral gene expression and the transitions from outer perianth to inner perianth to petaloid staminodes to stamens in *Nymphaea*; (2) gene expression and perianth differentiation in *Cabomba*, *Nuphar*, and *Nymphaea*; and (3) gene expression patterns and morphological convergence in *Nelumbo* and *Nymphaea*.

We surveyed expression profiles of several MADS-box gene homologues of the A, B, C, D, and E classes and the *GGM13* (B_{sister}) and *AGL6* clades (Becker and Theissen 2003; Martinez-Castilla and Alvarez-Buylla 2003; Nam et al. 2004; Theissen et al. 1996). As for a homologue of *APETALA3* (*AP3*), Stellari et al. (2004) reported alternative splicing of the *Nymphaea AP3* transcript. They found four different kinds of *Nymphaea AP3* transcript and suggested that there

might be diverse regulatory mechanisms in *AP3* across divergent taxa (Stellari et al. 2004). In our preliminary experiments, we also observed several bands corresponding to *AP3*, and therefore designed a specific primer set to investigate the expression pattern of specific kinds of *AP3* transcripts. We also compared our results here with expression patterns from other basal angiosperms including *Amborella* and *Persea* (Chanderbali et al. 2006; Kim et al. 2005; Zahn et al. 2005). This study will shed additional light on floral developmental genetics in early angiosperms.

Material and Methods

Plants

We collected samples from the following sources: *Cabomba caroliniana*, plants purchased from a local aquarium store, Gainesville, FL, USA (Yoo 10020, FLAS); *Nuphar advena*, Waccassasa River, near Waccassasa Preserve Area, Levy Co., FL, USA (Yoo & Koh 1000, FLAS); *Nymphaea odorata*, Waccassasa Preserve Area, Levy Co., FL, USA (Yoo & Koh 1001, FLAS); *Nymphaea capensis*, plants purchased from a local aquarium store, Gainesville, FL, USA (Yoo 10021, FLAS); and *Nelumbo nucifera*, cultivated at the Kanapaha Botanical Garden, Gainesville, FL, USA (Yoo & Koh 1002, FLAS). Floral buds or flowers from early developmental stages to anthesis were collected and preserved in liquid nitrogen and stored at -80℃.

RNA Extraction, RT-PCR, and Screening for Homologues of MADS-Box Genes

We extracted RNA from whole floral buds using the RNeasy Plant Mini Kit (Qiagen, Stanford, CA, USA). The modified method of Kim et al. (2004) was used for RNA isolation from *Nuphar advena.* This method, which consists of two parts, a CTAB DNA extraction protocol (Doyle and Doyle 1987) and subsequent use of the RNeasy Plant Mini Kit, increased

the amount of isolated RNA. Reverse transcription was performed by following the manufacturer's directions using Super-Script II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and polyT primer (5'-CCG GAT CCT CTA GAG CGG CCG C(T)17-3'). PCR reactions were performed using MADS gene-specific degenerate primers (5'-GGG GTA CCA AYM GIC ARG TIA CIT AYT CIA AGM GIM G-3') and the polyT primer used in reverse transcription (Kramer et al. 1998). PCR conditions were those employed by Kramer et al. (1998). PCR bands over 800 bp in size were excised from agarose gels and purified using the Geneclean Turbo Kit (QBio-Gene, Carlsbad, CA, USA). Purified DNAs were cloned using the TOPO TA Cloning Kit (Invitrogen), and plasmid DNAs were purified from cloned cells through the FastPlasmid Mini Kit (Eppendorf, Westbury, NY, USA). Sequences were determined by cycle sequencing reactions using the CEQ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA).

Phylogenetic Analysis for Sequence Identification

To determine the putative identity of newly obtained gene sequences, we applied a BLAST search followed by phylogenetic analysis. To assign our putative MADS-box genes to the appropriate subfamily, we added our sequences to a large data set of sequences representing major subfamilies of MIKCc-type MADS genes (Becker and Theissen 2003) and recently obtained sequences from basal angiosperms (Kim et al. 2005). A total of 186 amino acid sequences was aligned using CLUSTAL X (ver. 1.83) (Thompson et al. 1997) with manual adjustments by eye. Maximum parsimony analysis was performed using PAUP* 4.0b10 (Swofford 2002). The search strategy involved 10 random addition replicates with TBR branch swapping, saving all optimal trees. To assess support for each node, a bootstrap analysis

(Felsenstein 1985) was conducted using 100 bootstrap replicates, each with 10 random addition replicates and TBR branch swapping, saving all optimal trees.

Putative homologues of *Arabidopsis* ABCDE genes were denoted using the first two letters of the genus name and the first two letters of the species epithet of the species from which the sequence was isolated, followed by the *Arabidopsis* gene abbreviation and a number to indicate the gene copy detected, if more than one; for example, the *APETALA1* (*AP1*) homologue of *Cabomba caroliniana* is *Caca.AP1*.

Gene Expression Based on RQ-RT-PCR

To investigate the expression patterns of homologues of A-, B-, C-, D-, and E-class genes and *GGM13* (B_{sister}) and *AGL6* lineages in floral organs, we employed relative quantitative–reverse transcriptase–polymerase chain reaction (RQ-RT-PCR) because of its reliability in studies of floral genes (i.e., Adam et al. 2007; Chanderbali et al. 2006; Kim et al. 2005; Kramer et al. 1998; Kramer et al. 2003).

For RQ-RT-PCR, we dissected each floral organ from buds collected just before anthesis for all taxa except *Nymphaea odorata*. For *N. odorata*, we used two different developmental stages, an early developmental stage (female-premeiotic; prior to stage 9; Buzgo et al. 2004a) and flowers at anthesis (stage 10; Buzgo et al. 2004a), to trace changes in gene expression patterns. Young leaves were used for comparison with perianth parts and reproductive organs. For *Cabomba*, two leaf tissues (submerged and floating leaves) were included. Floating leaves are produced by flowering shoots and are axillary to the inflorescence. In contrast to petiolate and di- and trichotomously dissected submerged leaves, floating leaves are peltate and the lamina is entire (Fig. 4-1*A*) (Fassett 1953; Ørgaard 1991) .

Total RNAs were extracted from each organ sample using the RNeasy Plant Mini Kit (Qiagen). Extracted RNA was treated with DNase to remove potential contamination by genomic DNA (DNase-free kit from Ambion, Austin, TX, USA). Reverse transcription using RNA from each floral part was performed following the manufacturer's directions using SuperScriptTM II RNase H-reverse transcriptase (Invitrogen). For reverse transcription, we used a random-hexamer instead of the polyT primer because the 18S ribosomal RNA gene was used as an internal control. RQ-RT-PCR was performed using a gene-specific primer pair (Table 4-1), the 18S rRNA gene primer pair (internal control), and a competitive primer pair to the 18S rRNA gene primers (competimers) following the protocol of QuantumRNA (Ambion). For *AP3* homologues from *Nymphaea*, we designed a specific primer set, AP3-4 and AP3-5, to reduce the number of bands. The reverse primer, AP3-4, is from the sequence of exon 7 of *Nyod.AP3*, and the forward primer, AP3-5, is designed from sequences of exons 5 and 6 (Fig. 4-2). Using this primer set, two classes of *AP3* transcript, class I and class VI, were detectable (Fig. 4-2). The optimal ratio of the 18S primer pair to competimers was tested for each gene to obtain a similar level of PCR signal between the 18S rRNA and that of each gene. The optimal ratio ranged from 3:7 to 4:6 for the genes that we surveyed. PCR reactions for all genes were performed with 27 or 28 cycles at 95℃ (30 sec), 56℃ (30 sec), and 72℃ (30 sec) using an Eppendorf Mastercycler (Brinkmann, Westbury, NY, USA) with 50 ng of total cDNA template. Twenty μℓ from each PCR reaction were run in a 2% (w/v) agarose gel containing 10^{-4} % (w/v) ethidium bromide in TAE buffer. Gel images were analyzed using KODAK 1D Image Analysis Software (Kodak, Rochester, NY, USA). At least three replicates of RQ-RT-PCR were performed for each gene, and the relative PCR intensity of the specific gene to that of the 18S rRNA gene was calculated for each gene. The identity of each PCR product was confirmed by sequencing. To compare

relative gene expression levels among the floral organs of each taxon, we applied the quantification method of Kim et al. (2005). Expression levels were designated as follows: -, not expressed; +, <0.1; ++, 0.1-0.4; +++, 0.4-1 (Kim et al. 2005). The genes with +++ were highly expressed relative to the internal control in every PCR reaction. The genes with ++ were consistently expressed in all PCR reactions, but their expression level was relatively low. The genes with + were variable in their gene expression levels, ranging from very weak expression to no expression in some PCR reactions.

Results

Homologues of MADS-Box Genes in Nymphaeales

We report here the following homologues of MADS-box genes in Nymphaeales: homologues of *APETALA1* (*AP1*), *Caca.AP1* from *Cabomba caroliniana*, *Nenu.AP1* from *Nelumbo nucifera*; homologues of *APETALA3* (*AP3*), *Caca.AP3*, *Nyod.AP3* from *Nymphaea odorata*, *Nyca.AP3* from *Nymphaea capensis*, *Nenu.AP3-1*, *Nenu.AP3-2* from *Nelumbo nucifera*; homologues of *PISTILLATA* (*PI*), *Caca.PI*, *Nyod.PI*, *Nyca.PI*, *Nenu.PI*; homologues of *AGAMOUS* (*AG*), *Caca.AG*, *Nyod.AG1-1*, *Nyod.AG1-2*, *Nyod.AG2*, *Nyca.AG1*, *Nyca.AG2*, *Nenu.AG* ; homologues of *AGAMOUS-LIKE11* (*AGL11*), *Nyod.AG3*, *Nyca.AG3*; homologues of *AGAMOUS-LIKE2/4/9* (*AGL2/4/9*; *SEP1/2/3*), *Caca.AGL2-1*(*Caca.SEP1-1*), *Caca.AGL2-2*(*Caca.SEP1-2*), *Caca.AGL2-3*(*Caca.SEP1-3*), *Nuad.AGL4*(*Nuad.SEP2*), *Nuad.AGL 9* (*Nuad.SEP3*) from *Nuphar advena*, *Nyod.AGL2* (*Nyod.SEP1*), *Nenu.AGL2* (*Nenu.SEP1*), *Nenu.AGL9* (*Nenu.SEP3*); homologues of *AGAMOUS-LIKE6* (*AGL6*), *Caca.AGL6-1*, *Caca.AGL6-2*, *Caca.AGL6-3*, *Nuad.AGL6*, *Nyod.AGL6*, *Nenu.AGL6*; and homologues of B_{sister} (B_s), *Caca.*B_s*1* and *Caca.*B_s*2*.

Gene annotation is based on our phylogenetic analysis, and the subfamilial placement of each gene was determined based on >70% bootstrap support (Fig. 4-3). From this study, we report a newly identified orthologue of *AP1* (A function in *A. thaliana*) from *Cabomba* and *Nelumbo*, orthologues of *AP3* and *PI* (B function in *A. thaliana*) from *Cabomba*, *Nymphaea*, and *Nelumbo*, orthologues of *AG* (C function in *A. thaliana*) from *Cabomba*, *Nymphaea*, and *Nelumbo*, an orthologue of *AGL11* (D function in *A. thaliana*) from *Nymphaea*, orthologues of *AGL2/4/9* (E function in *A. thaliana*) from *Cabomba*, *Nuphar*, *Nymphaea*, and *Nelumbo*, orthologues of *AGL6* (tepals, carpels, and ovule development in some angiosperms) from *Cabomba*, *Nuphar*, *Nymphaea*, and *Nelumbo*, and orthologues of B_{sister} (ovule and female gametophyte development in *A. thaliana*, *Petunia*, and *Gnetum*) from *Cabomba*.

Analysis of Expression Profile by RQ-RT-PCR

In *Cabomba*, *Caca.AP1* was expressed in all floral organs and also leaves. Expression of *Caca.PI* was detected in sepals, petals, and stamens, and *Caca.AG* was expressed in stamens and carpels. *Caca.AP3*, however, was expressed in all floral organs and in all leaves, with the strongest expression in sepals, petals, and stamens. The two homologues of B_{sister} genes from *Cabomba* were expressed in carpels only. In addition, the three *AGL2* homologues, *Caca.AGL2-1*, *Caca.AGL2-2*, and *Caca.AGL2-3* (Fig. 4-3) exhibited almost identical gene expression patterns, with strong signal in all floral organs, although *Caca.AGL2-3* was slightly expressed in floating leaves (Fig. 4-4*A*). The three *AGL6* homologues were expressed in different tissues. *Caca.AGL6-1* and *Caca.AGL6-2* were detected in all floral organs, although the expression levels of *Caca.AGL6-1* were very low in stamens and carpels. *Caca.AGL6-3* was expressed in carpels only (Fig. 4-4*A*).

For *Nuphar*, *Nuad.AP1* was strongly expressed in sepals, carpels, and leaves, and transcripts of *Nuad.AG* were detected in petals, stamens, and carpels. Signals of both *Nuad.AGL4* and *Nuad.AGL9* were detected in all floral organs, and *Nuad.AGL6* was expressed in sepals and carpels (Fig. 4-4*B*).

The gene expression patterns of sepals (1st whorl) and outermost petals (2nd whorl) of *Nymphaea* were very similar for most of the genes we investigated (Fig. 4-4*C*; Tables 4-2, 3) at both developmental stages studied. However, two paralogues of *Nyod.AG1* and *Nyod.AG3* showed different gene expression patterns between sepals and outermost petals (Fig. 4-4*C*). *Nyod.AG1-1* and *Nyod.AG1-2* were expressed in both of these organs at an early developmental stage although *Nyod.AG1-1* showed relatively low expression level in petals relative to that of sepals. However, at anthesis, neither *Nyod.AG1-1* nor *Nyod.AG1-2* was detected in sepals. *Nyod.AG3* was weakly expressed in sepals, but not in the outermost petals at the early developmental stage (Fig. 4-4*C*).

At both developmental stages investigated in *N. odorata*, petals and petaloid staminodes had identical expression patterns for all genes (Fig. 4-4*C*; Table 4-2), although there are differences in expression levels. For example, the expression level of the *AP3* transcript class I was higher in petals than in staminodes. There is no difference in expression for genes investigated here between outermost and innermost stamens (Fig. 4-4*C*; Table 4-2).

Three *AG* homologues were identified from *Nymphaea odorata* (Fig. 4-3). Both *Nyod.AG1-1* and *Nyod.AG1-2* are expressed in all floral organs at the early developmental stage, but their expression patterns are narrower at anthesis (i.e., no expression in sepals, carpels, and ovules). *Nyod.AG2* was expressed in stamens, carpels, and ovules at the early developmental stage investigated, and the expression level was relatively high in carpels. However, its

expression area changed at anthesis. The expression of *Nyod.AG2* was only detected in innermost petals and petaloid staminodes, but the expression level was relatively low in innermost petals (Fig. 4-4*C*; Table 4-2). Based on this expression pattern, *Nyod.AG2* may be a functional equivalent of *AG* of *Arabidopsis*. In contrast to the different gene expression patterns of the *AG* homologues of *N. odorata*, two *AG* homologues of *N. capensis*, *Nyca.AG1* and *Nyca.AG2*, show similar gene expression patterns to each other. They were expressed in all floral organs except sepals although *Nyca.AG1* was very weakly expressed in sepals (Fig. 4-4*D*). Expression of the *AGL11* homologues, *Nyod.AG3* and *Nyca.AG3*, was observed in ovules while *Nyod.AG3* was also expressed in sepals at the early developmental stage investigated (Fig. 4-4*C*, *D*).

In addition to the four kinds of *AP3* transcripts previously reported from *Nymphaea* (Stellari et al. 2004), we found two more classes, classes V and VI (Fig. 4-2). We examined the expression patterns of two classes of *Nymphaea AP3* transcript, class I and class VI, which were detected with the AP3-4 and AP3-5 primer set (Fig. 4-2). These two transcripts show different gene expression patterns. The class I *Nyod.AP3* transcript was expressed in all floral organs and leaves at the early developmental stage studied although the expression level in staminodes and ovules was relatively low. However, at anthesis, expression was not observed in carpels (ovules) or leaves. The class VI *Nyod.AP3* transcript was detected in stamens and carpels (ovules) only at the early developmental stage. However, at anthesis, this transcript was found in all floral organs except carpels (ovules).

In *Nelumbo*, gene expression patterns differed between the two sepals and the outermost petals, especially for the two *AP3* homologues, which belong to *euAP3* lineage (Fig. 4-4*E*). The outermost and inner petals exhibited the same expression profiles (Fig. 4-4*E*). *Nenu.AP1* was

expressed in all floral organs and leaves, and *Nenu.AGL2* and *Nenu.AGL9* were detected in all floral organs. *Nenu.AGL6* was also expressed in all floral organs, but the expression level was higher in sepals, petals, and carpels than in stamens and the staminal appendage (Fig. 4-4*E*). The *AG* homologue, *Nenu.AG*, was strongly expressed in stamens and carpels only. Expression of *Nenu.PI* was observed in all floral organs except carpels. Two *AP3* homologues showed different expression patterns: *Nenu.AP3-1* expression was detected in petals, the staminal appendage, stamens, and carpels, whereas *Nenu.AP3-2* was expressed in petals, the staminal appendage, and stamens (Fig. 4-4*E*).

Discussion

Comparison and Implication of Expression Patterns of MADS-Box Genes in Nymphaeales

To elucidate the molecular determination of floral development in Nymphaeales, we investigated the expression patterns of homologues of floral ogan identity (MADS-box) genes from across a diverse array of Nymphaeales: *Cabomba*, *Nuphar*, and *Nymphaea*. Most of the floral organ identity gene homologues detected in Nymphaeales are expressed in all floral organs (Fig. 4-4, Tables 4-1, 2). The expression pattern in each genus of Nymphaeales examined here differs, however, perhaps in correlation with their morphological differences. For example, *AP3*/*PI* homologues are similarly expressed in all floral organs in *Nuphar* and *Nymphaea*. However, in *Cabomba*, the *PI* homologue is not expressed in carpels but in sepals, petals, and stamens, while the *AP3* homologue is additionally expressed in leaves and carpels at relatively low levels. This expression pattern of B-class homologues from *Cabomba* can be explained by the shifting boundary model (Bowman 1997) or the sliding boundaries model (Kramer et al. 2003). In this model, when B-class gene expression is expanded to include the entire perianth, only a petaloid perianth results. In *Cabomba*, petals differ from sepals in having retarded

development, and in the presence of nectaries and the absence of trichomes (Endress 2001; Schneider et al. 2003; Warner et al. 2008). However, their overall morphologies are quite similar to each other, much like the petaloid tepals of many monocots. Thus, the shared expression profile observed for *PI* and *AP3* homologues in both perianth whorls of *Cabomba* is reasonable based on their similar petaloid appearance (i.e., *Tulipa*, Kanno et al. 2003; van Tunen et al. 1993; *Persea*, Chanderbali et al. 2006).

Transcripts of C-class homologues from *Cabomba* are observed in stamens and carpels only (Fig. 4-4*A*, Table 4-2), in agreement with data for eudicot models and monocots (Davies et al. 1999; Kang et al. 1995; Kater et al. 1998; Kempin et al. 1993; Schmidt et al. 1993; Yanofsky et al. 1990). A similar pattern of *AG* homologue expression was observed in *Nymphaea* (see below) and other basal angiosperms, including *Amborella* and magnoliids (Chanderbali et al. 2006; Kim et al. 2005). These results imply that *AG* homologues have a conserved function throughout the angiosperms. However, the *AG* homologue of *Nuphar* is also expressed in petals (Fig. 4-4*B*); one *AG* homologue is also expressed in tepals of *Persea* (Chanderbali et al. 2006) and in inner tepals of *Illicium* (Kim et al. 2005). The expression patterns of *Nuad.AG* will be discussed further below.

We identified three *AG* homologues in *Nymphaea*, and *Nyod.AG2* is thought to be a functional equivalent of *AG* based on the expression profile we observed in early development (Fig. 4-4*C*; Table 4-2). Two other *AG* homologues, *Nyod.AG1-1* and *Nyod.AG1-2*, are expressed in all floral organs early in development (Fig. 4-4*C*; Table 4-2). In addition to their different gene expression patterns, these three *AG* homologues are included in different subclades: *Nyod.AG1-1* and *Nyod.AG1-2* form a clade with *AG* homologues from other *Nymphaea* species, while *Nyod.AG2* is clustered separately in the C lineage (Fig.4-3). As a result, there are two subclades

of *AG* homologues in *Nymphaea*, and two *AG* homologues identified from *N. capensis*, *Nyca.AG1* and *Nyca.AG2*, are also included in each subclade. However, we could not identify a second *AG* homologue from *Nuphar* or *Cabomba*, indicating a duplication event might have occurred only in *Nymphaea*, although we examined only two species of *Nymphaea*. Therefore, to determine whether a duplication event in the C lineage of the *AG* subfamily is specific to *Nymphaea* (or to *Nymphaea* and other Nymphaeaceae not sampled here) or occurred earlier in the history of Nymphaeales, a more comprehensive search of representative species of each genus is required.

Because *Nyod.AG1* and *Nyod.AG2* are differentially expressed, there may be functional differentiation between these *AG* homologues in *Nymphaea*. To investigate this possibility, we examined the expression patterns of *Nyca.AG1* and *Nyca.AG2*: these two *AG* homologues are expressed in all floral organs except sepals (Fig. 4-4*D*), implying there is no differentiation between the two copies at the stage of development studied in *N. capensis*. Thus, to investigate expression differentiation among duplicate *AG* homologues in *N. odorata*, we need to exploit other methods, for example, *in situ* hybridization throughout various developmental stages.

AGL11 homologues (D lineage) from two *Nymphaea* species (*Nyod.AG3* from *N. odorata* and *Nyca.AG3* from *N. capensis*) and two B_{sister} homologues from *Cabomba* (*Caca.*B_s*1* and *Caca.*B_s*2*) were restricted in their expression to ovules only (Fig. 4-4*A*, *C*, *D*). Both D lineage and B_{sister} genes are known to specify ovule or female gametophyte identities (Becker et al. 2002; de Folter et al. 2006; Nesi et al. 2002; Rounsley et al. 1995; Tzeng et al. 2002), so the expression profiles of these three genes from Nymphaeales agree with these functions and further suggest that their function may be conserved throughout the flowering plants.

Three remaining genes, *SQUA* (*AP1*), *AGL2* (*SEP1*), and *AGL6,* are phylogenetically close (Becker and Theissen 2003; Kim et al. unpublished; Martinez-Castilla and Alvarez-Buylla 2003), but their expression profiles are quite diverse. *AGL2* homologues from *Cabomba*, *Nuphar*, and *Nymphaea* are expressed in all floral organs, similar to *AGL2* genes in other basal angiosperms and eudicot model plants (Chanderbali et al. 2006; Honma and Goto 2001; Kim et al. 2005; Pelaz et al. 2000; Zahn et al. 2005), suggesting that these genes may be conserved in their expression patterns, and possibly their function throughout the flowering plants. For *AP1* homologues, functional studies are very limited. To date, only *AP1* of *Arabidopsis* and *MtPIM* (*AP1* orthologue) of *Medicago truncatula* exhibit a "true" A function (Benlloch et al. 2006; Bowman et al. 1993; Irish and Sussex 1990; Litt and Irish 2003). In *Antirrhinum*, although the expression pattern of the *AP1* homologue *SQUA* is the same as that of *AP1*, mutant analysis failed to exhibit the A function of *SQUA* (Davies et al. 2006; Huijser et al. 1992; Taylor et al. 2002). In fact, recent study of the *ap1* mutant of *Arabidopsis* suggests that *AP1* function is not essential for sepal and petal development: over-expression of *AGL24* seems to sufficient to mask the defects of sepals and petals in the *ap1-1* mutant, thus, in the absence of *AGL24*, mutants partially recover their wild-type phenotypes (Yu et al. 2004). Therefore, together with the expression patterns of *AP1* homologues from other eudicots such as *Petunia* (Rijpkema et al. 2006), *Antirrhinum* (Davies et al. 2006), and *Gerbera* (Teeri et al. 2006), "true A function" of *AP1* is questionable.

AP1 homologues from *Cabomba* and *Nuphar* also displayed different expression patterns. In *Cabomba*, the *AP1* homologue is expressed in all floral organs and leaf tissues (Fig. 4-4*A*), while the transcript of the *AP1* homologue of *Nuphar* is detected in sepals, carpels, and leaves (Fig. 4-4*B*). Kim et al. (2005) earlier suggested that *AP1* homologues may have different

functions in basal angiosperm taxa than in eudicots based on the broader patterns of gene expression in these basal lineages; our data further support this idea. In fact, *AP1* is a product of gene duplication (Litt and Irish 2003): basal angiosperms and early-diverging eudicots have only *FUL-like* genes while core eudicots contain *euAP1* and *euFUL* genes due to a duplication event near the base of the core eudicots. Interestingly, this duplication event in the *AP1* lineage is apparently coupled with the origin of true sepals, although several studies have questioned the role of *AP1* homologues in conferring "true" A function gene (see above). Albert et al. (1998) estimated phylogenetic timing of the sepal/petal distinction using the phylogeny of Chase et al. (1993). Through optimization analyses, Albert et al. (1998) found that the characteristic sepaloid/petaloid bipartite state of *Arabidopsis* may have become fixed in the ancestral core eudicots. Thus, *euAP1* might have gained a new function in sepal formation after duplication of the *FUL-like* gene. This implies that basal angiosperms may not have a "true" A-function gene because this function did not evolve until after the duplication that yielded *euAP1* and *euFUL* genes. However, a *FUL-like* gene or its relatives may be involved in perianth development. *FUL-like* genes are expressed in all floral organs and leaf tissue in basal angiosperms, such as *Cabomba* and *Magnolia* (Kim et al. 2005).

AGL6 homologues are known to be expressed in inflorescence buds, tepals, carpels, and ovules of eudicots (Fan et al. 2007; Hsu et al. 2003; Ma et al. 1991; Rounsley et al. 1995). For Nymphaeales, similar patterns of expression are observed. For example, in *Nuphar*, the *AGL6* homologue is expressed in sepals (1st and 2nd whorls) and carpels only. Interestingly, this expression pattern is exactly the same as that obtained for the *AP1* homologue of *Nuphar* (Fig. 4-4*B*). In contrast, the *Nymphaea AGL6* homologue is detected in sepals, petals, petaloid staminodes, and ovules in early development. In *Cabomba*, three *AGL6* homologues were

identified (Fig. 4-3), and their gene expression patterns differ: *Caca.AGL6-1* and *Caca.AGL6-2* are expressed in all floral organs, although *Caca.AGL6-1* is very weakly expressed in stamens and carpels, and *Caca.AGL6-3* is detected in carpels only (Fig. 4-4*A*). These expression patterns indicate the possibility of subfunctionalization after duplication of *AGL6* homologues in *Cabomba*, as in *Arabidopsis* (Duarte et al. 2006). In *Arabidopsis*, there are two paralogues in the *AGL6* lineage, *AGL6* and *AGL13* (Martinez-Castilla and Alvarez-Buylla 2003; Rounsley et al. 1995): *AGL6* is expressed in inflorescence buds (Ma et al. 1991; Rounsley et al. 1995), whereas *AGL13* is expressed in ovules only (Rounsley et al. 1995). Before the duplication event, one copy of *AGL6* might have played a role in both perianth and ovule development. After the duplication event, however, the two copies of *AGL6* in *Cabomba* may have partitioned the function of the ancestral gene; one copy functions in perianth development, and the other functions in ovule development. Therefore, the complement of both copies can represent the functional capability of the ancestral gene (Lynch and Conery 2000).

These gene expression patterns suggest a possible role of *AGL6* homologues in perianth and ovule development in Nymphaeales; these roles may be present in all flowering plants (Chanderbali et al. 2006; Fan et al. 2007; Hsu et al. 2003; Kim et al. unpublished; Rounsley et al. 1995). Also, some *AGL6* homologues from basal angiosperms are expressed in perianth organs only (e. g., *Magnolia*, Kim et al. 2005, *Liriodendron*, Kim et al. unpublished, and *Persea*, Chanderbali et al. 2006), indicating that *AGL6* homologues may be candidates for genes involved in perianth development in basal angiosperms.

The expression patterns of *Nymphaea AGL2*, *AGL6*, *PI*, and *AG* homologues early in development are more similar to those observed for *Nuphar* floral gene homologues than those from *Cabomba*. This is reasonable considering the closer phylogenetic relationship of *Nymphaea*

and *Nuphar*, and their floral similarities. For example, A and B homologues of all three Nymphaeales examined here show broader gene expression patterns compared to those reported from eudicot model plants (Angenent et al. 1993; Davies et al. 1999; Goto and Meyerowitz 1994; Jack et al. 1992; Jack et al. 1994; Schwarz-Sommer et al. 1992; Tröbner et al. 1992; Yu et al. 1999). However, the expression patterns of C, D, E, and B_{sister} homologues are almost the same as those reported from eudicot models (Colombo et al. 1995; Davies et al. 1999; de Folter et al. 2006; Honma and Goto 2001; Nesi et al. 2002; Pelaz et al. 2000; Tzeng et al. 2002; Yanofsky et al. 1990). In particular, the expression patterns of homologues involved in female gametophyte development are conserved in those few angiosperms and gymnosperms investigated to date (Becker et al. 2002; de Folter et al. 2006; Zhang et al. 2004). Gene expression patterns observed in Nymphaeales further support the conservation of function of C, D, and *GGM13* homologues in all seed plants.

Perianth Differentiation in Nymphaeales and Transition of Petaloid Staminodes to Stamens in *Nymphaea*

In *Nymphaea* the sepals and outermost petals have similar gene expression patterns at both developmental stages examined, except for genes that belong to the *AG* subfamily (Fig. 4-4*C*; Table 4-1). Two *AG* homologues, *Nyod.AG1-1* and *Nyod.AG1-2*, are expressed in all floral organs early in development, but their expression is not detected in sepals at anthesis (Fig. 4-4*C*; Table 4-1). Also, *Nyod.AG2*, a putative functional equivalent of *AG* of *Arabidopsis*, is expressed in the innermost petals but not the rest of the perianth of *Nymphaea* at anthesis. *Nyod.AG3*, an *AGL11* homologue in *Nymphaea*, is expressed in sepals but not outer perianth members early in development. As a result, sepals, outermost petals, and innermost petals of *Nymphaea* differ slightly in their floral gene expression patterns at both developmental stages (Table 4-1). In

accordance with previous research (Crow and Hellquist 2000; Ito 1987; Judd et al. 2002; Schneider and Williamson 1993; Schneider et al. 2003; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994), Warner et al. (2008) noted that perianth parts of *Nymphaea* are morphologically and anatomically slightly differentiated (i.e., by presence or absence of trichomes and papillate cells on the surface). However, sepals and petals of *Nymphaea* lack the typical "sepal" and "petal" characteristics of eudicots (Warner et al. 2008). In eudicots, A and B class genes are responsible for perianth differentiation, for example, A class gene is required for sepal identity and B class gene for petal identity (Coen and Meyerowitz 1991; Goto and Meyerowitz 1994; Jack et al. 1992; Schwarz-Sommer et al. 1990). However, in *Nymphaea* A and B class genes show the same expression patterns across all perianth parts. In addition, *AGL6* homologues, potentially involved in perianth development, are also expressed in all perianth parts. Hence, the absence of a well-differentiated perianth in Nymphaeales coupled with broad expression of floral organ identity genes agrees with data for other basal angiosperms (Kim et al. 2005). The slight perianth differentiation seen in *Nymphaea* might be due to the differences detected in gene expression patterns of *AG* homologues.

Typically, the innermost stamens of *Nymphaea* are functional, but stamens show a gradual transition toward the perianth members; the apical portion of the anther is smaller, and the laminar filament area gets broader toward the perianth members (Fig. 4-1; Schneider and Williamson 1993; Zomlefer 1994; Wiersema and Hellquist 1998; Crow and Hellquist 2000; Judd et al. 2002). In our study, petaloid staminodes share their floral gene expression patterns with the innermost petals, suggesting that innermost petals might have originated from petaloid staminodes (or outer stamens). Previously, based on the morphological similarity between petals and petaloid staminodes (or outermost stamens), petals of some taxa in Nymphaeales were

thought to have originated from stamens (see Albert et al. 1998), and our results also support this idea. However, the expression patterns of floral genes in staminodes, such as *AG2*, *AGL6*, and *AP3* class VI transcript, differ from those observed in stamens even though staminodes also have anthers. Although their similar morphology indicates staminodes may have originated from stamens, their genetic similarities should be investigated using stamen-specific genes for clarification of their homology (see below).

In *Nymphaea*, most of the MADS-box genes (*Nyod.PI*, *Nyod.AP3*-class VI, *Nyod.AG1*, *Nyod.AGL11*, and *Nyod.AGL6*) are expressed in a smaller and narrower area later in development (anthesis) than earlier. In particular, B-class genes are not expressed in carpels and ovules at anthesis. Also, the expression patterns of *AG* homologues differ between developmental stages, and those differences might contribute to the development of slightly differentiated sepals and petals.

We also compared the floral developmental genetics of *Cabomba* and *Nuphar* with that of *Nymphaea*. The morphological features of *Cabomba* and *Nuphar* differ from *Nymphaea*. In *Cabomba*, there are two petaloid perianth whorls; the outer whorl of sepals is very similar to the petals that have nectariferous parts, which absorb UV strongly (Endress 2001). However, there is no difference in floral gene expression patterns between sepals and petals in *Cabomba* (Fig. 4-4*A*). When we consider that sepals and petals in *Cabomba* share characteristics such as the color (purplish white), texture, development (simultaneous initiation), and vasculature (one trace) (Ito 1986; Les et al. 1999; Schneider et al. 2003), possessing the same gene expression patterns for the genes examined is not surprising.

Unlike other members of Nymphaeales, *Nuphar* has two whorls of sepals, which differ slightly in coloration (Crow and Hellquist 2000; Endress 2001; Moseley 1965, 1972; Schneider

and Williamson 1993; Schneider et al. 2003; Warner et al. 2008; Wiersema and Hellquist 1998; Zanis et al. 2003; Zomlefer 1994). *Nuphar* also has a whorl of nectar-producing petals between sepals and stamens (Fig. 4-1*B*), which are sometimes considered staminodes (Judd et al. 2002; Kim et al. 2005; Warner et al. 2008). Based on floral gene expression profiles, no differences are detected between the two whorls of sepals (Fig. 4-4*B*; Fig. 5b in Kim et al. 2005). However, petals differ from sepals in the expression patterns of *AG*, *AGL6*, and *AP1* homologues. Transcripts of *AP1* and *AGL6* homologues are detected in sepals, but not petals of *Nuphar*. In particular, transcripts of the *AG* homologue are found in petals, but not sepals of *Nuphar* (Fig. 4-4*B*), while C-function homologues of *Nymphaea* and *Cabomba* are expressed in stamens and carpels only (Fig. 4-3*A*, *D)*. Also, petals of *Nuphar* exhibit the same gene expression patterns as those observed for stamens (Fig. 4-4*B*). Considering these gene expression patterns, the petals of *Nuphar* might have originated from stamens (andropetals, as in *Persea*, Chanderbali et al. 2006). In fact, the development of petals of *Nuphar* is initiated in double positions against each sepal primordium, just like the origins of stamens in *Cabomba* (Fig. 4-5; Endress 2001), suggesting a close developmental relatedness between petals of *Nuphar* and stamens of *Cabomba*. The two leading hypotheses for the origin of petals are derivation from bracts (bracteopetals) and from stamens (andropetals), respectively; basal angiosperms are considered to have a bract-derived perianth while eudicots have andropetals (Hiepko 1965; Takhtajan 1991). However, recently, Chanderbali et al. (2006) hypothesized that the tepals of *Persea* (Lauraceae) are derived from stamens, not from bracts. Also, bracteopetals are considered to be more common than previously recognized in the core eudicots (Ronse De Craene 2008). Therefore, together with the expression patterns of the floral organ identity genes, based on their stamen-like appearance, developmental similarities to stamens (Yoo et al. in prep.), and their position between sepals and stamens, petals

might be of staminal origin despite the presence of an abaxial nectary. If the "petals" of *Nuphar* are staminodes, the outer and inner sepals can be regarded as sepals and petals, respectively, based on their positions, as in other members of Nymphaeales.

In fact, distinguishing staminodes from petals in Nymphaeales is not clear because if petals are derived from stamens, petals, stamens, and staminodes would all be homologous. Although most Nymphaeaceae but not Cabombaceae are reported to have staminodes (Heinsbroek and Van Heel 1969; Judd et al. 2002; Les et al. 1999; Moseley 1958), a detailed study of staminodes has not been done. Moseley (1958) found that staminodes have the same number of vascular bundles as are found in stamens, but only *Barclaya* and *Victoria* follow this rule. Although he doubted the presence of staminodes in *Nymphaea* and *Nuphar*, he showed that there were only three vascular bundles in sepals, petals, and stamens in *Nymphaea*. Stamens of *Nuphar* have one trace while petals have three vascular bundles (Moseley 1958); however, traces of stamens and petals are from the same common system which supplies the stamens and carpels (Moseley 1958). In general, therefore, staminodes of Nymphaeaceae are similar to stamens in the number of vascular bundles, but no information is available for staminodes of *Ondinea* and *Euryale*. In this study, we examined two species, *Nuphar advena* and *Nymphaea odorata*, which have staminodes between petals and stamens. They share their gene expression patterns with stamens (*Nuphar*) and innermost petals (*Nymphaea*), indicating that staminodes ("petals") of *Nuphar* and innermost petals of *Nymphaea* might have originated from stamens and staminodes (outermost stamens), respectively. However, we investigated only two species of Nymphaeaceae with a few genes, so the entities and features of staminodes in Nymphaeaceae should be reevaluated both morphologically and developmentally. Also, using stamen-specific genes, their genetic

homology can be investigated further. Those comparative studies across diverse taxa of Nymphaeaceae will be helpful for elucidating the identity of staminodes.

Based on the gene expression data obtained for *Nymphaea*, *Cabomba*, and *Nuphar*, the morphological differences among them may be explained by different floral genetic programs. As a result, sepals and petals are not appropriate terms for the perianth members of Nymphaeales because they differ from typical sepals and petals of eudicot model organisms in morphology, development, and genetics. Endress (2006) noted that it is appropriate to use "sepaloid or petaloid tepals" to describe organs in *Nymphaea* due to the ambiguous floral organ identities in these plants. Therefore, considering phylogenetic, morphological, developmental, and genetic data, it might be better to use sepaloid or petaloid tepals, following Endress (2006). According to these terms, *Nymphaea* species have four sepaloid tepals and numerous petaloid tepals, and *Cabomba* species have only six petaloid tepals. *Nuphar* has sepaloid tepals in the first whorl and three petaloid tepals in the second whorl. The organs between the petaloid tepals and stamens in *Nuphar* might be additional whorls of petaloid tepals or petaloid staminodes.

Floral Developmental Genetics in *Nymphaea* and *Nelumbo*

To determine whether the floral morphological similarity of *Nelumbo* and *Nymphaea* is based on similarities in developmental genetics, we investigated the expression patterns of floral organ identity genes in *Nelumbo* and compared them to those obtained from *Nymphaea.* In *Nelumbo*, the expression pattern of *Nenu.AP1* is similar to that of other *FUL-like* genes that are *AP1* homologues in early-diverging eudicots and basal angiosperms: *FUL-like* genes are not true A-function genes, and their expression area is not restricted to sepals and petals, but extends to all floral organs and leaf tissue (reviewed in Litt 2007), and our results also follow those expression patterns.

The *PI* and *AP3* homologues of *Nelumbo, Nenu.PI*, *Nenu.AP3-1*, and *Nenu.AP3-2*, are commonly expressed in all petals (outer, inner, and with a staminal appendage) and stamens like the B-class genes of eudicot model plants. However, *Nenu.PI* is additionally expressed in sepals while *Nenu.AP3-1* is also detected in carpels. Considering that *PI* and *AP3* homologues function properly as heterodimers in *Arabidopsis* (Goto and Meyerowitz 1994; Honma and Goto 2001; Riechmann et al. 1996), *Antirrhinum* (Schwarz-Sommer et al. 1992; Zachgo et al. 1995), *Petunia* (Vandenbussche et al. 2004), and maize (Whipple et al. 2004), B-class homologue expression in sepals and carpels in *Nelumbo* is surprising; *Nenu.PI* and *Nenu.AP3-1* may not actually function in sepals or carpels, respectively, because both are required for proper function (Goto and Meyerowitz 1994; Honma and Goto 2001; Riechmann et al. 1996; Vandenbussche et al. 2004; Whipple et al. 2004; Zachgo et al. 1995). Therefore, together with developmental differences between sepals and petals (Hayes et al. 2000), this result suggests that differentiation of sepals and petals in *Nelumbo* might be due to different expression of B-class genes; expression of both *AP3* and *PI* homologues in petals, but expression of only the *PI* homologue in sepals.

In fact, similar phenomenon was observed in expression patterns of *PI* and *AP3* homologues of Ranunculaceae (Kramer et al. 2003): due to many duplications in B-class gene lineage in Ranunculaceae, some members of this family have several copies of *PI* and *AP3* homologues which expression patterns differ. However, considering their proper functioning as heterodimers, they are only functional in petaloid sepals, petals, and stamens like *Nelumbo* (Kramer et al. 2003). These variations observed in expression patterns of B-class homologues in *Nelumbo* and Ranunculaceae might be due to the unsettled ABC program in early-diverging eudicots. Also, as Kramer et al. (2003) suggested, lineage-specific duplication events and

subsequent diversification in their function could be plausible explanation, which should be further addressed with more detailed study.

The *AG* homologue from *Nelumbo*, *Nenu.AG*, shows the same expression pattern as that of the *AG* homologue from *Nymphaea*, further supporting the idea that the function of *AG* homologues may be conserved throughout the flowering plants (see above).

Homologues of the remaining three genes, *AGL6*, *AGL2*, and *AGL9*, show the same expression patterns in *Nelumbo* and *Nymphaea*. The expression patterns of *AGL2* and *AGL9* homologues in *Nelumbo* are similar to *AGL2* genes in other flowering plants (Chanderbali et al. 2006; Honma and Goto 2001; Kim et al. 2005; Pelaz et al. 2000; Zahn et al. 2005). The *AGL6* homologue from *Nelumbo* also shows similar expression patterns to those obtained from *Nymphaea*, indicating its involvement in perianth and carpel development. However, both *Nenu.AP1* and *Nenu.AGL6* show similar expression patterns, although *Nenu.AP1* is also expressed in leaf tissue.

In summary, in *Nelumbo* sepals are differentiated from petals based on their developmental sequence and gene expression (i.e., the expression pattern of a *PI* homologue, but not an *AP3* homologue). Unlike the undifferentiated tepals ("sepaloid and petaloid tepals") of *Nymphaea*, *Nelumbo* has differentiated sepals and petals. Also, petals with a staminal appendage share their expression patterns with outermost and inner petals in *Nelumbo*, indicating that petals with the staminal appendage are not homologous to stamens.

Therefore, even though there is morphological similarity between the perianth of *Nymphaea* and *Nelumbo*, the floral gene expression patterns of the two genera differ from each other: broad floral gene expression patterns are observed in B-class homologues from *Nymphaea*, but the expression of B-class homologues in *Nelumbo* follows the classic ABCDE eudicot model.

In general, however, the expression of other floral organ identity gene homologues in both *Nelumbo* and *Nymphaea* generally follows the eudicot model. Thus, based on different organ identity gene profiles, similar floral morphologies between *Nelumbo* and *Nymphaea* might be just morphological convergence.

Molecular Models of Floral Development in Nymphaeales

In this study, we focus on MADS-box genes because they play key roles in regulating floral organ identities. Expression of C- and E-class homologues in Nymphaeales generally follows the classic ABCDE eudicot model. However, B-class homologues of Nymphaeales show generally broad expression patterns compared to those obtained from eudicot model organisms. These results are consistent with the fading borders model (Buzgo et al. 2004; Buzgo et al. 2005) and may explain the intergradation of perianth organs, and of perianth to stamens in Nymphaeales. Similar results were found in other basal angiosperms with gradual differentiation of floral organs (Chanderbali et al. 2006; Kim et al. 2005). However, *Cabomba*, which has discrete whorls of floral organs with a perianth differentiated by position, fits the shifting boundary model (Bowman 1997) or the sliding boundaries model (Kramer et al. 2003).

Therefore, within Nymphaeales, both the fading borders model, which appears to be ancestral for the angiosperms (Kim et al. 2005), and the sliding boundaries model are present in divergent clades. However, further work is needed to evaluate the details of the fading borders model in Nymphaeales. No difference in signal strength is apparent between the margin and center of a gene's zone of activity, for examples, between sepaloid tepals and carpels. Similar results were obtained previously for *PI*/*AP3* in *Nuphar*, also with RT-RQ-PCR (Kim et al. 2005). However, using RNA *in situ* hybridization, both *PI*/*AP3* homologues were strongly expressed in stamens and staminodes ("petals" in this study), but also very weakly expressed in sepaloid and

petaloid tepals rather than highly expressed in all floral organs (Kim et al. 2005). This result shows that the fading borders model indeed might be applicable for the *PI*/*AP3* homologues of *Nuphar*, but the sensitivity of RNA *in situ* hybridization is needed to evaluate the details of the model's predictions. Therefore, to determine the details of the fading borders model in *Nuphar* and *Nymphaea*, further research is needed using additional developmental stages and additional genes, and greater sampling of species of Nymphaeales.

Origin of the Floral Parts in Nymphaeales

Since the first appearance of angiosperms in earth about 130 mya, origin of flower and their subsequent strong diversification have been a mystery for a long time, which is often called "Darwin's abominable mystery" (Crepet 1998, 2000; Frohlich 1999; Frohlich and Parker 2000; Frohlich 2003; Ma and dePamphilis 2000; Theissen et al. 2002; Theissen and Becker 2004; Theissen and Melzer 2007; Winter et al. 2002). Recently, based on the molecular and developmental genetics, several theories and hypothesis have been proposed for origin of flower; among them are the 'out-of-male' hypothesis, the 'out-of-female' hypothesis, and the mostly male theory (Frohlich and Parker 2000; Frohlich 2003, 2006; Theissen et al. 2002; Theissen and Becker 2004; Theissen and Melzer 2007). The first two theories were proposed based on the fact that gymnosperms, the closest relatives of angiosperms, have orthologues of B-class genes and their expression patterns are distinct in reproductive organs (Theissen et al. 2002; Theissen and Becker 2004). For example, B-class homologues were expressed only in male reproductive units (Becker et al. 2000; Becker et al. 2003; Fukui et al. 2001; Mouradov et al. 1999; Sundström et al. 1999; Winter et al. 1999), while C/D-class homologues were expressed in both male and female reproductive units (Jager et al. 2003; Rutledge et al. 1998; Tandre et al. 1998; Winter et al. 1999). These expression patterns facilitate the 'out-of-male' or 'out-of-female' hypothesis, explaining

that hermaphroditic flower originated from either a male cone or a female cone, respectively, and changes in B gene expression played a critical role in evolution of the other reproductive organ (Theissen et al. 2002; Theissen and Becker 2004; Theissen and Melzer 2007). For example, in the 'out-of-male' hypothesis, B genes were expressed in male cones, but down-regulation of B genes in the upper region of male cones resulted in development of the female structures there. In the 'out-of-female' hypothesis, ectopic expression of B gene in lower region of female cones led to development of male reproductive organs (Theissen et al. 2002). The mostly male theory was developed based on the *LEAFY* duplications prior to the split between angiosperms and gymnosperms and the loss of one copy in angiosperm lineages (Frohlich and Parker 2000). In this theory, two copies of *LEAFY*, *LEAFY* and *NEEDLY*, were required for specifying male and female cones in gymnosperms, respectively. The loss of *NEEDLY* in angiosperms caused to retain more genes relatively active in male cones, and the minimum set of female genes were restricted in ovule identity (Frohlich and Parker 2000; Frohlich 2003, 2006). Therefore, in general this theory is similar to the 'out-of-male' hypothesis in respect to derivation of flower organization more from the male structure than from the female structure (Frohlich and Parker 2000; Frohlich 2003, 2006). However, in the mostly male theory, the female structure developed ectopically in male cones (Frohlich and Parker 2000; Frohlich 2003, 2006). Although these theories do not propose the mechanism for perianth development, later Baum and Hileman (2006) suggested that sterilization of the outer stamens led to evolution of perianth organs.

Our data, especially the expression patterns of B-class homologues in Nymphaeales, fit the 'out-of-male' hypothesis. First, *PI* and *AP3* homologues of *Nuphar* are expressed all floral organs, but the expression of *PI* homologue is not detected in carpels later in development (Kim et al. 2005). This is also observed in B-class homologues of *Nymphaea*; *Nyod.PI* and *Nyod.AP3* are expressed

in all floral organs early in development while their expression are not detected in carpels later in development (Fig. 4-4*C*). Therefore, broad gene expression patterns of B-class homologues with restriction of their expression in carpels later in development support the idea that down-regulation of B-class homologues might have resulted in female reproductive units in the upper region of male structures. Surprisingly, similar expression pattern was also observed in *PI* of *Arabidopsis*: unlike *AP3*, *PI* transcripts of *Arabidopsis* were detected in the second, third and fourth whorls of flowers at stage 3 (petal initiation), but *PI* was not detected in the fourth whorl after stage 4 (stamen initiation), indicating that PI expression early in development may not be related to carpel specification (Goto and Meyerowitz 1994). However, this feature observed in *Arabidopsis* might be vestige of ancestral feature which might have been a rule for flowering plants. Furthermore, perianth organs of Nymphaeales share their B gene expression patterns with stamens (Fig. 4-4*A*, *C*; Kim et al. 2005). In addition, Yoo et al. (in prep.) also suggested that perianth organs might have originated from stamens based on analysis of global gene expression profiles using microarray. Thus, together with microarray data, our data indicate that the 'out-of-male' hypothesis, which assumes that all floral parts might have derived from male reproductive units, might be true for ancestors of Nymphaeaceae.

Table 4-1. Primer information used in this study

Taxon	Gene name	Forward sequence Name: sequence	Reverse sequence Name: sequence
GLO subfamily			
Cabomba	*Caca.PI*	*CcPI-1: GAACCAAGAGCTGGAGAGAA*	*CcPI-2: ACGGTTGCATCGCCGCCA*
Nymphaea	*Nyod.PI*	*Nyod.PI-3: ATCAGTGTCCTCTGTGACGC*	*Nyod.PI-4: CTGCTGCAAGTTAGGCTGTA*
Nelumbo	*Nenu.PI*	*Nenu.PI: CTCAGCAACGAAATAGACAG*	*Nenu.PI-2: GCTGGATTGGCTGCACACG*
DEF subfamily			
Cabomba	*Caca.AP3*	*CcAP3-3: GTTGAGTTACACCGAGCTG*	*CcAP3-4: TCATGCTAGCCTCAAGTCA*
Nymphaea	*Nyod.AP3*	*Nyod.AP3-5: GAAAAAGATAAGGTTAGCCG*	*Nyod.AP3-4: CACGCAGATTAGGATGGCTG*
Nelumbo	*Nenu.AP3-1*	*Nenu.AP3-1: CGATCTGAGTGTGGACGAGC*	*Nenu.AP3-2: CATCCTGAAGATTGGGCTG*
	Nenu.AP3-2	*Nenu.AP3-3: CGATATGAGCATCGAAGAAC*	*Nenu.AP3-4: CAACCTGAAGATTAGGATG*
AG subfamily			
Cabomba	*Caca.AG-1*	*CcAG-7: ACCTTGAAGTGAAGCTGGAG*	*CcAG-8: GAGGCTCTCCAGTCATCATA*
	Caca.AG-2	*CcAG-9: ATGCTCAATATTATCAACAGG*	*CcAG-8: GAGGCTCTCCAGTCATCATA*
Nuphar	*Nuad.AG*	*Nuad.AG-5: ATGCTGGGTGAAGGAATCAG*	*Nuad.AG-6: TCATCCAAGTTGTAGTGCCG*
Nymphaea	*Nyod.AG1-1*	*Nyod.AG4-1: AGAATAAATTGGAGAGAAGC*	*Nyod.AG4-2: TCCATTAGATTGATGTGCTG*
	Nyod.AG1-2	*Nyod.AG2-3: GTGAGAATGAAAGAGCGCAGC*	*Nyod.AG2-4: TACGACAATGAGACAACGGC*
	Nyod.AG2	*Nyod.AG-1: CTCCAACTCGGGAACCGTTAC*	*Nyod.AG-4: ATGCTGCTGCTGCTGCTGATG*
	Nyod.AG3	*Nyod.AG3-1: ATCCTTCAGAATGCGAACAG*	*Nyod.AG3-2: TCAGCAACCTTGGCTCGAA*
	Nyca.AG1	*Nyca.AG1-1: GAGACCTCAGATCTTTAGAG*	*Nyca.AG1-2: TCATCCAAGTTGCAAGGCAG*
	Nyca.AG2	*Nyca.AG2-3: ACTTAGAAGGCAAACTGGAG*	*Nyca.AG2-4: TCATCCAAGTTGAAGGGC*
	Nyca.AG3	*Nyca.AG3-3: GACCAAGTTGAGGCAGCAG*	*Nyca.AG3-4: GGTTGGAGTAGTGGATCATG*
Nelumbo	*Nenu.AG*	*Nenu.AG-1: GCTCTTAGCACTATGACTG*	*Nenu.AG-2: CTTGGCGAGAGTAATGGTG*

Table 4-1. Continued

Taxon	Gene name	Forward sequence Name: sequence	Reverse sequence Name: sequence
SQUA subfamily			
Cabomba	*Caca.AP1*	*CcAP1-1: CATCAGACTCCAGCATAATG*	*CcAP1-2: ATAGACTCCTGCATCAGCTG*
Nuphar	*Nuad.AP1*	*NuadAP1-3: GTGAAGATCTCGAGCCATTGAG*	*NuadAP1-4: GCTAGTCTGAGTTTGCAGGTG*
Nelumbo	*Nenu.AP1*	*Nene.AP1-1: TAGAGCAACAGCTTGACAC*	*Nenu.AP1-2: GTGAGTTGGTCCGAGCATG*
AGL2 subfamily			
Cabomba	*Caca.AGL2-1*	*CcSEP-1: TCTCTGCGATGCTGAGGTC*	*CcSEP-2a: CATATACTGTGTCTTGGTAG*
	Caca.AGL2-2	*CcSEP-1a: GTACCAGAAGTGTAGCTATG*	*CcSEP-2a: GAACGTACTGGATGTGCTG*
	Caca.AGL2-3	*CcSEP-3: TTGCGATGCGGAGGTCG*	*CcSEP-4: TGAGGTCAGCAAGTTGATC*
Nuphar	*Nuad.AGL4*	*Nuad.AGL4-1: CATCTTGAGCAGCAGCTAGAG*	*Nuad.AGL4-2: CAGTTATCTGCTCTTGCGCA*
	Nuad.AGL9	*Nuad.AGL9-1: ACTGGAGCGGTATCAGAAGT*	*Nuad.AGL9-2: CTTGTTAGCTTCGATCAGCA*
Nymphaea	*Nyod.AGL2*	*Nyod.SEP-1: AGCTACTGTGCCATCTCGAGA*	*Nyod.SEP-2: ATGTGCTGCGCACTGTTATC*
Nelumbo	*Nenu.AGL2*	*Nenu.AGL2-1: GCTTGAACAGCTTGAGCATC*	*Nenu.AGL2-2: GTTATCTGATCTGGACCAAC*
	Nenu.AGL9	*Nenu.AGL9-1: CTACAGCGGTCACAGAGGA*	*Nenu.AGL9-2: GCTCACACTCTACAGGATG*
AGL6 subfamily			
Cabomba	*Caca.AGL6-1*	*CcAGL6-1: TGATGGAGCAGATGGATGA*	*CcAGL6-2: ATGAAGTTGCTCTCAGGAG*
	Caca.AGL6-2	*CcAGL6-3: CATTAGACAACAGCATAGC*	*CcAGL6-4: GCTCTGAGAACATGACCA*
	Caca.AGL6-3	*CcAGL6-5: TACGATGCATTTGACAACAG*	*CcAGL6-6: CATGCGATTCTAGCTGCA*
Nuphar	*Nuad.AGL6*	*Nuad.AGL6-1: GCAAAGCTGAAGGCAAGATA*	*Nuad.AGL6-2: CATGTCCTTGTGATTCTAGCT*
Nymphaea	*Nyod.AGL6*	*Nyod.AGL6-1: CAACACTGCTGCTGCTGCGA*	*Nyod.AGL6-2: GTTCACGTCTCCAAGGTGAC*
Nelumbo	*Nenu.AGL6*	*Nenu.AGL6-1: ACCAGTGCGTCCGACCGTGA*	*Nenu.AGL6-2: TGATGTCTCCAAGATGACGT*
GGM13 subfamily			
Cabomba	*Caca.Bs1*	*CcBs-1: GAGGATCTTGCCACTCTCAC*	*CcBS-2: TGGTGGATGGCTGCATGATC*
	Caca.Bs2	*CcBs-1: GAGGATCTTGCCACTCTCAC*	*CcBS-4: ATGTACGGCCGCAGGATC*

Table 4-2. Comparison of floral gene expression patterns from different developmental stages of *Nymphaea odorata*

Gene	Leaves	Sepals	Outer petals	Innermost petals	Staminodes	Stamens	Innermost stamens	Carpels	Ovules
Nyod.PI[a]	-	+++	+++		+++	+++		+++	+++
Nyod.PI[b]	-	+++	+++	+++	+++	+++	+++	-	-
Nyod.AP3									
Class I[a]	+++	+++	++		+	++		+++	++
Class I[b]	-	+++	+++	+++	+++	+++	+++	-	-
Class IV[a]	-	-	-		-	+++		+++	+++
Class IV[b]	-	++	++	++	+++	++	++	-	-
Nyod.AG1-1[a]	-	+++	++		++	+++		++	++
Nyod.AG1-1[b]	-	-	++	+++	+++	+++	+++	-	-
Nyod.AG1-2[a]	-	++	++		+	++		+++	+
Nyod.AG1-2[b]	-	-	+++	+++	+++	+++	+++	-	-
Nyod.AG2[a]	-	-	-		-	++		+++	++
Nyod.AG2[b]	-	-	-	++	+++	+++	+++	-	-
Nyod.AG3[a]	-	+++	-		-	-		-	+++
Nyod.AG3[b]	-	-	-	-	-	-	-	-	+++
Nyod.AGL2[a]	-	+++	++		++	+++		+++	+++
Nyod.AGL2[b]	-	+++	+++	+++	+++	+++	+++	++	+++
Nyod.AGL6[a]	-	+++	++		++	-		-	+++
Nyod.AGL6[b]	-	+++	+++	+++	+++	-	-	-	-

[a]Gene expression patterns from an early developmental stage
[b]Gene expression patterns at anthesis

Table 4-3. Summary of expression patterns of floral genes in Nymphaeales and *Nelumbo* just prior to anthesis

Taxa	Gene	Leaves	Sepals 1st	Petals 2nd	Inner stamens 3rd	Carpels 4th
GLO subfamily						
Cabomba	*Caca.PI*	-	+++	+++	+++	-
Nuphar	*Nuad.PI* [a]	-	+++	+++	+++	++
Nymphaea	*Nyod.PI*	-	+++	+++	+++	+++
Nelumbo	*Nene.PI*	-	+++	+++	+++	-
DEF subfamily						
Cabomba	*Caca.AP3*	+	+++	+++	+++	+
Nuphar	*Nuad.AP3-1* [a]	-	+++	+++	+++	+++
	Nuad.AP3-2 [a]	-	+++	+++	+++	+++
Nymphaea	*Nyod.AP3*(class I)	+++	+++	+++	++	+++
	Nyod.AP3(class VI)	-	-	-	+++	+++
Nelumbo	*Nene.AP3-1*	-	-	++	+++	+++
	Nene.AP3-2	-	-	+++	+++	-
AG subfamily						
C lineage						
Cabomba	*Caca.AG*	-	-	-	+++	+++
Nuphar	*Nuad.AG* [a]	-	-	+++	+++	+++
Nymphaea	*Nyod.AG1-1*	-	+++	++	+++	++
	Nyca.AG1	-	-	+++	+++	+++
	Nyod.AG1-2	-	++	++	++	+++
	Nyod.AG2	-	-	-	+++	+++
	Nyca.AG2	-	-	++	+++	+++
Nelumbo	*Nene.AG*	-	-	-	+++	+++
D lineage						
Nymphaea	*Nyod.AG3*	-	+++	-	-	ovules
	Nyca.AG3	-	-	-	-	ovules
SQUA subfamily						
Cabomba	*Caca.AP1*	+++	+++	+++	+++	+++
Nuphar	*Nuad.AP1*	+++	+++	-	-	+++
Nelumbo	*Nene.AP1*	++	+++	+++	++	++

Table 4-3. Continued

Taxa	Gene	Leaves	Sepals 1st	Petals 2nd	Inner stamens 3rd	Carpels 4th
AGL2 subfamily						
AGL2 lineage						
Cabomba	*Caca.AGL2-1*	-	+++	+++	+++	+++
	Caca.AGL2-2	-	+++	+++	+++	+++
	Caca.AGL2-3	++	+++	+++	+++	+++
Nuphar	*Nuad.AGL2*[b]	-	+++	+++	+++	+++
	Nuad.AGL4	-	+++	+++	+++	+++
Nymphaea	*Nyod.AGL2*	-	+++	+++	+++	+++
Nelumbo	*Nene.AGL2*	-	+++	+++	+++	+++
AGL9 lineage						
Nuphar	*Nuad.AGL9*	-	+++	+++	+++	+++
Nelumbo	*Nene.AGL9*	-	++	+++	+++	+++
AGL6 subfamily						
Cabomba	*Caca.AGL6-1*	-	+++	+++	+	+
	Caca.AGL6-2	-	+++	+++	+++	+++
	Caca.AGL6-3	-	-	-	-	+++
Nuphar	*Nuad.AGL6*	-	+++	-	-	+++
Nymphaea	*Nyod.AGL6*	-	+++	++	-	ovules
Nelumbo	*Nene.AGL6*	-	++	+++	+	++
GGM13 subfamily	*Caca.Bs1*	-	-	-	-	+++
Cabomba	*Caca.Bs2*	-	-	-	-	+++

NOTE- For comparison, the expression patterns of *Nymphaea odorata* were obtained from an early developmental stage.
[a]Data from Kim et al. (2005)
[b]Data from Zahn et al. (2005)

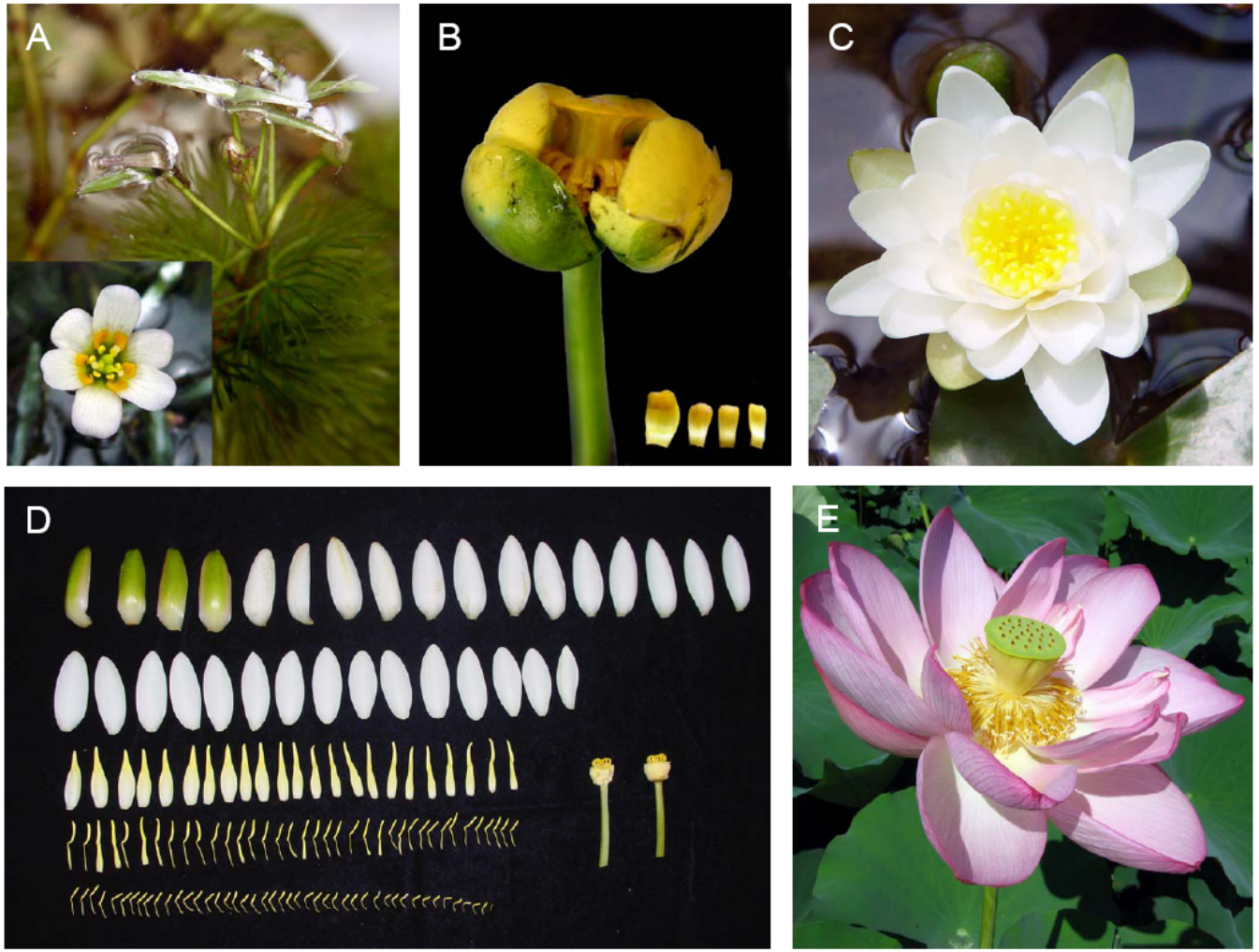


Figure. 4-1. Photographs of flowers of Nymphaeales and *Nelumbo*. *A*, *Cabomba Caroliniana*; peltate floating leaves and di- and trichotomously dissected submerged leaves, and flower. *B*, *Nuphar advena*; flower and petals. *C*, *Nymphaea odorata*, flower. *D*, Dissected floral parts of *N. odorata*; sequence showing from four green sepals to 28 white petals (the 1st and 2nd rows) and from petaloid staminodes (the 3rd row) to functional stamens (the 4th and 5th rows), and two views of carpels and ovules are presented (photo credit: S. Kim). *E*, *Nelumbo nucifera*, flower.

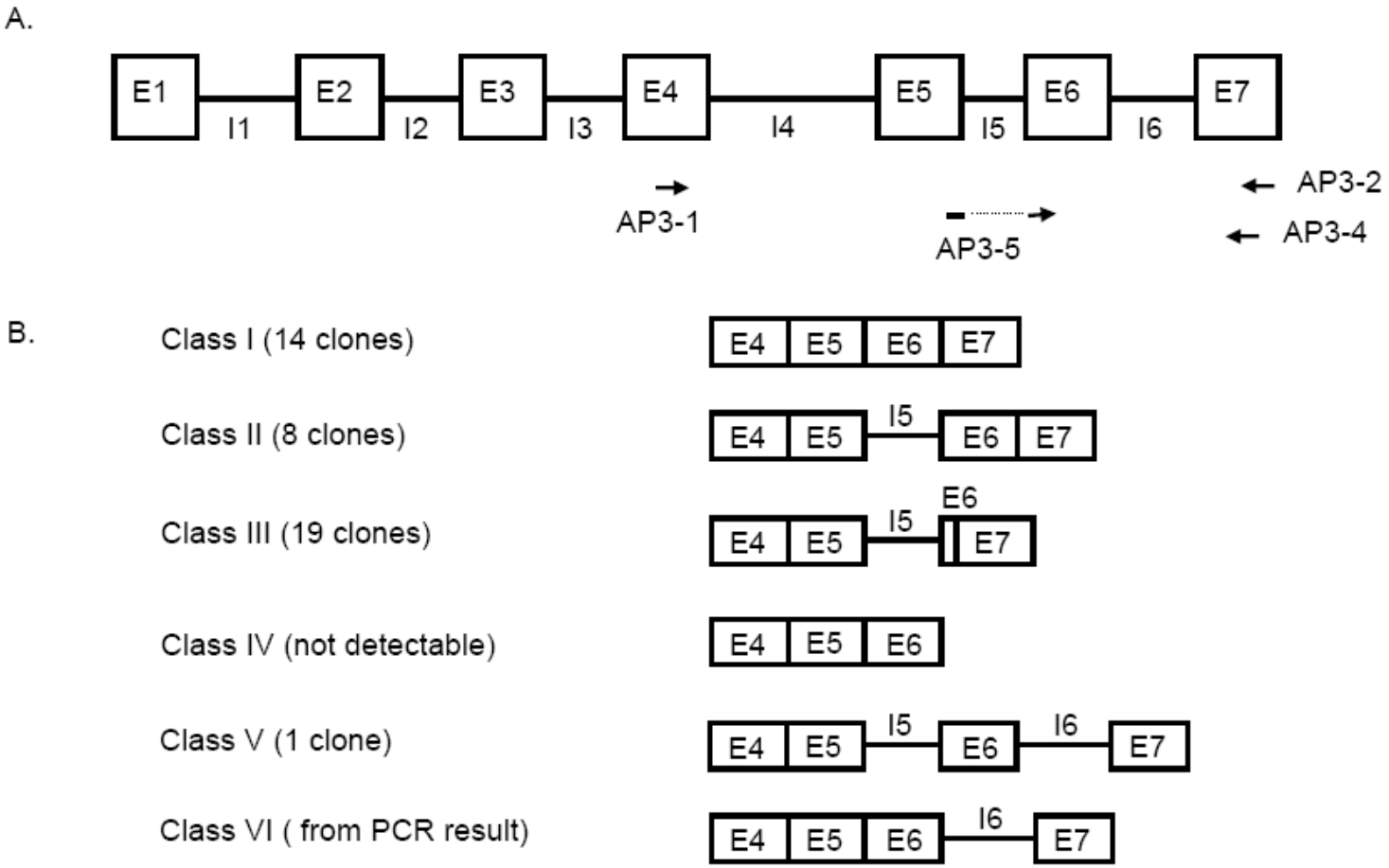


Figure. 4-2. Alternatively spliced transcript of *Nyod.AP3*. *A*, Intron/exon structure of the *Nyod.AP3* genomic sequences. Designations of the exons and introns are based on comparison to genomic structure of *NymAP3* (Stellari et al. 2004) and *Arabidopsis AP3* (Jack et al. 1992). *B*, Inferred structure of cDNA splicing variants (Stellari et al. 2004). The number of clones for each class is shown in parentheses.

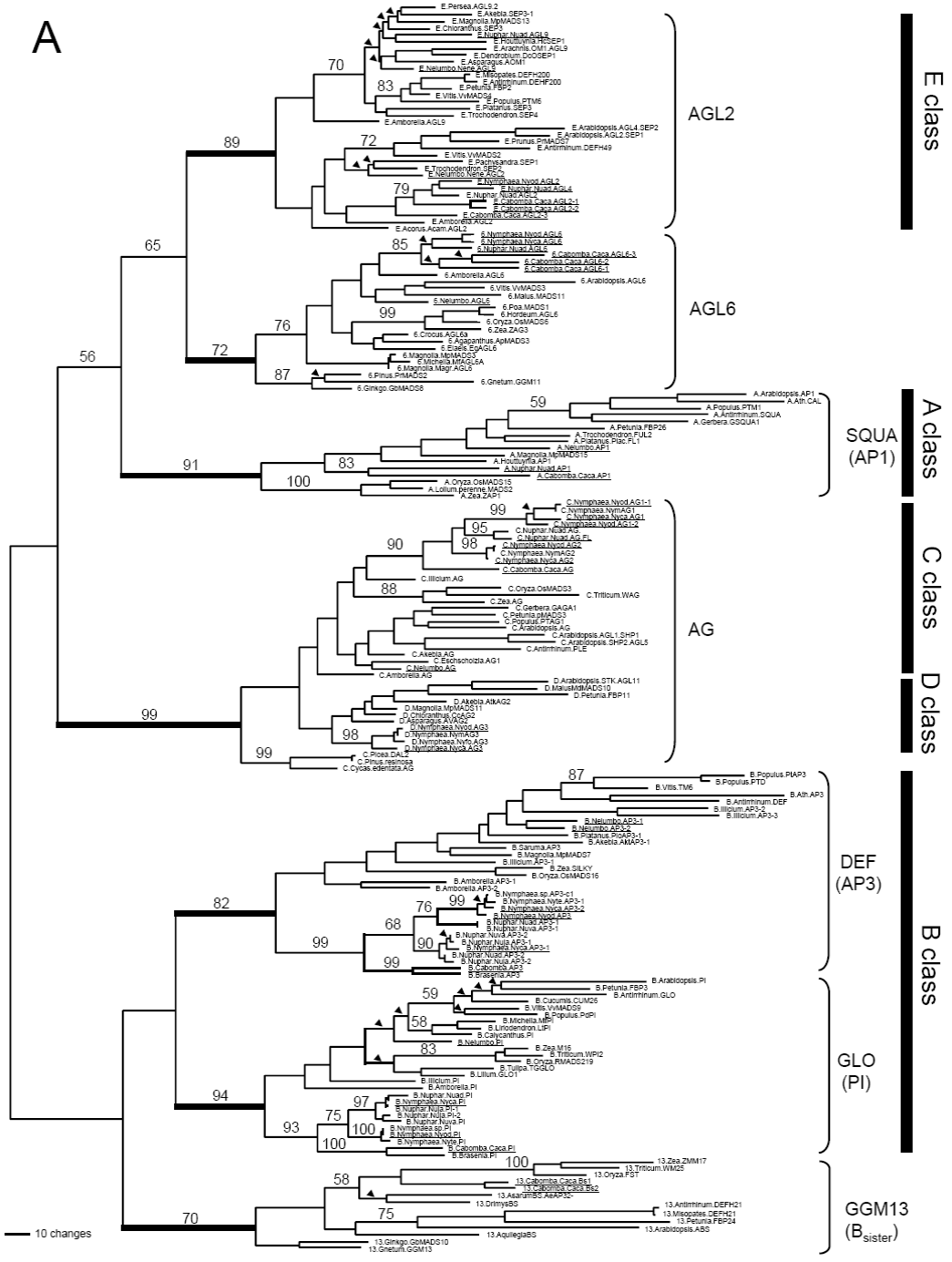


Figure. 4-3. Phylogenetic analyses using 186 MADS-box gene homologues. *A*, One of the most parsimonious trees. Triangle symbol indicates the collapsed branch in the strict consensus tree of 1355 shortest trees from a maximum parsimony analysis of MADS genes (6815 steps, consistency index=0.3859 and retention index=0.7568). MADS-box gene homologues newly identified and used in this study (underlined) are placed as a member of a well-supported major clade of MADS-box subfamily (thickened nodes). *B*, Enlarged tree of *AGL2*, *AGL6*, and *SQUA* lineages. *C*, Enlarged tree of *AG* lineage. *D*, Enlarged tree of *DEF*, *GLO*, and B_{sister} lineages.

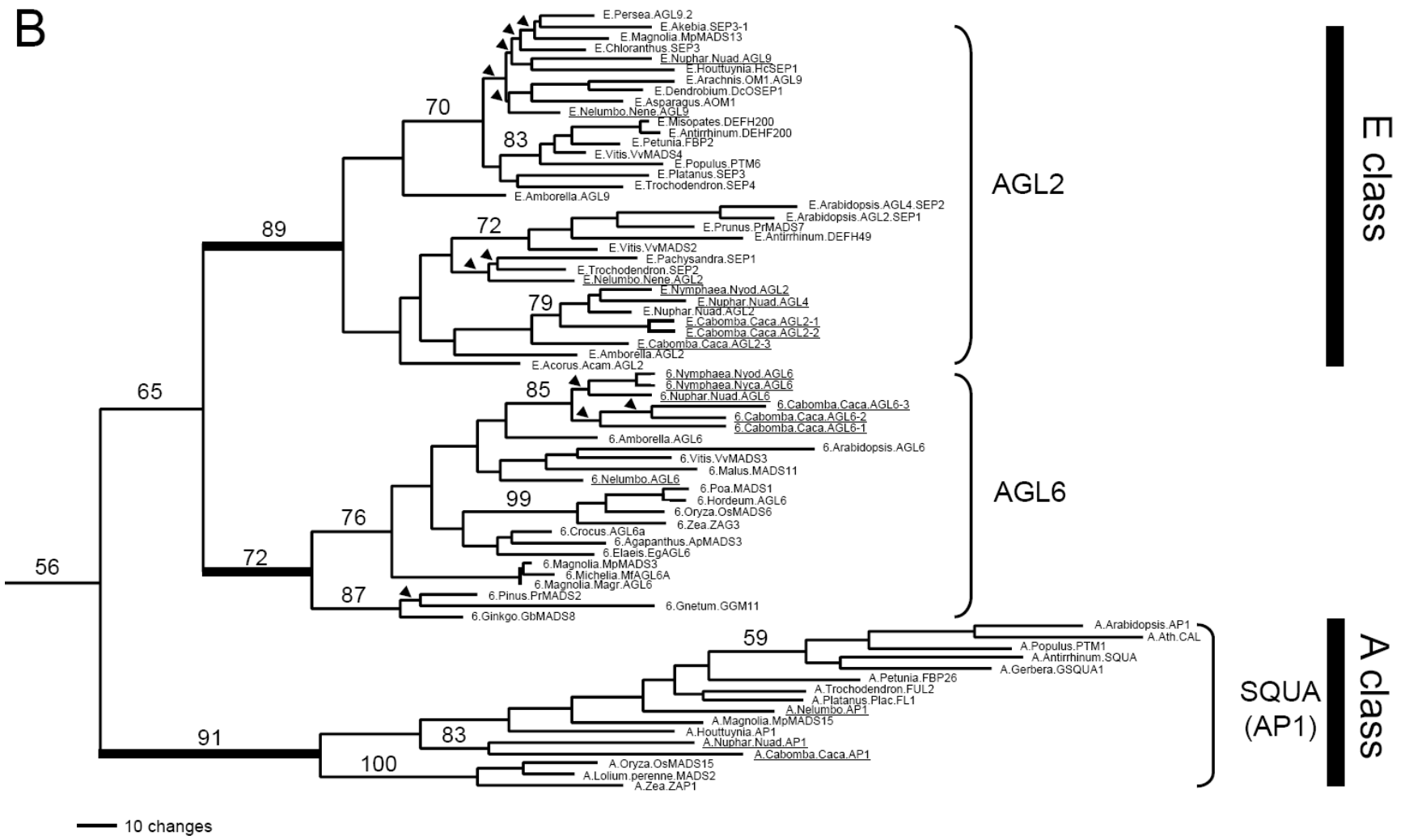


Figure. 4-3. Continued.

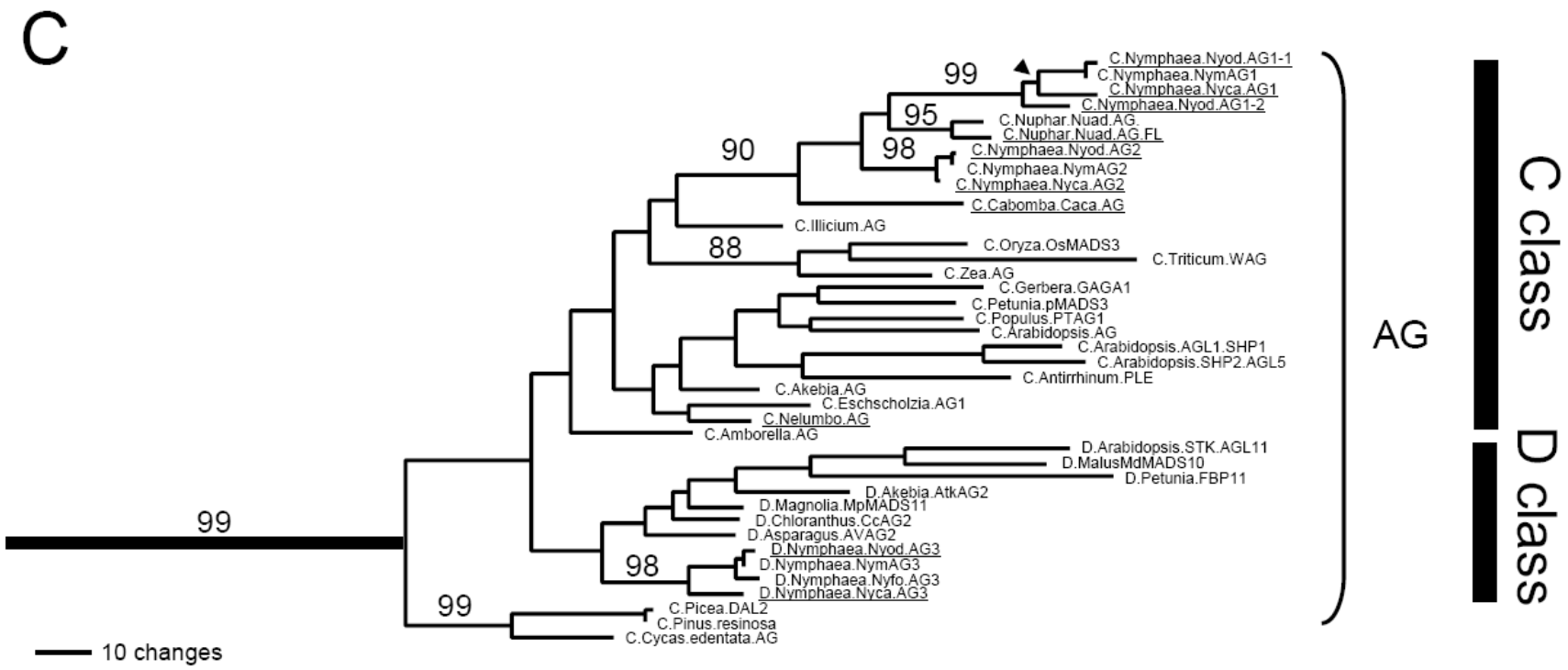


Figure. 4-3. Continued

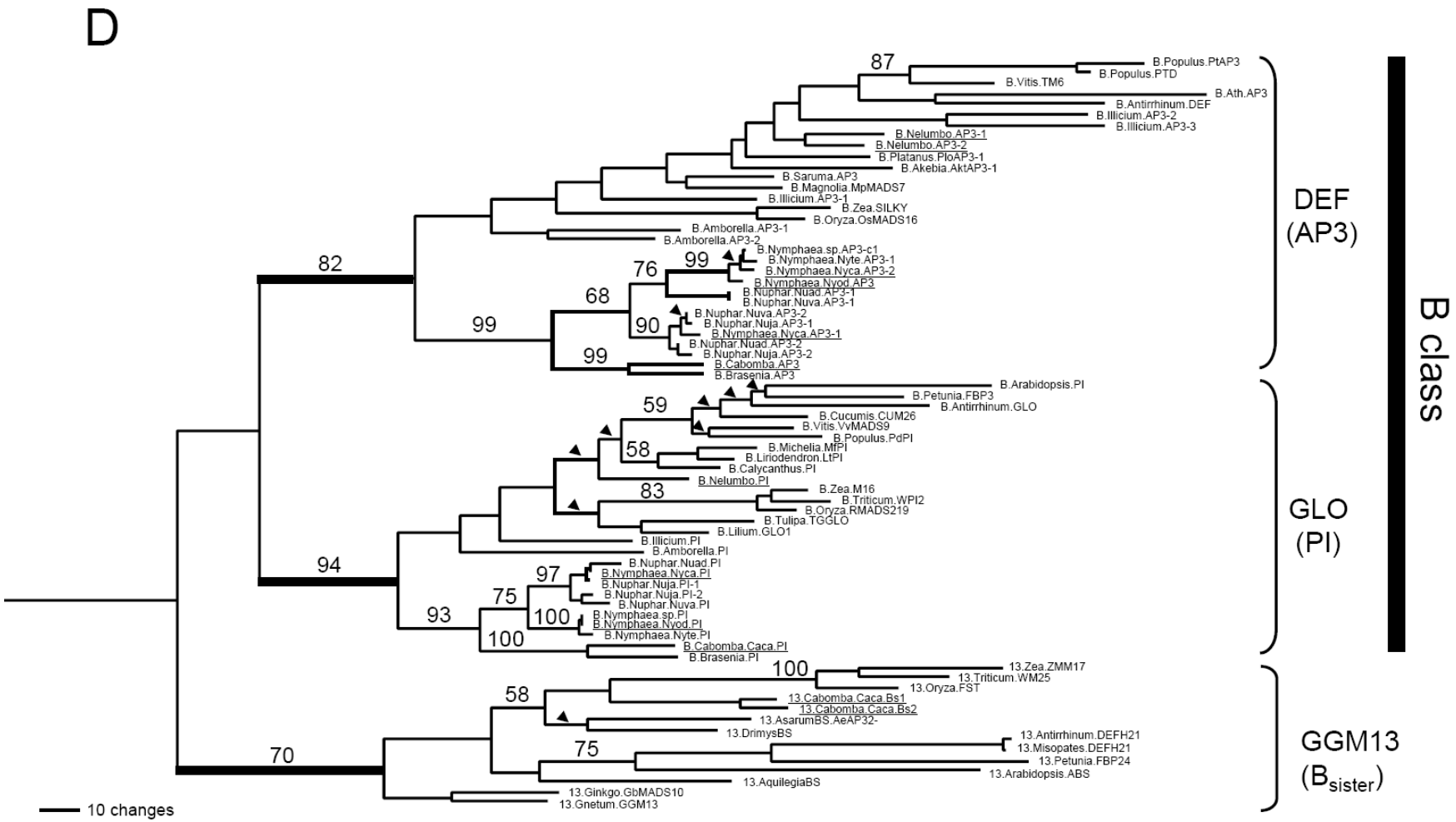


Figure. 4-3. Continued.

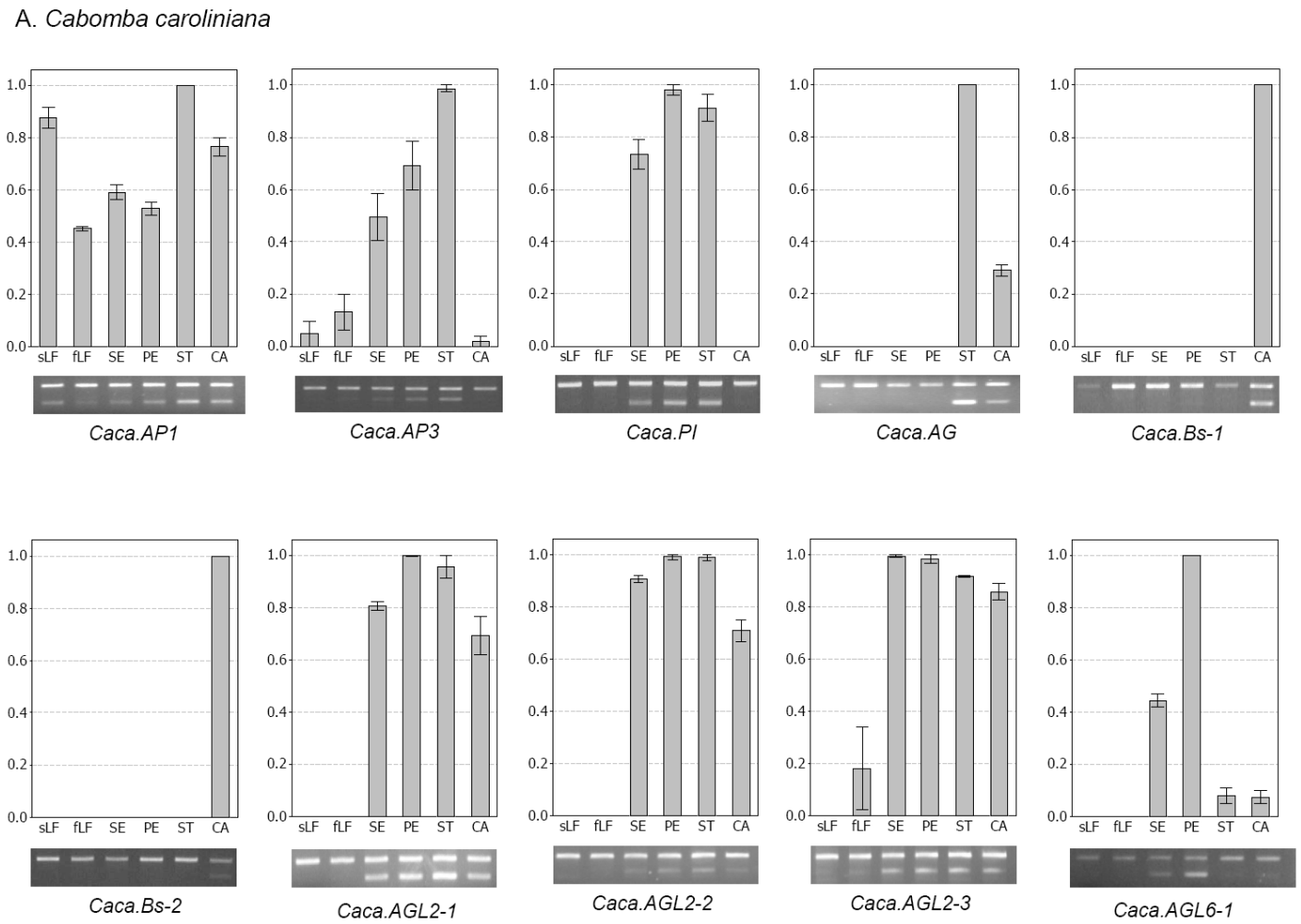
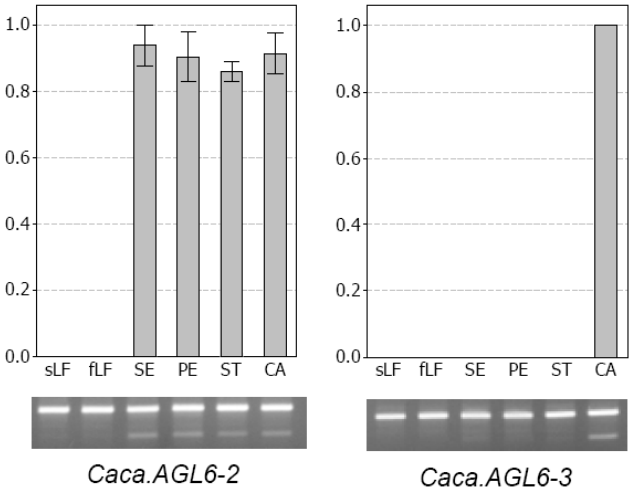


Figure. 4-4. Relative quantitative RT-PCR results of floral MADS-box gene homologues in Nymphaeales and *Nelumbo*. Standard deviations are indicated for each value. sLF, submerged leaves; fLF, floating leaves; SE, sepals; PE, petals; ST, stamens; CA, carpels; OSE, outer sepals; ISE, inner sepals; LF, leaves; OPE, outer petals; IPE, inner petals; SN, staminodes; ST1, outermost stamens; ST2, innermost stamens; OV, ovules; FA; floral apex; SA, staminal appendages. In *Nymphaea odorata*, E shows expression patterns from early developmental stage, and A indicates expression patterns from at anthesis.

A. *Cabomba caroliniana* (continued)



B. *Nuphar advena*

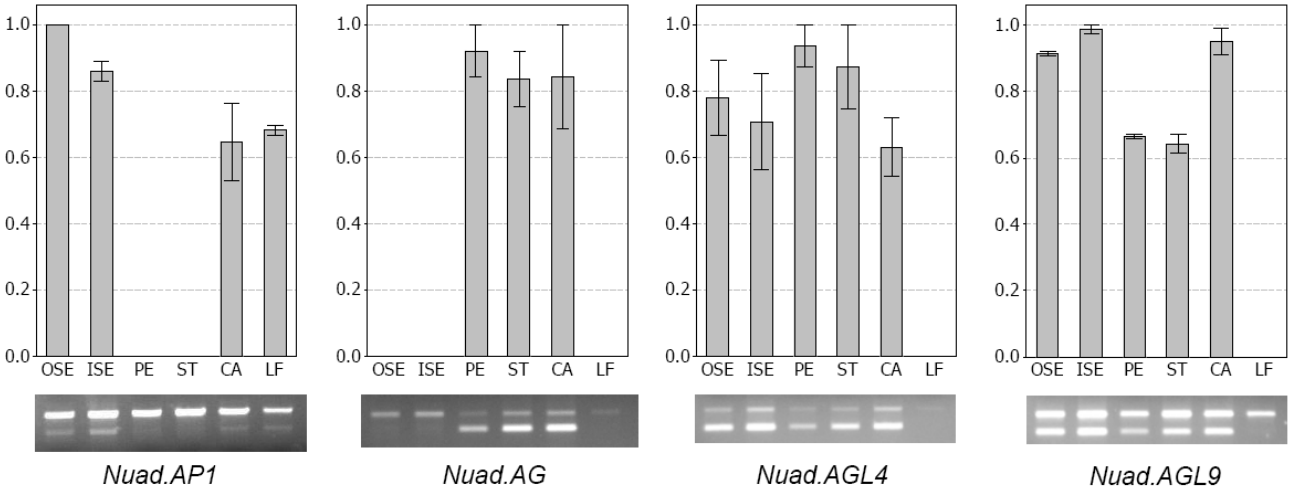


Figure 4-4. Continued.

C. *Nymphaea odorata*

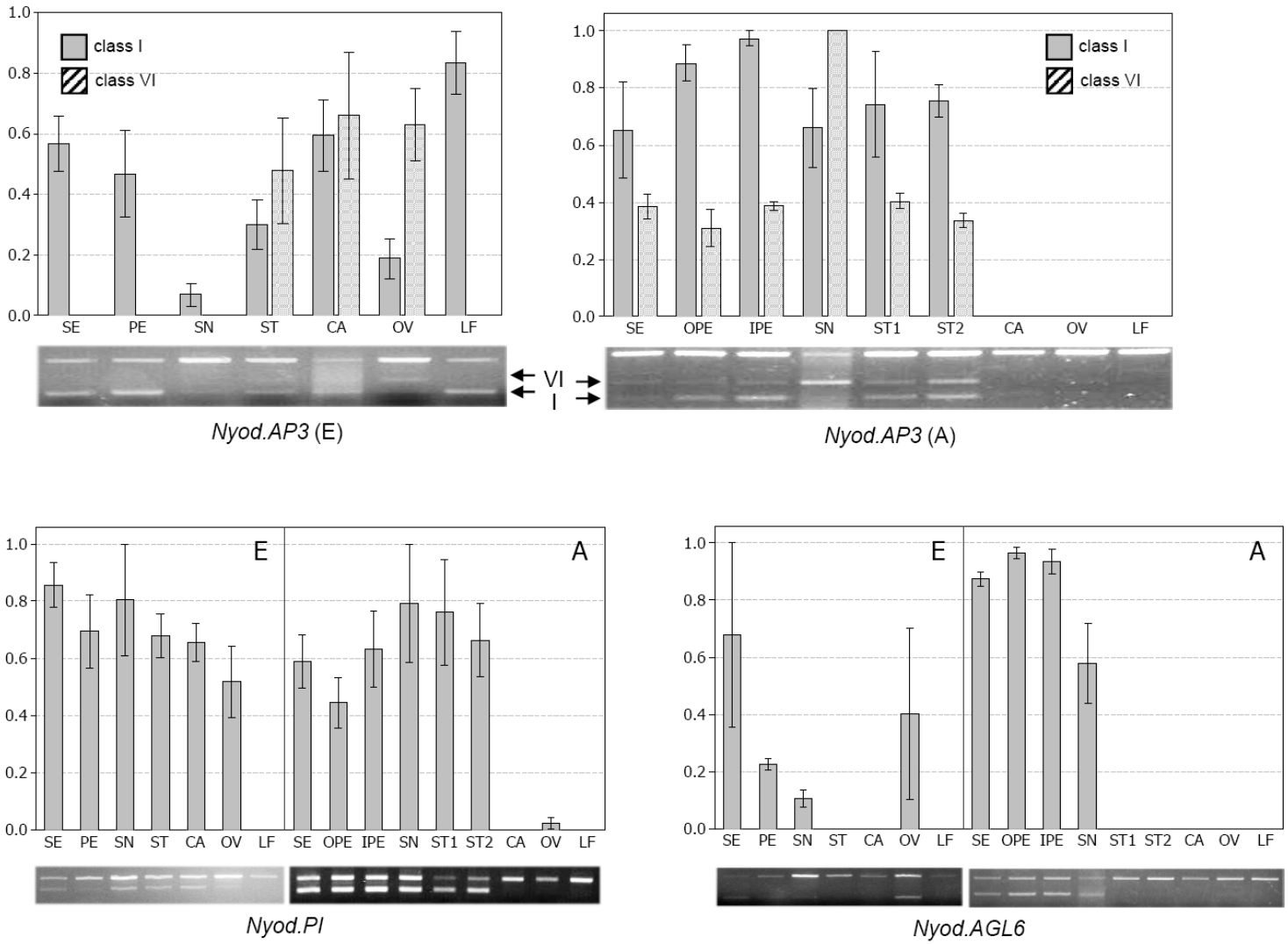


Figure 4-4. Continued.

C. *Nymphaea odorata* (continued)

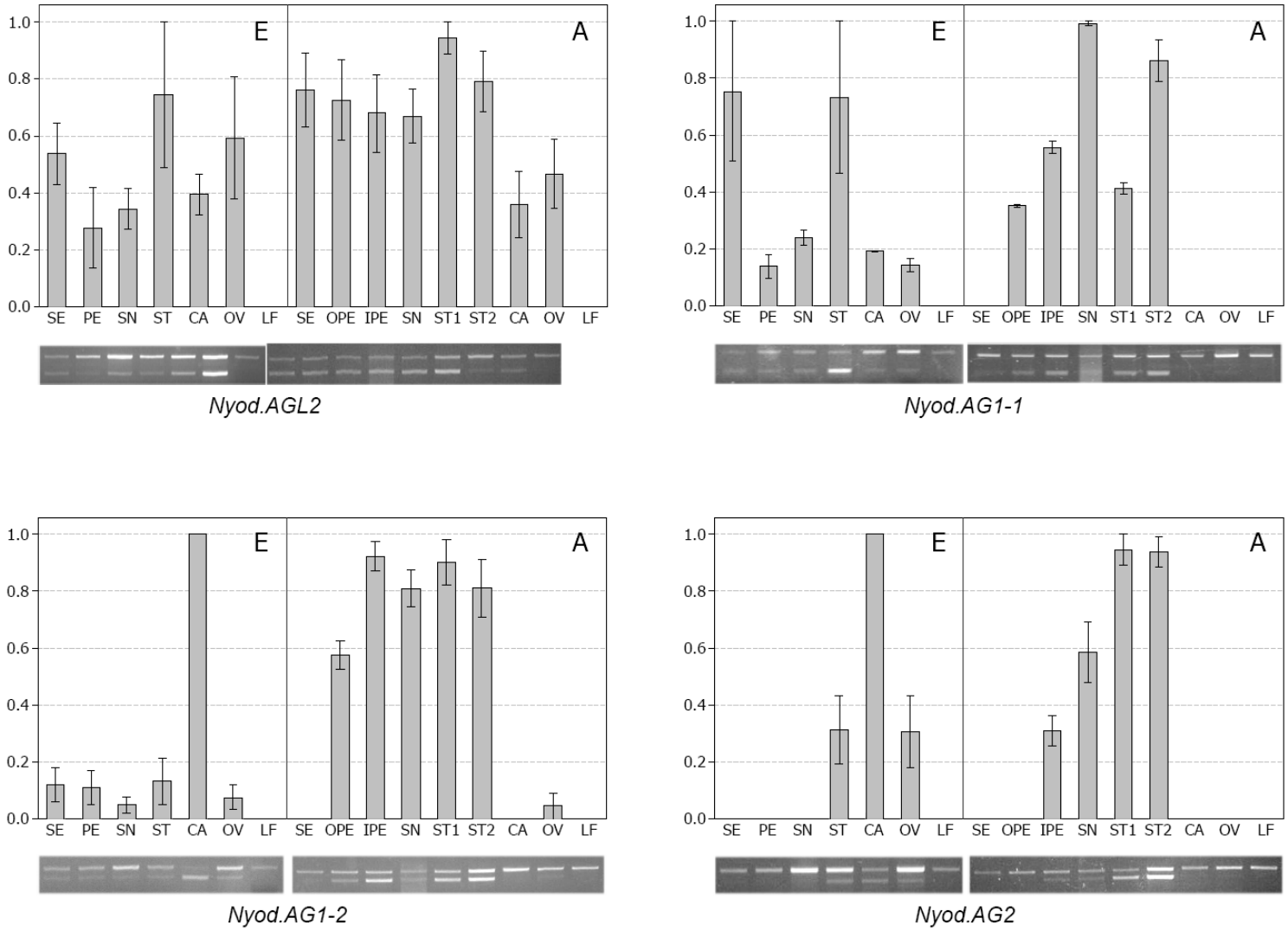
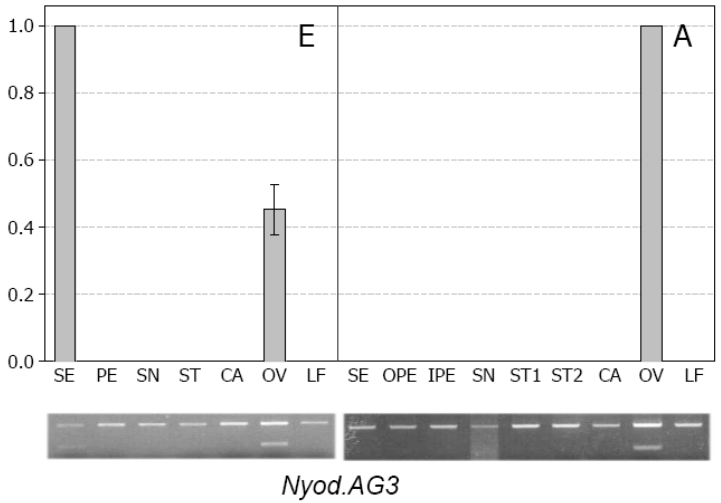


Figure 4-4. Continued.

C. *Nymphaea odorata* (continued)



D. *Nymphaea capensis*

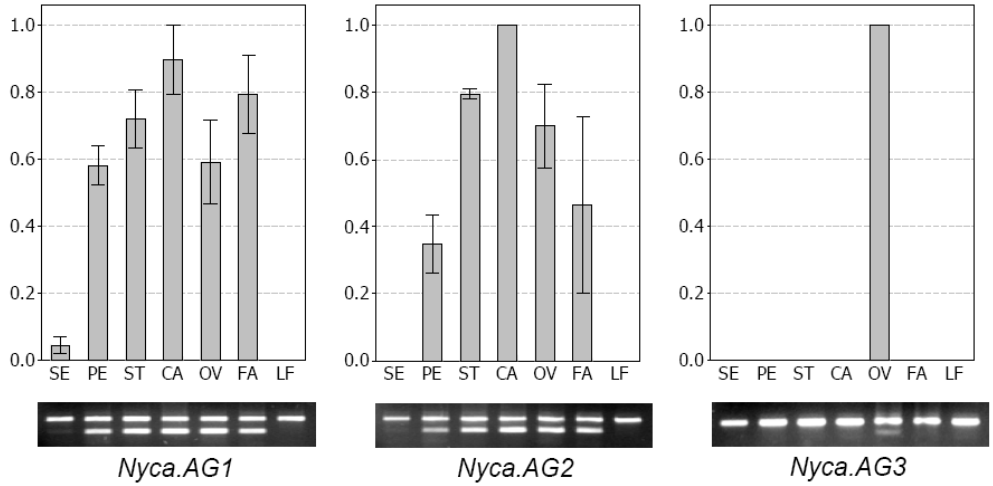


Figure 4-4. Continued.

E. *Nelumbo nucifera*

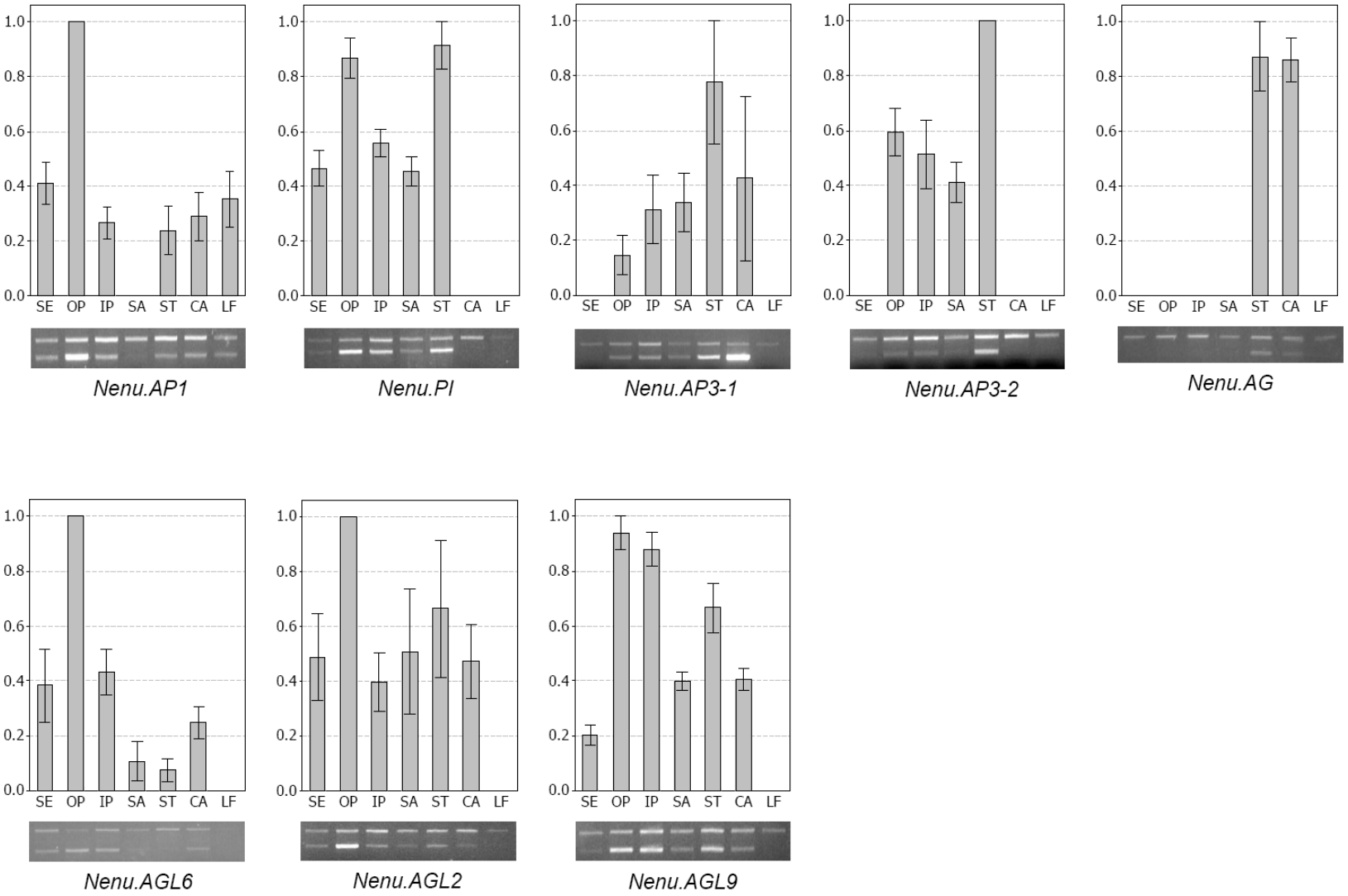


Figure 4-4. Continued.

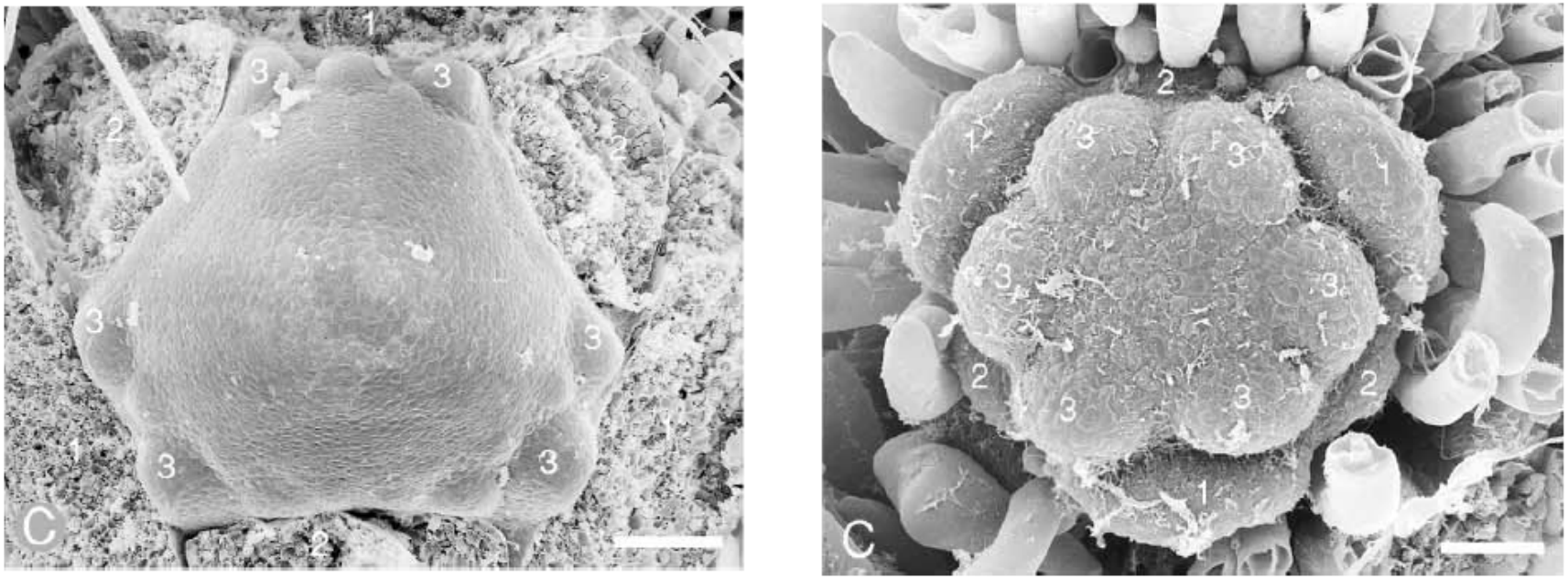


Figure. 4-5. Developmental features of *Nuphar* and *Cabomba*. *A*, Petal initiation (3) in *Nuphar advena*. *B*, Stamen initiation (3) in *Cabomba caroliniana*. Images from Endress (2001).

CHAPTER 5
ANALYSIS OF THE FLORAL TRANSCRIPTOME OF A BASAL ANGIOSPERM, *NUPHAR ADVENA* (NYMPHAEACEAE)

Introduction

Extensive genetic analyses, particularly of *Arabidopsis* and *Antirrhinum* floral mutants, have led to the development of the ABCDE model for the molecular mechanism controlling floral organ identity: A and E class genes control sepal identity; A, B, and E class genes control petal identity; B, C, and E class genes control stamen identity; C and E class genes control carpel identity; D and E class genes control ovule identity (Coen and Meyerowitz 1991; Colombo et al. 1995; Ditta et al. 2004; Pelaz et al. 2000). . In *Arabidopsis*, A function is provided by *APETALA1* (*AP1*) and *APETALA2* (*AP2*), B function by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), C function by *AGAMOUS* (*AG*), and E function by multiple *SEPALLATA* gene products (*SEP1- 4*). Some aspects of the ABCDE model are conserved across eudicot and monocot angiosperms (i.e., *Petunia hybrida* (Angenent et al. 1993); *Silene latifolia* (Hardenack et al. 1994); *Gerbera hybrida* (Yu et al. 1999); *Oryza sativa* (Fornara et al. 2003; Kater et al. 2006; Kyozuka et al. 2000); *Pisum sativum* (Taylor et al. 2002); *Zea mays* (Whipple et al. 2004); *Magnolia grandiflora* (Kim et al. 2005), *Persea americana* (Chanderbali et al. 2006), *Akebia trifoliata* (Shan et al. 2006); *Elaeis guineensis* (Adam et al. 2007); *Taihangia rupestris* (Lü et al. 2007); *Vitis vinifera* (Poupin et al. 2007)). All of the ABCDE genes are transcription factors, and are thought to interact with genes involved in the establishment of floral meristem identity or floral organ formation (Jack 2004; Theissen 2001b; Wellmer et al. 2006). However, relatively few downstream targets have been identified, possibly due in part to limitations of genetic approaches based on mutant analyses (Wellmer et al. 2006). Also, many duplication events leading to functional redundancy may hinder elucidation of functional roles in flower

development (Arora et al. 2007; Cho et al. 2002; Moore et al. 2005; Moore and Purugganan 2005; Wellmer et al. 2006).

Recent studies of gene expression profiling using microarray techniques provide valuable information for understanding the transcriptional programs of plants on a genome-wide scale (Alves-Ferreira et al. 2007; Arora et al. 2007; Birnbaum et al. 2003; Cho et al. 2002; Wellmer et al. 2004; Wellmer and Riechmann 2005; Wellmer et al. 2006). In particular, several studies identified genes with potentially important roles in flower development (Gomez-Mena et al. 2005; Hennig et al. 2004; Schmid et al. 2003; Schmid et al. 2005; Wellmer et al. 2006; Zhang et al. 2005). For example, many potential signaling components, such as protein kinases and other putative signaling proteins, and transcription factors, were up-regulated during reproductive development (Hennig et al. 2004). Among the latter are members of the YABBY, MADS box, and MYB gene families, consistent with genetic studies of floral organ development (Bowman 2000; Golz and Hudson 1999; Ng and Yanofsky 2001; Theissen et al. 2000). Other studies have targeted the expression profiles of specific intra-floral organs and/or tissues, for example, stamens (Alves-Ferreira et al. 2007; Wang et al. 2005; Wellmer et al. 2004), stigma (Li et al. 2007), female gametophyte (Yu et al. 2005), and embryo sac (Jones-Rhoades et al. 2007).

Microarray-based analyses are also useful for comparing the expression profiles of duplicated genes or members of gene families within organisms (Arora et al. 2007; Schmid et al. 2005; Wellmer et al. 2006). For example, Schmid et al. (2005) showed that large gene families, such as WRKY and MADS transcription factors, which are important developmental regulators, exhibited correlated expression patterns; many WRKY genes are preferentially expressed in leaves while many MADS-box genes are mainly expressed in flowers and fruits. Wellmer et al. (2006) also showed that in *Arabidopsis* closely related genes in gene families are highly

correlated in their temporal gene expression patterns, and the majority of those genes is up-regulated during certain developmental stages.

As illustrated by the studies above, nearly all transcriptome analyses of floral development have so far focused on *Arabidopsis* (Gomez-Mena et al. 2005; Hennig et al. 2004; Schmid et al. 2003; Schmid et al. 2005; Wellmer et al. 2004; Wellmer et al. 2006; Zhang et al. 2005). Here, we report the floral transcriptome profiling of *Nuphar advena*, a member of the Nymphaeales (water lilies) which, together with Hydatellaceae (Saarela et al. 2007), lie sister to all other extant angiosperms but *Amborella* (Fig. 5-1) (Borsch et al. 2005; Hilu et al. 2003; Jansen et al. 2007; Löhne and Borsch 2005; Leebens-Mack et al. 2005; Mathews and Donoghue 1999; Moore et al. 2007; Qiu et al. 1999; Qiu et al. 2005; Soltis et al. 1999; Soltis et al. 2000; Soltis et al. 2005; Zanis et al. 2002; Zanis et al. 2003). Nymphaeales consist of two families, Cabombaceae and Nymphaeaceae, and reconstructions of floral evolution suggest that a small number of floral parts and whorled phyllotaxy (trimery) are ancestral characteristics (Borsch et al. 2008; Les et al. 1999; Zanis et al. 2003), whereas flowers with large number of floral parts, e.g. *Nymphaea*, *Victoria*, and other water lilies, are derived in Nymphaeaceae (Borsch et al. 2008; Les et al. 1999; Zanis et al. 2003). *Nuphar* is sister to all other genera of Nymphaeaceae (Borsch et al. 2007; Borsch et al. 2008; Löhne et al. 2007; Les et al. 1999), and has trimerous flowers of moderate size; the ancestral character states of Nymphaeales (Les et al. 1999). The flower of *Nuphar advena* consists of two perianth whorls (three sepals and three petals), numerous staminodes, numerous stamens, and numerous carpels in a syncarpous gynoecium (Fig. 5-2). The two perianth whorls differ in color: the sepals in the outer whorl are green, and the petals in inner whorl are yellow (Fig. 5-2; Padgett et al. 1999; Warner et al. 2008). The staminodes are smaller

than the sepals and petals, but broader than the stamens, with a nectary on their adaxial surface (Fig. 5-2).

We have used floral ESTs collected by the Floral Genome Project (Albert et al. 2005; http://fgp.bio.psu.edu/fgp/index.html) to conduct the first investigation of the floral transcriptional profile in *Nuphar*, one of the basalmost angiosperm lineages. Specifically, we assessed gene expression levels in both young and medium-aged floral buds, as well as sepals, petals, stamens, carpels, fruits, relative to leaves, to provide an assessment of the genes involved in floral development in one of the basal-most angiosperms. Also, we conducted comparative analyses of similar data sets for *Arabidopsis* (Schmid et al. 2005) and *Persea* (Chanderbali et al. submitted), the latter a member of the large magnoliid clade of basal angiosperms, to infer evolutionary trends in floral transcriptomes.

Materials and Methods

Sample Preparation, Probe Labeling and Microarray Hybridization

Tissues were collected from four individuals (biological replicates) of *Nuphar advena* in Pennsylvania, USA. The tissues include young leaf tissue, "young" floral buds (Ybd) at the pre-microsporangia initiation stage, "medium-aged" floral buds (Mbd) at the pre-meiotic stage, and sepals, petals, stamens, and carpels, dissected from flowers at anthesis. Total RNA was extracted from all tissue samples using the RNeasy Plant Mini Kit (Qiagen, Stanford, CA, USA). Both the quantity and quality of RNA were assessed using a 2100 Agilent Bioanalyzer. RNA transcripts were labeled using the Low-input RNA labeling kit (Agilent Inc.) and hybridized to the arrays according to the manufacturer's protocol.

Microarray Design

We used custom microarrays produced by Agilent Technologies (Palo Alto, CA), containing 10,187 60-mer oligonucleotide probes, targeting 6,220 unique *Nuphar* floral transcripts collected from a pre-meiotic floral cDNA library by the Floral Genome Project (Albert et al. 2005, Soltis et al. 2007; http://fgp.bio.psu.edu/fgp/index.html). The oligonucleotide probes were *in situ* synthesized and randomly arranged on the arrays. For quality control checks of hybridization, 544 Agilent controls were included in the arrays. We measured expression level in different developmental stages of the flower (pre-microsporangia initiation stage vs. the pre-meiotic stage in floral buds), sepals, petals, stamens, carpels, fruits, and young leaves, using an interwoven double loop for eight tissues with 16 arrays (Fig. 5-3), which minimizes the variance of pair-wise comparisons between any two tissues and efficiently detects differentially expressed genes.

Data Acquisition and Statistical Analysis

Microarrays were scanned with an Agilent DNA microarray scanner using Agilent's Feature Extraction Software 9.1.3 (Agilent Inc.). Raw data were imported into the Bioconductor package Limma and processed as previously described (Chanderbali et al. submitted). After quality control checks for hybridization, arrays were background corrected and loess normalized within arrays and Aq normalized between arrays (Smyth et al. 2006; Yang and Thorne 2003). To identify significantly differentially expressed genes among the eight tissue samples, we employed a one-way empirical Bayes ANOVA using single channel analysis while considering correlation between channels at each spot (Smyth 2004; Smyth et al. 2005). Data for all genes showing differential expression were assembled by hierarchical clustering (Eisen et al. 1998), as implemented by de Hoon et al. (2002), into groups with similar gene expression patterns.

Specifically, $\log_2$ ratios relative to leaf for 3,333 genes with a differential expression probability greater than 95% (p-value <0.05) were read into CLUSTER 3.0 (de Hoon et al. 2002) and hierarchical clustering performed via centroid linkage of Pearson correlations. The cluster results were visualized using Java TreeView (Saldanha 2004). We also searched $\log_2$ transformed expression data of differentially expressed genes for transcripts with at least two-fold up-regulation in floral organs relative to leaves to identify genes significantly up-regulated in particular floral organs and/or floral stages.

Comparison of Microarray Data with Relative Quantitative Reverse Transcription PCR (RT-RQ-PCR) Data

We compared our microarray data with RT-RQ-PCR data for *Nuphar* from Kim et al. (2005), Zahn et al. (2005), and Yoo et al. (in prep.) for *Nuphar* homologs of several floral organ identity genes; *AGAMOUS* (*AG*), *PISTILLATA* (*PI*), *APETALA3* (*AP3*), *AGAMOUS-LIKE 2* (*AGL2; SEP1*), *AGAMOUS-LIKE 6* (*AGL6*), and *APETALA1* (*AP1*). From our microarray data for *Nuphar*, we used the absolute amount of gene expression in each tissue and then compared those expression patterns relative to leaves. In this way we assessed whether gene expression patterns from microarray and RT-RQ-PCR are consistent with each other.

Comparative Floral Transcriptomics

To investigate evolutionary patterns in floral transcriptomes we compared the expression profiles of differentially expressed *Nuphar* genes with those of their putative homologs in the model angiosperm *Arabidopsis*, representing the derived eudicot lineage Brassicaceae, and *Persea*, of the magnoliid clade of basal angiosperms. In the absence of reliable gene family phylogenies, except for MADS box genes (Kim et al. 2005), we based gene homology assignments on amino acid sequence similarity (best reciprocal BLAST E score < 10^{-5}). We

combined the AtGenExpress (Development) expression data for *Arabidopsis* (Schmid et al. 2005) and the *Persea* data set (Chanderbali et al. submitted) with our *Nuphar* data set based on putative homology to construct two separate data sets: (1) *Nuphar* and *Arabidopsis* homologs (*Nuphar* subset 1), and (2) *Nuphar*, *Persea* and *Arabidopsis* homologs (*Nuphar* subset 2). Each data set was subjected to hierarchical clustering analyses, pair-wise plots of gene expression levels in floral organs within species to compare the extent of correlation in their transcriptional profiles, and sorted by organ of primary positive expression ratios relative to leaves to compare the relative extent of expression domains. Finally, we searched for genes with organ-specific expression in accordance with the criteria of Chanderbali et al. (submitted), where organ-specific expression is defined as an at least two-fold up-regulation in the target floral organ and less than 1.5-fold increase in all others.

Results

Genes Expressed Differentially in Reproductive Organs

We identified 3,333 differentially expressed genes among the eight tissue samples ($P < 0.05$; FDR = 0.38%), of which 2,165 were up-regulated in floral tissues, including 831 at two-fold or higher expression levels relative to leaves (Table 5-1). Thus, 53.6% (3,333 out of 6,220) and 13.4% (831 out of 6,220) of the genes surveyed are differentially expressed and up-regulated in floral tissues relative to leaves with a minimum of two-fold higher expression levels. These values found in *Nuphar* are low relative to those obtained from gene expression profiles of *Persea*, which was studied using a similar research strategy: 77.9% of genes (4,744 out of 6,086) showed differential expression, and 17.8% of them (1,084 out of 6,086) were up-regulated in floral tissues and/or stages relative to leaves. We identified only 22 and 23 genes at least 2-fold up-regulated in carpels and stamens of *Nuphar* whereas 68 and 105 such genes were up-

regulated in carpels and stamens of *Persea*, respectively (Table 5-1). Most of the genes up-regulated in *Nuphar* floral organs are primarily expressed in perianth organs and stamens (35.8%), followed by perianth organs alone (20.3%). Therefore, the most common pattern was observed in both perianth members and stamens with 88 genes, followed by both sepals and petals, and all floral organs (Table 5-1).

Among the genes down-regulated in *Nuphar* flowers are transcription factors such as members of the bHLH, homeobox, SBP (SQUAMOSA-pROMOTER BINDING PROTEIN), and WRKY families (Table 5-2). Several are related to leaf morphogenesis in *Arabidopsis*, for example, homologs of *CURLY LEAF* (Kim et al. 1998), *BRASSINOSTEROID-INSENSITIVE 2* (Perez-Perez et al. 2002), and *FASCIATA 2* (Kaya et al. 2001) (Table 5-2). However, several genes related to flower development are also down-regulated in some reproductive organs. For example, a homolog of *ABNORMAL FLORAL ORGANS*, required for flower organ formation and development in *Arabidopsis* (Chen et al. 1999), is down-expressed in all reproductive organs except Ybd, indicating an exclusively early role in *Nuphar*.

As observed for non-floral genes, many genes up-regulated in *Nuphar* flowers are transcription factors of the bHLH, bZIP, MADS, and Myb-related gene families (Table 5-3). All the *Nuphar* MADS-box genes examined, except the *AP1* homolog, are up-regulated in reproductive organs. bZIP transcription factors are diverse in their functions, including light and stress signaling, floral transition, and seed development (Jakoby et al. 2002; Nijhawan et al. 2008). Eight members of the bZIP family are differentially expressed in *Nuphar*, all mainly expressed in Ybd. *NAC-LIKE* (*NAP, ACTIVATED BY AP3/PI*) plays a role in cell morphogenesis and leaf senescence (Guo and Gan 2006; Li et al. 2004) and floral development in *Arabidopsis*, particularly in petals and stamens (Sablowski and Meyerowitz 1998), but the *Nuphar* homolog is

expressed in early floral stages (Ybd and Mbd) and not in mature stamens and petals. *KANADI1* (*KAN1*) is known to regulate leaf and carpel polarity in *Arabidopsis* and is expressed temporarily on the abaxial side of initiating floral-organ primordia (Kerstetter et al. 2001). The *Nuphar KAN1* homolog is expressed in petals, stamens, and carpels.

Comparison of Microarray Data with RT-RQ-PCR Data

Previously reported RT-RQ-PCR data for *Nuphar* homologs *AG*, *PI*, *AP3*, *AP1*, and *SEP1* (Kim et al. 2005, Zahn et al. 2005) generally agree with the expression patterns obtained in our microarray experiments (Fig. 5-4). This is especially true for homologs of *AP3* and *PI*. Transcripts of a *Nuphar AP1* homolog were barely detectable in floral organs with RQ-RT-PCR, and the same holds true in our microarray data (Fig. 5-4). The *Nuphar AG* homolog was only detected in stamens and carpels by RQ-RT-PCR techniques, and is strongly up-regulated in stamens and carpels relative to leaves according to the microarray datas, but it is also up-regulated, albeit at lower expression levels, in sepals and petals (Fig. 5-4). Similarly, the *Nuphar AGL6* homolog was detected in sepal, petals, and carpels with RQ-RT-PCR (Yoo et al. in prep) whereas some stamen expression was detected with microarrays, again at lower levels compared to the other floral tissues (Fig. 5-4).

Hierarchical Clustering

Hierarchical clustering sorted differentially expressed genes into florally "up-regulated" and "down-regulated" clusters (Fig. 5-5). Within the up-regulated cluster, two major subsets accommodate genes expressed primarily in carpels, including the *Nuphar* homolog of *AG*, and genes expressed broadly across the flower but primarily in stamens, sepals and petals including homologs of *AP3*, *PI*, *SEP1*, and *AGL6*. The *AP1* homolog was placed in the "down-regulated" cluster together with most genes primarily expressed during the earliest developmental stages

sampled. A second probe targeting the *PI* homolog detected a similar expression pattern and was also placed in the "down-regulated" cluster (Fig. 5-5). Clustering of tissues separated the two floral buds from mature organs suggesting extensive turnover in transcriptional programs through developmental stages, and grouped sepals with petals, followed by stamens, then carpels in the hierarchy of floral organ relationships (Fig. 5-5).

Comparative Floral Transcriptomics

Hierarchical Clustering: Among the differentially expressed *Nuphar* genes, 2,449 have homologs in the AtGenExpress data set for *Arabidopsis* , of which 957 are also present in the *Persea* data set. Hierarchical clustering of the two matrices generated gene clusters characterized by up regulation in *Nuphar* but down-regulation in *Arabidopsis* and vice versa (Fig. 5-7*A*,) and up-regulation in either *Nuphar*, *Persea*, or both, but down-regulation in *Arabidopsis,* and vice versa (Fig. 5-7*B*,). We observed that a very small proportion of the genes show similar expression patterns between *Nuphar* and *Arabidopsis* (Fig. 5-7*A*). Those genes are involved in metabolism and energy production, and this is also found in *Nuphar* subset 2 (*Nuphar*, *Arabidopsis*, and *Persea*).

Floral organs clustered into groups in accordance with species, with *Nuphar* and *Persea* flowers transcriptionally closer to each other than either is to *Arabidopsis* flowers. Also, in both *Nuphar* and *Persea*, perianth organs cluster together, followed by stamens, as in analyses of the total *Nuphar* (Fig. 5-7*A*) and *Persea* (Chanderbali et al. submitted) data sets. However, *Arabidopsis* petals always cluster with stamens, and sepals are either placed distant from the other floral organs (Fig. 5-7*A*; *Nuphar-Arabidopsis* data set) or next to the petal/stamen cluster (Fig. 5-7*B*; *Nuphar-Persea-Arabidopsis* data set).

Investigation of Spatial Gene Expression Patterns: Three analyses were conducted to examine spatial gene expression patterns across floral organs. First, in scatter plots comparing the transcriptional profiles of all pairs of floral organs (Fig. 5-8), *Arabidopsis* floral organs are more divergent from each other than those of *Nuphar* and *Persea*. Petals and stamens share the highest similarity among *Arabidopsis* organs, but with rather low correlations (r^2=0.44), compared to almost linear correspondence between petals (inner tepals) and sepals (outer tepals) for *Nuphar* and *Persea* perianth organs, correlations of r^2=0.93 and 0.96, respectively (Fig. 5-8). Perianth organs and stamens are also highly correlated in the two basal angiosperms, with r^2=0.79 and 0.73 for *Nuphar* and r^2=0.63 and 0.67 for *Persea* comparing stamens and outer and inner perianth whorls, respectively. However, low correlation between carpels and other floral organs of *Persea* are observed (i.e., r^2<0.4).

Next, to examine the relative expression domains of each floral organ, we sorted expression level of genes with positive $\log_2$ value according to organ of primary expression. In all three data sets (*Nuphar*, *Nuphar* subsets 1 and 2), *Nuphar* genes showed extended expression domain from one organ to adjacent organs (Fig. 5-9*A*-*C*). *Persea* genes also exhibited similar trends to those of *Nuphar*, while in *Arabidopsis* there is little "spill over" into adjacent organs (Fig. 5-9*B*, *C*) (see also Chanderbali et al. submitted).

Organ-specific expression in *Nuphar*, in accordance with the criteria of Chanderbali et al. (submitted), identified 22 carpel-specific genes, 23 specific to stamens, and 11 and eight specific to petals and sepals, respectively. In our search for genes specific to combinations of adjacent floral organs, we identified two genes specific to carpels and stamens, one specific to stamens and petals, 50 specific to petals and sepals, and 88 specific to perianth organs and stamens. Similar analyses for subsets 1 and 2 of the *Nuphar* data set are presented in Fig. 10. In general,

we find stronger representation of genes with organ-combination-specific than with organ-specific expression in *Nuphar*, with perianth organs plus stamens accounting for ~43% of the observed instances, followed by sepals plus petals (Fig. 5-10*A*-*B*). In *Persea*, approximately 33% of the genes exhibiting up-regulation are co-expressed in outer + inner tepals and perianth organs + stamens (Fig. 5-10*A*-*B*). However, in *Arabidopsis* a relatively low number of genes is co-expressed in carpels/stamens and stamens/petals (Fig. 5-10*A*-*B*).

Discussion

Genes Expressed Differentially in Reproductive Organs

From the microarray experiments, we found that in *Nuphar* the number of genes differentially expressed and up-regulated in floral tissues relative to leaves (with a minimum of two-fold higher expression), is low relative to the number obtained from gene expression profiles of *Persea* (Table 5-1). The difference between the expression profiles of *Nuphar* and *Persea* is mainly due to the low number of genes expressed in carpels and stamens of *Nuphar*. In fact, most of the genes up-regulated in floral tissues and/or stages in *Nuphar* are found in perianth members, but only a very small number of the genes are found in carpels and stamens in *Nuphar*. In contrast, in *Persea* a majority of the genes up-regulated in reproductive organs is found in stamens and carpels (Table 5-1). This finding can be explained by floral developmental features of *Nuphar*. Although a cDNA library was constructed using the floral buds from premeiotic stages, perianth members of *Nuphar* at premeotic stages are relatively larger than stamens and carpels. Therefore, there is a greater possibility of including more genes related to perianth development in the library. This inference is further supported when we examined the genes up-regulated in specific floral organs in the combined data set from *Nuphar*, *Arabidopsis*, and *Persea* (*Nuphar* subset 2). Even though we have the same gene homologs in *Nuphar* subset 2 for

these three species, in *Nuphar* most of the genes are up-regulated in perianth members and stamens, while the majority of the genes are up-regulated in stamens and carpels in *Persea* and carpels in *Arabidopsis* (Fig. 5-10*B*). Therefore, the difference in the proportions of the genes differentially expressed between *Nuphar* and *Persea* can be explained by their different floral morphologies.

When we checked for down- or up-regulated genes in reproductive organs, we found that some of them may have a different function in *Nuphar* compared to their function in *Arabidopsis*. For example, *AFO* is required for floral organ formation and development (Chen et al. 1999); thus, it is mainly expressed in the very early stage of floral development. In our experiment, this gene homolog is weakly expressed in Ybd, the stage at which all initial floral organ formation takes place, so down-regulation in reproductive organs is consistent with expression patterns in a later stage. Also, a homolog of *BARELY ANY MERISTEM 2* (*BAM2*, At3g49670), a regulator of early anther development in *Arabidopsis* (Hord et al. 2006), is also down-regulated in all reproductive tissues in *Nuphar*. However, since we only included floral tissues before microsporangia initiation, we would not expect to see expression in our samples. Importantly, other gene homologs in *Nuphar*, such as *NAP*, *KAN1*, *VRN5*, and *TUF*, are expressed in different floral organs compared to their function in *Arabidopsis*. Thus, the homologs of these genes may have different functions in *Nuphar* than in *Arabidopsis*; the roles of these genes in flower development in *Nuphar* should be addressed using other approach.

In general, microarray data for homologs of floral organ identity genes in *Nuphar* confirm expression patterns obtained in earlier RQ-RT-PCR experiments (Yoo et al. in prep.). In those instances of inconsistency, microarray data suggested broader expression patterns than RQ-RT-PCR data for homologs of *AP1*, *AG*, and *AGL6*. Whether these reflect greater sensitivity, un-

specific binding, or experimental variation in cDNA populations, remains unclear. RQ-RT-PCR uses sequence-specific primer sets to amplify target cDNA, whereas microarray experiments are based on hybridization between probes and target cRNA. Therefore, if duplicate gene copies with sufficient sequence similarity exist in the cDNA population, the probe may bind to both and wider gene expression patterns could be detected if duplicates collectively exhibit broader gene expression domains. Similar results would be obtained if microarray probes target conserved motifs. Also, expression levels measured by RQ-RT-PCR are relative to that of an internal control, therefore, direct comparison between these two experiments may only be partially reliable. When we tried to examine the expression patterns of *Nuphar AP1* and *AGL6* homologs without the internal control, those transcripts were detected in all floral tissues even though there is variation in signal intensities as shown in microarray data (data not shown). However, as for an *AG* homolog from *Nuphar*, we still could not detect any *AG* transcripts in sepals and petals. Closer examination of microarray expression levels in floral tissues relative to leaf tissue, shows that the expression levels are relatively high in stamens and carpels (Fig. 5-4), suggesting that expression levels in sepal and petals may lie below the threshold sensitivity of RQ-RT-PCR. However, the broader expression pattern observed in microarrays might be due to the non-specificity of the *Nuphar AG* probe, since it is designed between the MADS-box and I region. However, BLAST analyses of the probes against NCBI nr database only hit other members of the *AG* gene lineage; therefore, there is a higher possibility that it binds other *AG* homologs in the transcriptome of *Nuphar* sepals and petals, than more distantly related MADS-box genes.

Hierarchical Clustering

The four main gene clusters correspond to distinct spatial expression patterns. The *AG* cluster contains the *Nuphar* homolog of *AG* and most of the genes with two-fold or higher

expression levels in carpels relative to leaves (Fig. 5-6). The *AG* homolog from *Nuphar* is strongly up-regulated in all floral buds and floral tissues, although its expression is restricted to stamens and carpels in RQ-RT-PCR data (Yoo et al. in prep.). Other genes highly expressed in carpels are related to diverse functions, such as carbon metabolism (At1g23760, At1g48100, At5g66460), stress response (At1g56340, At3g58450), and systemic interaction with the environment (At2g46370, At3g25230).

The *AP3*/*PI*/*AGL2*/*AGL6* module contains the largest number of genes among the four regulatory modules, and *AGL6* homolog of *Nuphar* was separated from the main cluster with other two genes, *serine carboxypeptidase-like 45 precursor* (*SCPL45*, At1g28110) and expressed protein (At2g35880) (Fig. 5-5). These three genes exhibited a two-fold higher expression in all floral tissues and carpels relative to leaves (Fig. 5-6). In *Arabidopsis*, *SCPL45* is expressed everywhere, including seedlings, young leaves, flowers, and fruits (Fraser et al. 2005). However, its expression is very weak in old leaves and mature organs, such as stem and root. A *SCPL45* homolog from *Nuphar* is also up-regulated in all floral organs and medium-sized floral buds, but not in leaves. *AGL6* (At2g45650) is particularly highly expressed in sepals, petals and carpels of *Arabidopsis* (Schmid et al. 2005), and this expression pattern is also shown by its *Nuphar* homolog (Fig. 5-4).

Most of the genes in the *AP3*/*PI*/*AGL2*/*AGL6* module were expressed primarily in petals, sepals and petals, or both perianth members and stamens (Fig. 5-6). Together with homologs of floral organ identity genes, such as *AP3*, *PI*, and *AGL2*, MYB or MYB-related transcription factors are up-regulated in perianth members and/or stamens relative to leaves (two-fold higher expression level).

The *AP1* module includes an *AP1* homolog of *Nuphar*, and most of the genes in this module were up-regulated in floral buds (Fig. 5-6). Some of them are involved in meristem activity or flowering transition in *Arabidopsis*. First, *AP1* is an A-function gene and helps to regulate sepal identity in *Arabidopsis* (Bowman et al. 1993), but its inferred function (based on its expression pattern) differs in *Nuphar*. It is up-regulated during early flower development relative to leaves, especially at the pre-microsporangia initiation stage, but not in floral organs. Also, *ATSKP1* (At1g75950), which is mainly involved in meristem activity is included in this module. Porat et al. (1998) showed that mRNA of *ATSKP1* accumulated in all of the plant meristems, including inflorescence and floral meristems, and our data is consistent with their observation.

The last module contains a *PI* homolog of *Nuphar*, and most of the genes showed similar expression patterns to those of genes in the *AP3*/*PI*/*AGL2* module. However, genes included in the *PI* module were also highly expressed in floral buds. In contrast to a *PI* homolog in the *AP3*/*PI*/*AGL2* module, this *PI* shows relatively high expression in both floral buds rather than floral tissues of *Nuphar*. Considering we include only one *PI* homolog in the array, this observation might be due to the non-specificity of probes. The probe in the *PI* module is designed from the MADS-box region, so it can potentially hybridize to other MADS-box genes that are highly expressed in early floral developmental stages. However, the probe in the *AP3/PI/AGL2* module is designed from sequences between the K-box and the C-terminal region, so it is more *PI*-specific and its expression pattern is more accurate. For example, most of the genes from the *AP3*/*PI*/*AGL2*/*AGL6* module are mainly expressed in perianth members and stamens (Fig. 5-6). However, when we examine gene expression in detail, there can be differences in the gene expression level between the floral organs. For example, *AGL6*

(At2g45650) is highly expressed in all floral organs of *Arabidopsis* (Schmid et al. 2005), and this expression pattern is also observed in *Nuphar*. However, the expression level of an *AGL6* homolog in stamens is half the expression level of other floral organs (Fig. 5-4). Similar expression patterns are shown by the *AGL6* homologs of *Persea* and *Hordeum* from microarray data (Chanderbali et al. submitted; Druka et al. 2006). In *Persea*, two *AGL6* homologs are highly expressed in outer and inner tepals and carpels, but show relatively low expression in stamens (Chanderbali et al. submitted). Also, an *AGL6* homolog of *Hordeum* is up-regulated in inflorescence, bracts, and carpels, but the expression level is three times less in anthers (Druka et al. 2006). These data indicate that *AGL6* homologs in basal angiosperms might differ in their function from that of the model eudicot *Arabidopsis*. In *Arabidopsis*, there are two paralogs in the *AGL6* lineage, *AGL6* and *AGL13* (Martinez-Castilla and Alvarez-Buylla 2003; Rounsley et al. 1995): *AGL6* is expressed in inflorescence buds (Duarte et al. 2006; Ma et al. 1991), whereas *AGL13* is expressed in ovules only (Rounsley et al. 1995). Thus, before the duplication event, one copy of *AGL6* might have played a role in both perianth and ovule development. For example, Mena et al. (1995) showed that two *AGL6* homologs, *ZAG3* and *ZAG5*, are present in maize, and *ZAG3* functions in inflorescence and carpel development although this gene is expressed in sterile floral organs that correspond to sepals and petals of eudicots. *ZAG5* is homologous to *ZAG3*, so it is suggested that these two genes were duplicated via polyploidy and subsequently diverged (Mena et al. 1995). However, the function of *ZAG5* is not well known. Thus, we cannot tell whether divergence in function of *AGL6* homologs occurs in maize. In other monocots, *Oncidium* and *Hyacinth*, *AGL6* homologs are detected in floral buds, perianth members (lip or tepals), and carpels, but not in stamens (Fan et al. 2007; Hsu et al. 2003). In addition, through transgenenic experiments, these two genes have been shown to play a role in

the regulation of flower transition and organ formation, as does *AP1* in *Arabidopsis* (Fan et al. 2007; Hsu et al. 2003). In Nymphaeales, *AGL6* homologs from *Nymphaea* and *Cabomba* are also expressed in perianth members and carpels (Yoo et al. in prep.). In other basal angiosperms, *AGL6* transcripts were detected only in perianth members (*Magnolia* and *Liriodendron*, Kim et al. 2005, *Persea*, Chanderbali et al. 2006). Together with these reported expression patterns, *AGL6* homologs might contribute to perianth development in basal angiosperms (and monocots) (as a putative A-function gene) because they are mainly expressed in perianth members (Kim et al. unpubl. data; Yoo et al. in prep). However, *AGL6* homologs are also involved in carpel development in most basal angiosperms and monocots; thus, more extensive study of expression throughout the angiosperms is needed to evaluate the idea of *AGL6* homologs as A-function genes which promote perianth organ identity in basal angiosperms.

In the *AP3*/*PI*/*AGL2*/*AGL6* module, together with homologs of floral organ identity genes, such as *AP3*, *PI*, and *AGL2*, MYB or MYB-related transcription factors are up-regulated in perianth members and/or stamens relative to leaves (two-fold higher expression level). This result is consistent with the study of Henning et al. (2004), which showed that many potential signaling components, such as protein kinases, transcription factors, and other putative signaling proteins, were up-regulated during reproductive development. However, most genes in this module do not have *Arabidopsis* homologs, so it is hard to infer their function.

Another MADS-box gene homolog exhibiting a different expression pattern from its function in *Arabidopsis* is an *AP1* homolog of *Nuphar*. Originally, *AP1* was identified as an A-function gene to regulate sepal identity in *Arabidopsis* (Bowman et al. 1993). However, a recent study of *ap1* mutants of *Arabidopsis* suggests that *AP1* function is not essential for sepal and petal development: over-expression of *AGL24* seems to be responsible for defects of sepals and

petals in the *ap1-1* mutants. Thus, in the absence of *AGL24*, *ap1* mutants partially recover their wild type phenotypes (Yu et al. 2004). Therefore, together with the expression patterns of *AP1* homologs from other eudicots such as *Petunia* (Rijpkema et al. 2006), *Antirrhinum* (Davies et al. 2006), and *Gerbera* (Teeri et al. 2006), the identity of the true A function gene is questionable.

The *AP1* homolog from *Nuphar* also displayed different expression patterns from *AP1* in *Arabidopsis*. The *AP1* homolog from *Nuphar* is up-regulated during early flower development relative to leaves, especially at the pre-microsporangia initiation stage, but not in floral organs. This expression pattern is consistent with several recent studies, suggesting that *AP1* homologs may not be floral organ identity genes (Chen et al. 2008; Huijser et al. 1992; Shan et al. 2007; Taylor et al. 2002; Zhang et al. 2008). Rather than being expressed only in sepals and petals, most *AP1* homologs are expressed in both vegetative and reproductive organs, showing the highest expression in inflorescence and floral meristem (Shan et al. 2007). Our microarray data and RQ-RT-PCR data (Yoo et al. in prep.) also show a similar pattern; the *AP1* homolog is expressed in both vegetative and reproductive organs, exhibiting the highest expression in floral buds. Thus, considering that *AP1* is involved in both vegetative and reproductive development in other species, our result for *Nuphar* is consistent with other studies (Chen et al. 2008; Huijser et al. 1992; Shan et al. 2007; Taylor et al. 2002; Zhang et al. 2008) (Shan et al. 2007).

The four modules obtained in the *Nuphar* floral transcriptome appear to exploit similar gene contents, for example, MADS-box genes and other transcription factors, to those of *Arabidopsis*, and suggest their functional conservation in major regulatory elements between these two species (Fig. 5-6). However, we have only focused this discussion on genes related to floral meristem and organ formation, so further comparison between *Nuphar* and *Arabidopsis* is required (see below).

Comparative Floral Transcriptomics

Hierarchical Clustering: From *Nuphar* subsets 1 and 2, we found that the majority of the genes showing similar expression patterns is involved in metabolism and energy production. Also, MADS-box gene homologs from *Nuphar, Persea*, and *Arabidopsis* exhibit similar expression patterns (Fig. 5-7*B*). However, other genes regulating flower development, such as *NAP*, *AFO*, are expressed in different floral organs in the three species, indicating that those genes function differently in these three species. This results suggest that even though the major elements in floral organ formation work in similar ways among these species, other genes acting downstream of these key floral regulators act differently in these three species. Therefore, modification and refinement in downstream gene function may be responsible for floral diversification across the angiosperms.

Arrays also clustered into three groups that correspond to species. In contrast to *Nuphar* and *Persea*, which perianth members are clustered together with stamens, in *Arabidopsis* petals and stamens are clustered together with carpels in *Nuphar* subset 1 and with sepals in *Nuphar* subset 2 (Fig. 5-7*A, B*). Considering that the branch lengths between the cluster of petals and stamens and other organs are relatively short, this difference may be meaningless. Petals in basal angiosperms are thought to have been derived from bracts (bracteopetals), while petals of eudicots are considered to be derived from of stamens (andropetals) (Endress 2001; Hiepko 1965; Takhtajan 1991). However, the petals (inner tepals) of *Nuphar* and *Persea* are less differentiated from the sepals (outer tepals) than observed in most eudicots, so the petals of these basal taxa may be not homologous to petals of *Arabidopsis*. Furthermore, the two perianth whorls of *Nuphar* and *Persea* clusterd with stamens, indicating that the perianth organs of these two species may have originated from stamens (i.e., the petals would therefore be andropetals).

Although this result contradicts the classic view of bracteopetals in basal angiosperms, other studies also supported the idea of andropetals in *Nuphar* (Yoo et al. in prep.) and *Persea* (Chanderbali et al. 2006).

Investigation of Spatial Gene Expression Patterns: To examine whether there is any relationship between gene expression patterns of floral organs, we drew scatter plots using the transcriptional profiles of floral organs in *Nuphar, Persea,* and *Arabidopsis*. We observed a strong linearity between perianth members of *Nuphar* and *Persea*, and this similarity in transcriptional profile might reflect their largely undifferentiated morphology, although their position and some subtle morphological characters at least in *Nuphar* can distinguish these two whorls. However, *Persea* differs from *Nuphar* in the low correlation between carpels and other floral organs in the former (i.e., $r^2<0.4$), suggesting different floral transcriptomic programs are exploited in carpels. In contrast to *Nuphar* and *Persea*, *Arabidopsis* showed a very weak correlation among the floral organs ($0.07<r^2<0.44$), thus it has more divergent transcriptional profiles relative to the other two species, representing an exploitation of different floral transcriptomes.

Next, we examined the relative expression domains of each floral organ. In *Nuphar* and *Persea*, which have less differentiated perianth members compared to *Arabidopsis*, genes with peak expression in sepals (outer tepals) are also strongly expressed in petals (inner tepals), indicating these perianth members may share genes. In both *Nuphar* and *Persea*, genes with peak expression in carpels seem to have unique expression patterns (Fig. 5-9*A*, *B*). In contrast to *Nuphar* and *Persea*, each organ of *Arabidopsis* has unique peak expression patterns. However, genes strongly expressed in petals are also expressed in sepals and stamens, although the reverse does not seem to be true (Fig. 5-9*A, B*).

The trends observed above were shown in a numerical way. There are large differences in the genes strongly expressed in one floral organ, but with lower expression in all other floral organs among three species. For *Nuphar*, a large number of genes is expressed in petals/sepals and petals/sepals/stamens, indicating that in *Nuphar* these three organs share very similar patterns of expression. Significantly, 88 genes are shared by these three organs, indicating a strong similarity among three organs. A similar pattern is observed in *Persea*; approximately 30% of the genes with up-regulation are co-expressed in outer and inner tepals and both perianth members and stamens (63 and 67 genes, respectively; Fig. 5-10*A*). In *Persea*, however, 105 genes are up-regulated only in stamens, indicating that stamens of *Persea* have a more unique transcriptome than do stamens of *Nuphar* (see below).

In *Arabidopsis* a relatively low number of genes is co-expressed in carpels/stamens and stamens/petals (Fig. 5-10*A*, *B*). In particular, more than 2.5 times many genes are co-expressed in petals/stamens compared to the number of genes co-expressed in petals/stamens, supporting the idea of andropetal orgin in eudicot.

The three species compared here (*Nuphar*, *Persea*, and *Arabidopsis*) show differences in spatial expression patterns of floral genes, and those patterns are correlated with different floral morphologies. For example, *Arabidopsis*, a derived core eudicot, has well-differentiated floral organs, and the number of genes expressed in more than one floral organ is very small, indicating that each floral organ has a unique floral transcriptional program. This likely typifies most eudicots. In *Persea*, a magnoliid, similar floral transcriptional programs characterize the morphologically undifferentiated inner and outer tepals and these programs differ from those for carpels and stamens. Although stamens share a number of genes with perianth members, many genes are exclusively up-regulated in stamens of *Persea*. This pattern in *Persea* may be

explained by morphology; *Persea* stamens possess well-differentiated anthers and filaments like *Arabidopsis*, so more genes are possibly associated with microsporogenesis. In *Nuphar*, which exhibits only modest differentiation between sepals and petals, petals appear to have a slightly different floral transcriptional program from sepals. However, the high proportion of genes expressed in both petals and sepals also implies that differentiation between them is not extensive; similarly, the morphological distinction between sepals and petals is also slight in *Nuphar* (see Warner et al. 2008). In addition, the higher proportion of genes commonly expressed in perianth members and stamens compared to the number of genes expressed exclusively in stamens suggests that the floral developmental program in stamens of *Nuphar* is not unique, as it is in *Persea* and *Arabidopsis*. We infer that differences among these species in spatial gene expression patterns among floral organs are consistent with their different floral morphologies.

In summary, the similarities of transcription profiles among floral organs indicate that *Nuphar* and *Persea* exploit the floral transcriptome in a similar way while *Arabidopsis* employs a much more divergent and refined program, which appears to have originated well after the origin of the flower itself. However, *Nuphar* and *Persea* also show differences in some spatial gene expression patterns, although they are similar in some aspects of floral morphology. For example, both share the same merosity (trimery in both species) and largely undifferentiated perianth members. In *Nuphar*, most of the floral genes are involved in the development of perianth members and stamens, and the floral transcriptional programs of these organs overlap substantially. However, *Persea* also exhibits different patterns from *Nuphar*; although inner and outer tepals share similar gene expression patterns in *Persea*, most of the genes are also

expressed in stamens and carpels. Thus, each floral organ (tepals, stamens, and carpels) of *Persea* is under a relatively unique transcriptional program compared to *Nuphar*.

Floral organ identity genes, such as *PI*, *AP3*, *AG*, and *AGL6*, showed similar expression patterns in *Nuphar*, *Persea*, and *Arabidopsis*, but most of the genes investigated exhibited different expression patterns (Fig. 5-7). This result suggests that even though the major elements in floral organ formation work in similar ways among species, other genes acting downstream of these key floral regulators act differently in these three species. Therefore, modification and refinement in downstream gene function may be responsible for floral diversification across the angiosperms.

Table 5-1. Number of genes up-regulated (two-fold higher expression level) in *Nuphar* floral tissue relative to leaves

Organ	No. of genes	Organ	No. of genes
Sepals	43	Petals	113
Sepals + Carpels	2	Petals + Fruits	1
Sepals + Fruits	4	Petals + Stamens	32
Sepals + Petals	101	Petals + Stamens + Carpels + Fruits	1
Sepals + Petals + Carpels	5	Petals + Stamens + Fruits	3
Sepals + Petals + Fruits	13	Stamens	73
Sepals + Petals + Stamens	124	Stamens + Carpels	2
Sepals + Petals + Stamens + Carpels	16	Stamens + Carpels + Fruits	1
Sepals + Petals + Stamens + Carpels +Fruits	25	Stamens + Fruits	13
Sepals + Petals + Stamens + Fruits	48	Carpels	23
Sepals + Stamens	30	Carpels + Fruits	7
Sepals + Stamens + Fruits	10	Fruits	65
		Buds	76

Table 5-2. Genes down-regulated in reproductive organs of *Nuphar*. Genes in red are involved in leaf morphogenesis. TFs=transcription factors, Mbd: medium-aged floral buds at the pre-meiotic stage, Ybd: young floral buds at the pre-microsporangia initiation stage.

Ath Homolog	Description	Expression area
At5g42700	ABI3-VP1 family (TFs)	All reproductive organs
At5g58280	ABI3-VP1 family	Sepal, petal, stamen
At2g41710	AP2-EREBP Family	All reproductive oragns except Mbd
At4g37750	AINTEGUMENTA (ANT), AP2-EREBP family (TFs)	All reproductive organs
At1g30330	ARF6, ARF family (TFs)	All reproductive oragns except carpel
At5g62000	Auxin Responsive Factor 2 (ARF2), ARF family (TFs)	All floral tissues
At1g05805	AtbHLH128, bHLH family (TFs)	All reproductive oragns except sepal, petal
At1g22490	AtbHLH94, bHLH family (TFs)	All reproductive oragns except Ybd
At1g63650	ENHANCER OF GLABRA 3 (EGL3, AtbHLH3), bHLH family (TFs)	All reproductive oragns except Ybd
At3g26744	INDUCER OF CBF EXPRESSION 1 (ICE1, AtbHLH116), bHLH family (TFs)	All reproductive organs
At4g01460	AtbHLH57, bHLH family (TFs)	All reproductive organs
At4g09820	TRANSPARENT TESTA 8 (TT8, AtbHLH42), bHLH family (TFs)	Ybd, sepal, petal, stamen
At1g06070	AtbZIP69, bZIP family (TFs)	Sepal, petal, stamen
At2g26580	YABBY5, C2C2-YABBY family (TFs)	Ybd, stamen, carpel, fruit
At2g45190	ABNORMAL FLORAL ORGANS (AFO, YAB1, FIL), C2C2-YABBY family (TFs)	All reproductive oragns except Ybd
At3g44750	C2H2 family (TFs)	Ybd, sepal, petal, stamen
At3g48430	C2H2 family (TFs)	Sepal, petal, stamen
At3g12680	ENHANCER OF AG-4 1 (HUA1), C3H family (TFs)	All floral tissues
At1g52150	ATHB15, Homeobox family (TFs)	All reproductive organs
At1g62990	KNOTTED-LIKE HOMEOBOX OF ARABIDOPSIS THALIANA 7 (KNAT7), Homeobox family (TFs)	All reproductive organs
At1g73360	HOMEODOMAIN GLABROUS11 (HDG11), Homeobox family (TFs)	All reproductive organs
At2g34710	PHABULOSA (PHB), Homeobox family (TFs)	All reproductive oragns except Ybd

Table 5-2. Continued

Ath Homolog	Description	Expression area
At3g61150	HOMEODOMAIN GLABROUS1 (HDG1), Homeobox family (TFs)	All floral tissues
At5g11510	AtMYB3R4, MYB family (TFs)	All reproductive organs
At5g53200	TRIPTYCHON (TRY), WRKY family (TFs)	All floral tissues except fruit
At1g08560	SYNTAXIN OF PLANTS 111 (SYP111, KNOLLE)	All reproductive organs
At1g09570	PHYTOCHROME A (PHYA)	All floral tissues
At1g48410	ARGONAUTE 1 (AGO1)	All reproductive organs except Mbd
At1g70940	PIN-FORMED 3 (PIN3)	All reproductive oragns except Ybd
At2g19520	FVE	All reproductive organs
At2g23380	CURLY LEAF (CLF)	All reproductive oragns except fruit
At2g42260	UV-B-INSENSITIVE 4 (UVI4)	All reproductive oragns except Ybd
AT3G15670	late embryogenesis abundant protein (LEA protein)	all floral tissues
At3g19820	DWARF 1 (DWF1)	All reproductive oragns except Ybd
At3g49670	BARELY ANY MERISTEM 2 (BAM2)	All reproductive oragns except Ybd
At4g08980	FBW2	all floral tissues
At4g18710	BRASSINOSTEROID-INSENSITIVE 2 (BIN2)	All floral tissues except fruit
At4g32551	LEUNIG (LUG)	All floral tissues
At4g39400	BRASSINOSTEROID INSENSITIVE 1 (BRI1)	All reproductive oragns except Ybd
At5g08370	Arabidopsis thaliana ALPHA-GALACTOSIDASE 2 (AtAGAL2)	All reproductive organs
At5g58230	MULTICOPY SUPRESSOR OF IRA1 (MSI1)	All floral tissues
At5g64630	FASCIATA 2 (FAS2)	All reproductive oragns except Ybd
At2g42200	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 9 (SPL9), SBP family (TFs)	All reproductive oragns except Ybd
At3g60030	SPL12, SBP family (TFs)	All reproductive oragns except Ybd
At5g50670	SPL13, SBP family (TFs)	All reproductive oragns except Ybd
At5g08330	TCP family (TFs)	Stamen, carpel, fruit

Table 5-2. Continued

Ath Homolog	Description	Expression area
At2g37260	TRANSPARENT TESTA GLABRA 2 (TTG2, AtWRKY44), WRKY family (TFs)	All reproductive organs
At4g26640	AtWRKY20, WRKY family (TFs)	All floral tissues
At5g26170	AtWRKY50, WRKY family (TFs)	All reproductive oragns except Mbd
At5g28650	AtWRKY74, WRKY family (TFs)	All floral tissues
At4g24660	ATHB22, ZF-HD family (TFs)	All reproductive organs

Table 5-3. Genes up-regulated in reproductive organs of *Nuphar*. Genes in red are involved in flower development. TFs=transcription factors, Mbd: medium-aged floral buds at the pre-meiotic stage, Ybd: young floral buds at the pre-microsporangia initiation stage.

Ath Homolog	Description	Expression area
At1g22490	AtbHLH94, bHLH family (TFs)	Ybd
At2g20180	PHY-INTERACTING FACTOR 1 (PIF1, AtbHLH15), bHLH family (TFs)	All reproductive organs except fruit
At3g23210	AtbHLH34, bHLH family (TFs)	All reproductive organs except Ybd
At1g58110	bZIP family (TFs)	Ybd
At2g40950	AtbZIP17, bZIP family (TFs)	Ybd
At3g23210	PHYTOCHROME INTERACTING FACTOR 3-LIKE 5 (PIL5), bZIP family (TFs)	Mbd, Fruits
At3g58120	AtbZIP61, bZIP family (TFs)	Ybd
At5g06950	AtbZIP20 (AHBP-1B), bZIP family (TFs)	Ybd
At5g39660	CYCLING DOF FACTOR 2 (CDF2), C2C2-Dof family (TFs)	Mbd, Ybd
At1g02040	C2H2 family (TFs)	Stamens
At5g14140	C2H2 family (TFs)	Petal, stamen, fruits
At1g30500	CCAAT-HAP2 family (TFs)	Sepal, petal, stamen
At2g01060	G2-like family (TFs)	Mbd
At5g16560	KANADI1 (KAN1), G2-like family (TFs)	Petal, stamen, carpel
At1g27050	ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 54 (ATHB54), Homeobox family (TFs)	Sepal, petal, stamen
At1g69120	APETALA1, MADS family (TFs)	Mbd, Ybd
At2g45650	AGAMOUS-LIKE6, MADS family (TFs)	All reproductive organs
At3g54340	APETALA3, MADS family (TFs)	All reproductive organs
At4g18960	AGAMOUS, MADS family (TFs)	All reproductive organs
At5g15800	AGAMOUS-LIKE2, MADS family (TFs)	All reproductive organs
At5g20240	PISTILLATA, MADS family (TFs)	All reproductive organs
At3g47600	AtMYB94, MYB family (TFs)	Sepal, petal, stamen, fruit

Table 5-3. Continued

Ath Homolog	Description	Expression area
At1g01060	LATE ELONGATED HYPOCOTYL 1 (LHY1), MYB-related family (TFs)	Sepal, petal, stamen
At1g74840	MYB-related family (TFs)	Stamen, carpel
AT3G09600	MYB-related family (TFs)	Sepal, petal
At5g52660	MYB-related family (TFs)	All reproductive orgasn except fruit
At1g69490	NAC-LIKE, ACTIVATED BY AP3/PI (NAP), NAC family (TFs)	Mbd, Ybd
At1g10940	ARABIDOPSIS SERINE/THREONINE KINASE 1 (ASK1)	All reproductive organs except Ybd
At1g19270	DA1, LIM domain-containing protein	Sepal, petal, stamen
At3g15354	SPA1-RELATED 3 (SPA3)	All floral tissues except fruit
At1g10670	ACLA-1	Sepal, petal, stamen
At3g24440	VERNALIZATION 5 (VRN5), a PHD finger protein	Sepal, petal, stamen
At4g11150	VACUOLAR ATP SYNTHASE SUBUNIT E1 (TUF)	All floral tissues except fruit

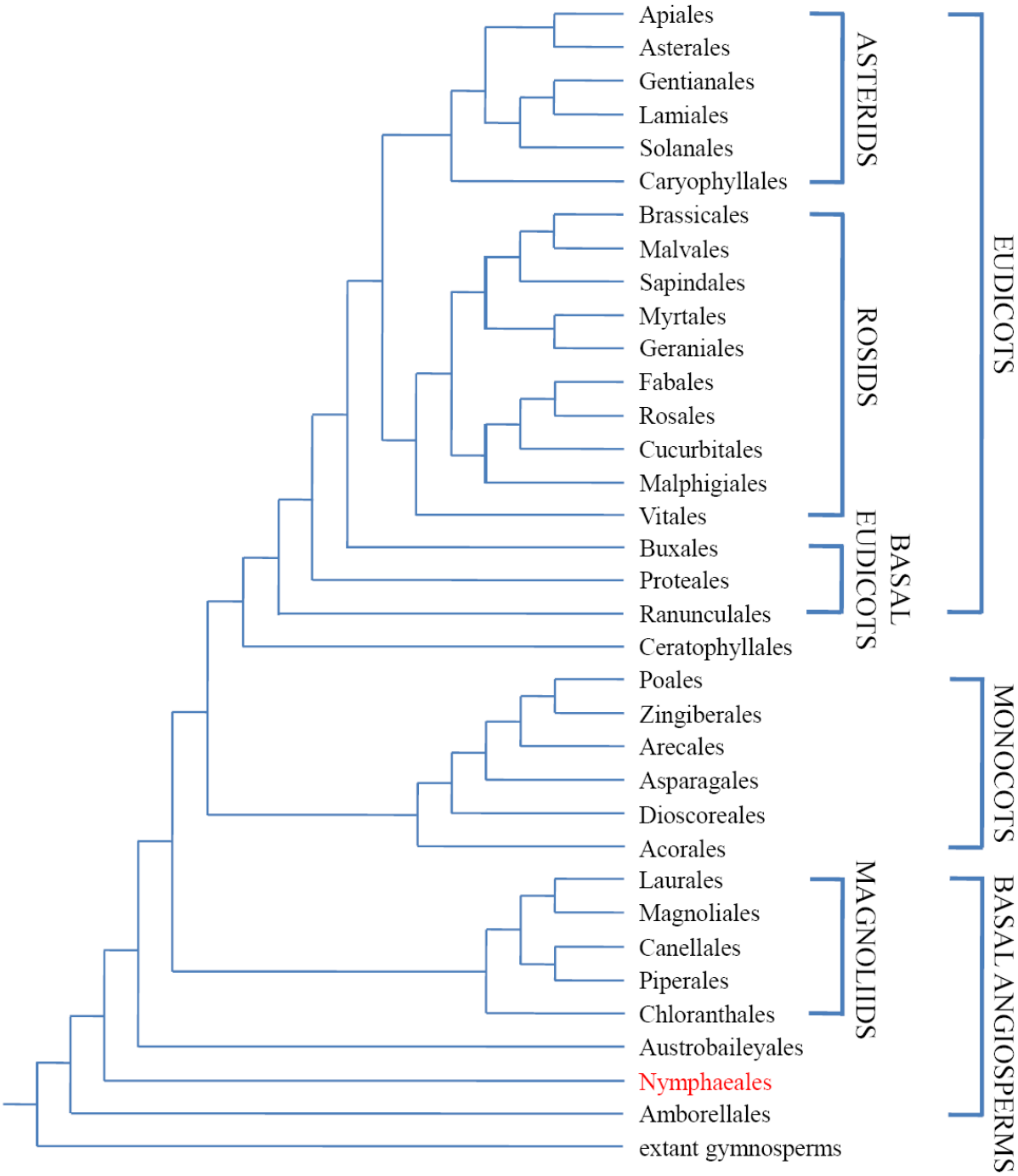


Figure 5-1. Summary tree for angiosperms. This tree is modified from the plastid genome trees of Jansen et al. (2007) and Moore et al. (2007), and only a few representatives of asterids and rosids are included. *Nuphar advena* belongs to Nymphaeales (red) which are the sister to all extant angiosperms except *Amborella* (Amborellales).

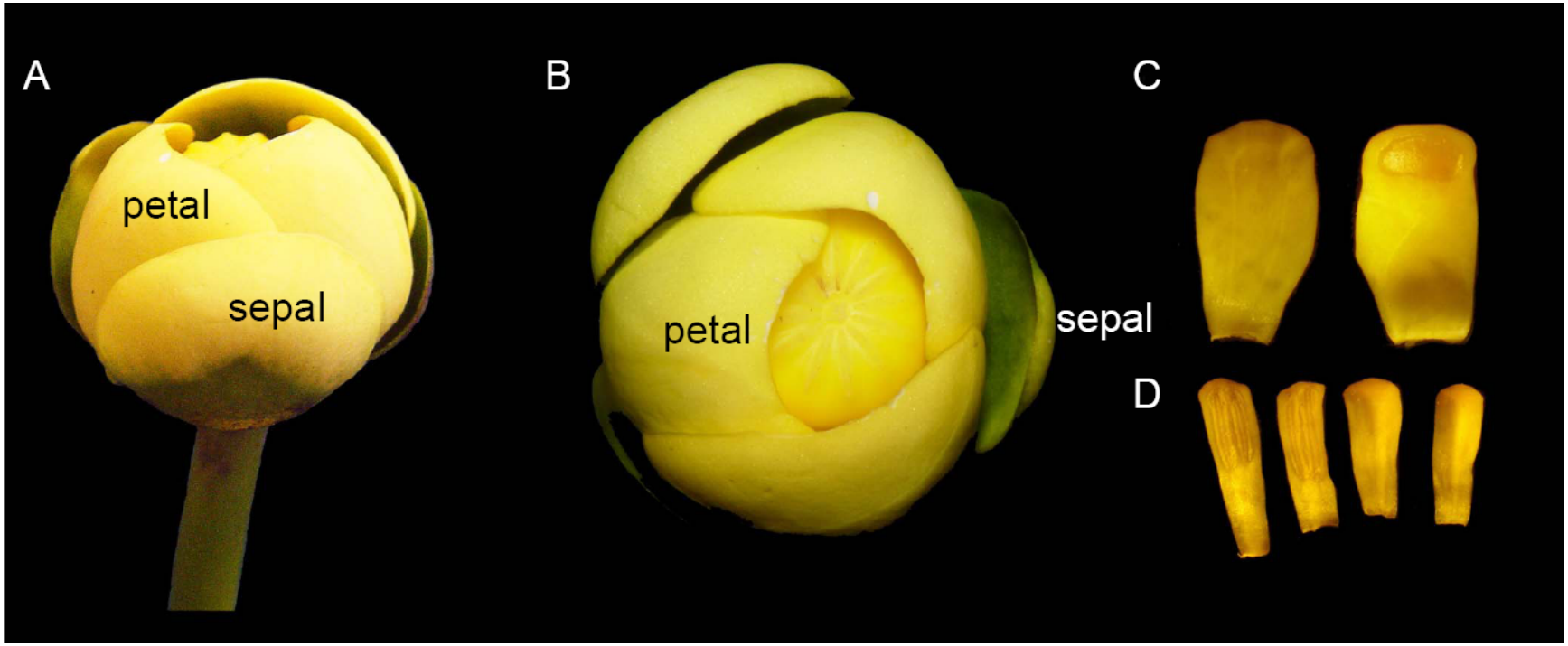


Figure 5-2. Flower of *Nuphar advena*. *A*, Side view of whole flower. *B*, Three sepals in the first whorl and three petals in the second whorl. *C*, staminodes with nectary, adaxial (left) and abaxial (right) view. *D*, stamens with anther, adaxial (two left) and abaxial (two right) view.

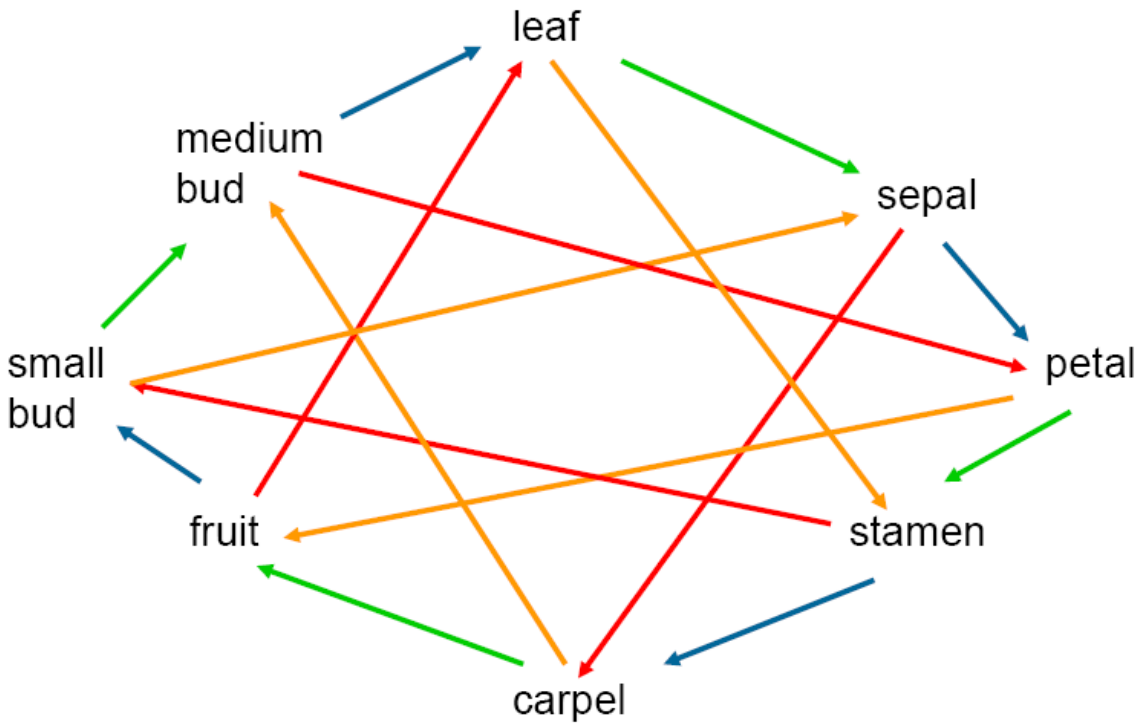


Figure 5-3. Double loop design for *Nuphar* microarray experiments. Each line indicates a single array, and different color means different biological replicates. The tissue at the head of the arrow is labeled red, and the tissue at the tail is labeled green.

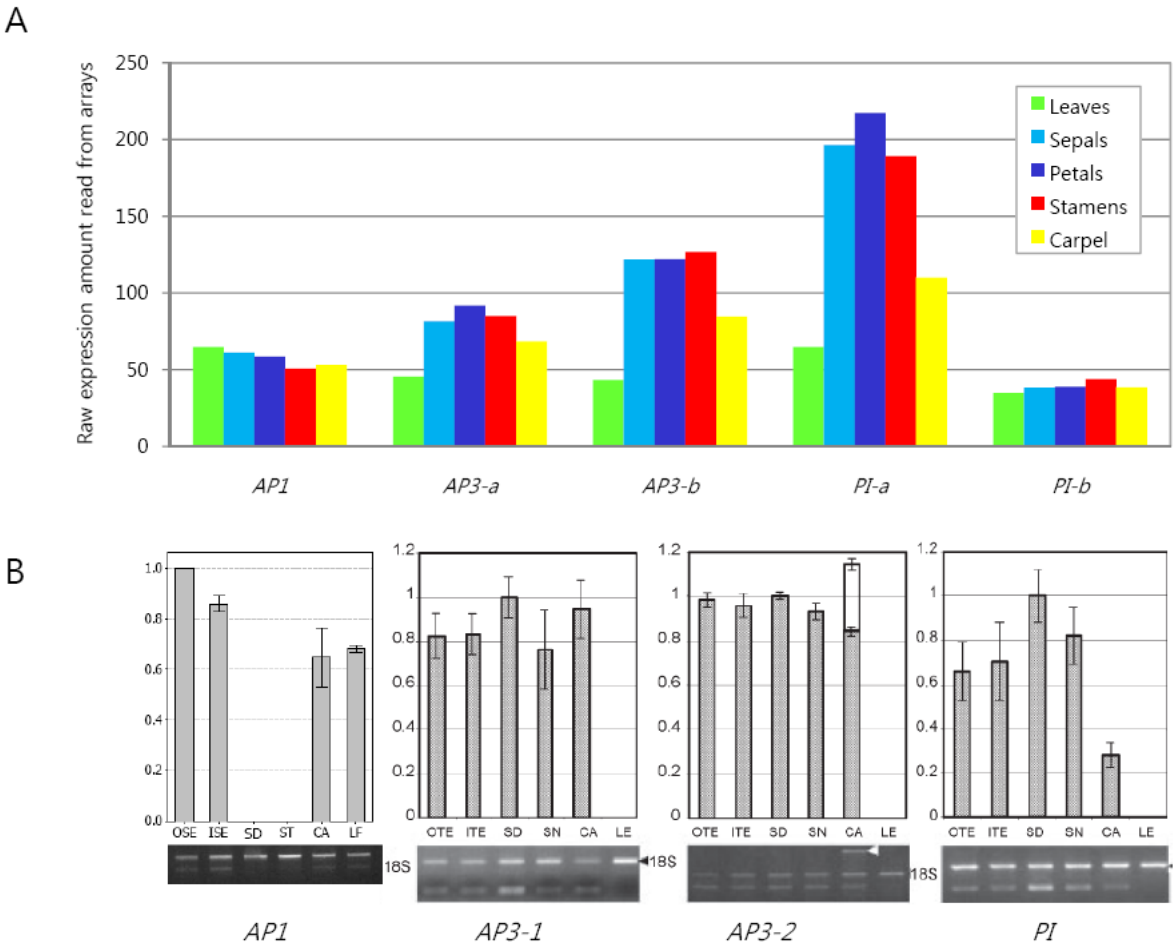


Figure 5-4. Comparison of microarray data with RQ-RT-PCR-data. *A*. Microarray data of *AP1*, *AP3-a*, *AP3-b*, *PI-a*, and *PI-b*. For *AP3* and *PI*, two different probes were designed from one sequence. *B*. RQ-RT-PCR data of *AP1* (Yoo et al., in prep.), *AP3-1*, *AP3-2*, and *PI* (Kim et al., 1995). *C*. Microarray data of *AG*, *AGL2-a*, *AGL2-b*, and *AGL6*. For *AGL2*, two different probes were designed from one sequence. *D*. RQ-RT-PCR data of *AG* (Yoo et al., in prep.), *AGL2* (Zahn et al., 1995), *AGL6* (Yoo et al., in prep). For all RQ-RT-PCR data, the expression level was relative to 18S rRNA internal control, so the maximum expression level is 1.

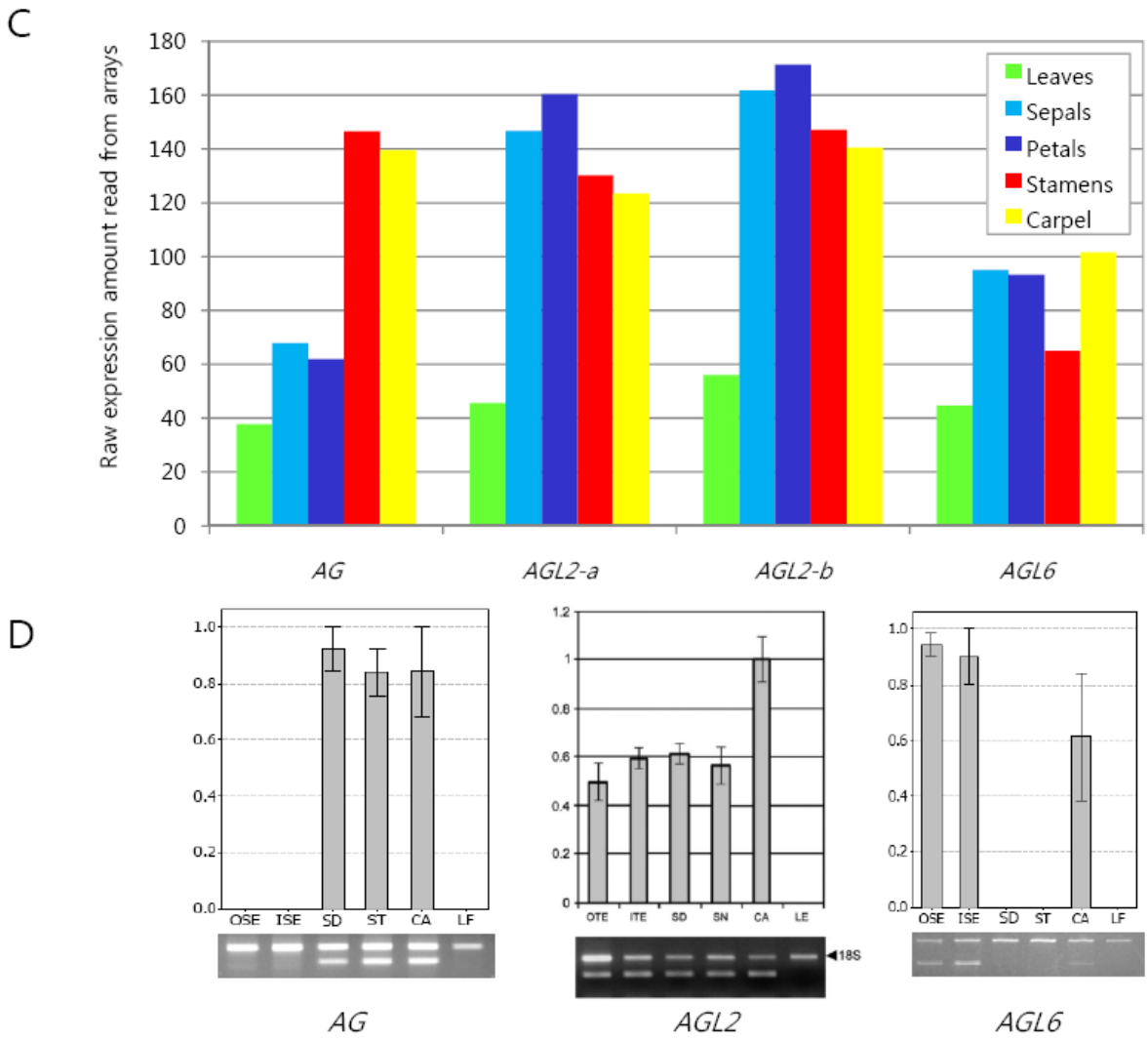


Figure 5-4. Continued.

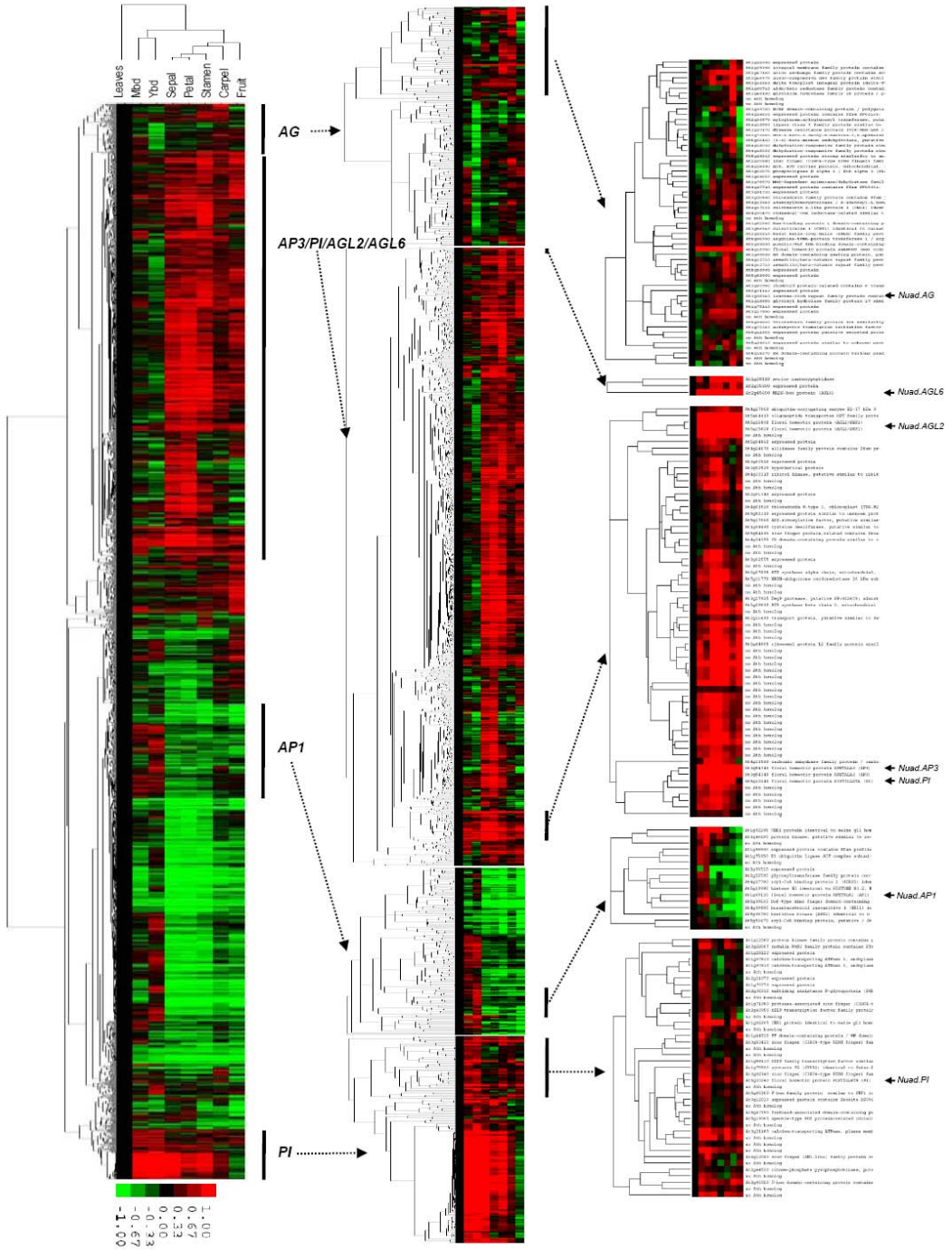


Figure 5-5. Hierarchical clustering displays "up-regulated" or "down-regulated" gene clusters based on similarity of expression patterns. Identified five regulatory modules are sequentially enlarged in the next of dendrogram. The color scale is presented below dendrogram; red indicates up-regulation, and green shows down-regulation in each floral organ relative to leaf. Top, array tree; left, gene tree. Mbd: medium-aged floral buds at the pre-meiotic stage, Ybd: young buds at the pre-microsporangia initiation stage.

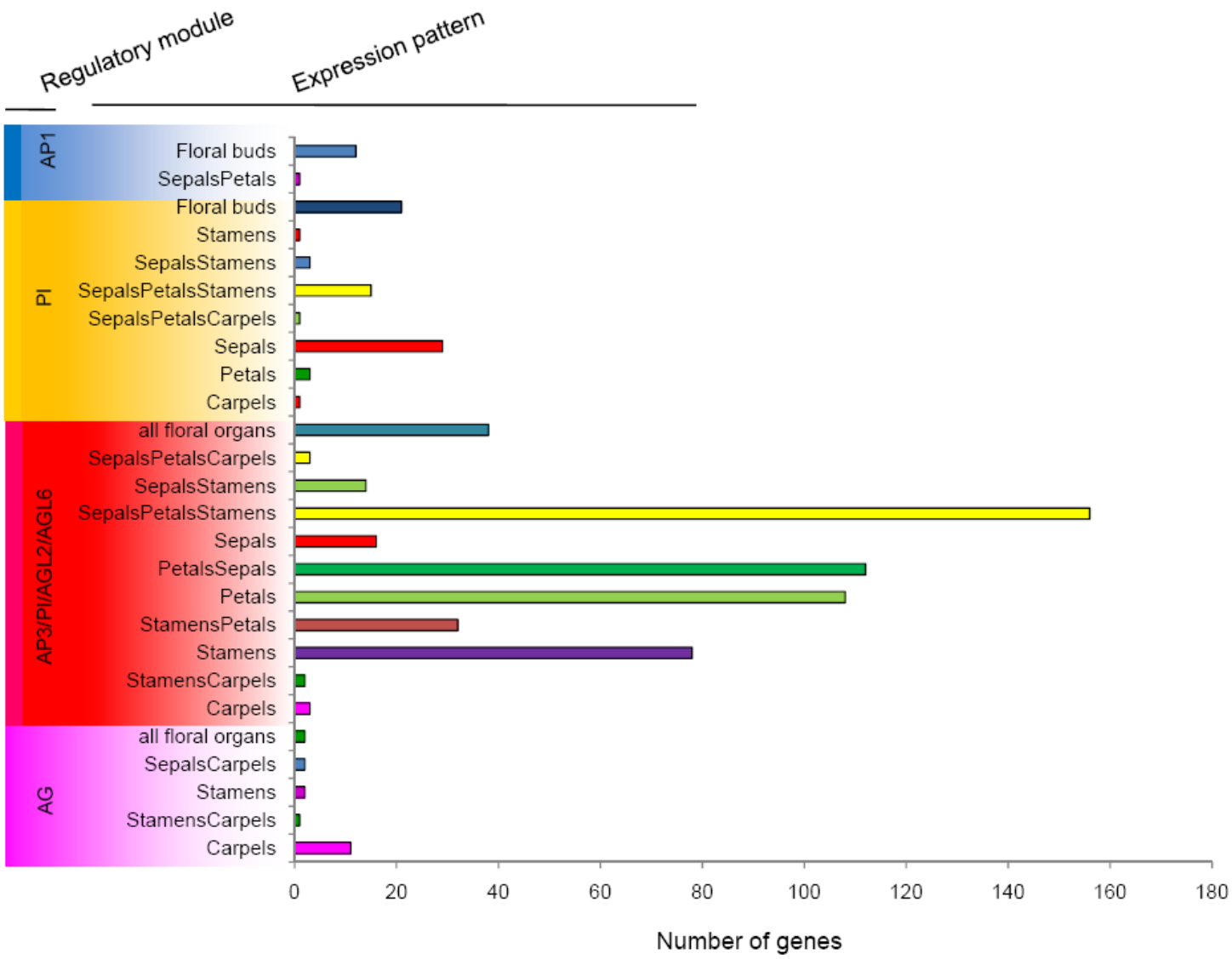


Figure 5-6. Number of genes up-regulated in *Nuphar* floral tissue at identified four regulatory modules.

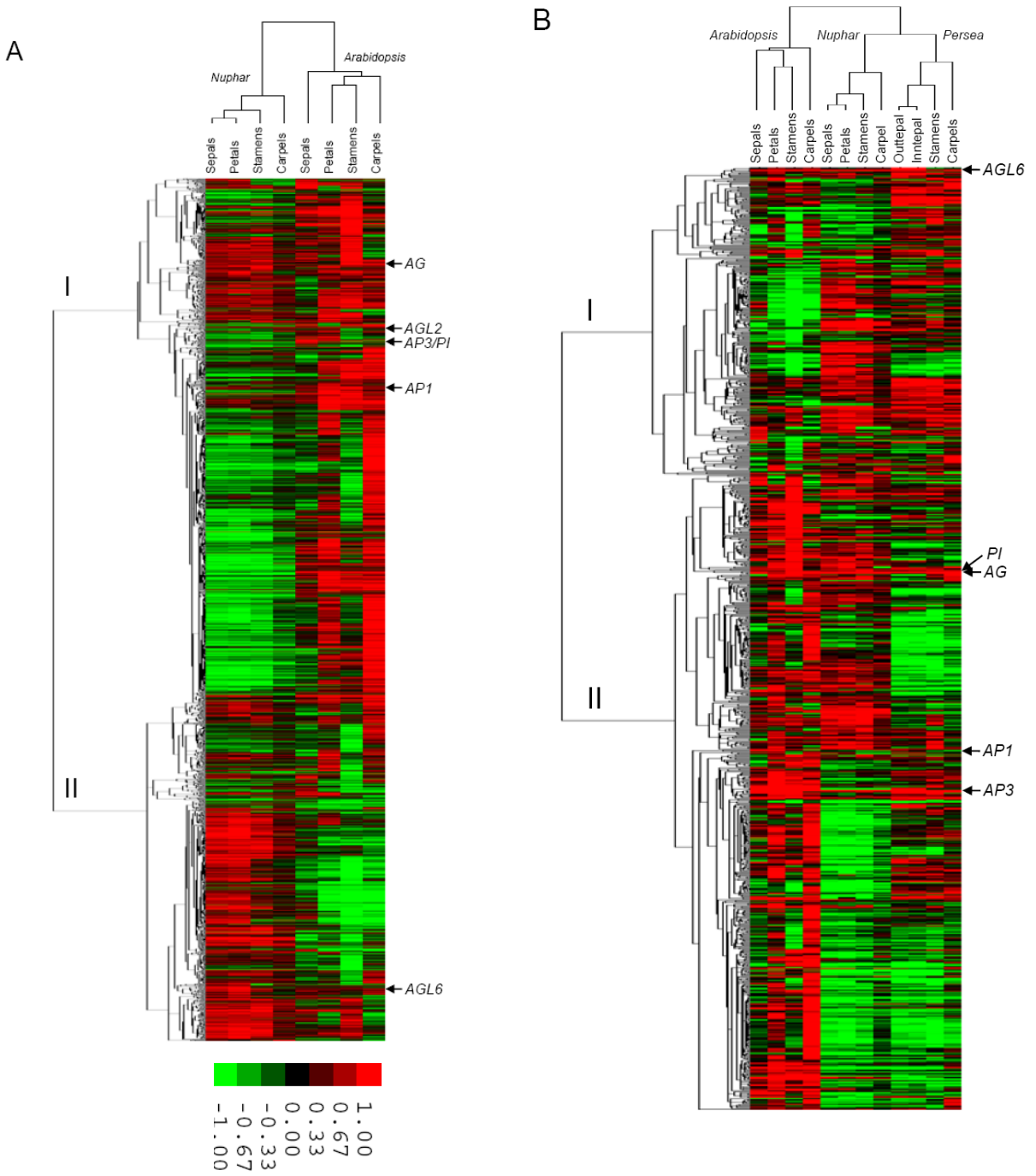


Figure 5-7. Hierarchical clustering results of combined data sets of *Arabidopsis*, *Nuphar*, and *Persea* data set. *A*, clustering result from comparison of *Nuphar* with *Arabidopsis* only. I: up-regulation of *Arabidopsis* genes, II: down-regulation of *Arabidopsis* genes. *B*, clustering result from comparison of *Nuphar* with *Persea* and *Arabidopsis*. I: down-regulation of *Arabidopsis* genes, II: up-regulation of *Arabidopsis* genes. The color scale is presented below dendrogram; red indicates up-regulation, and green shows down-regulation in each floral organ relative to leaf. Top, array tree; left, gene tree.

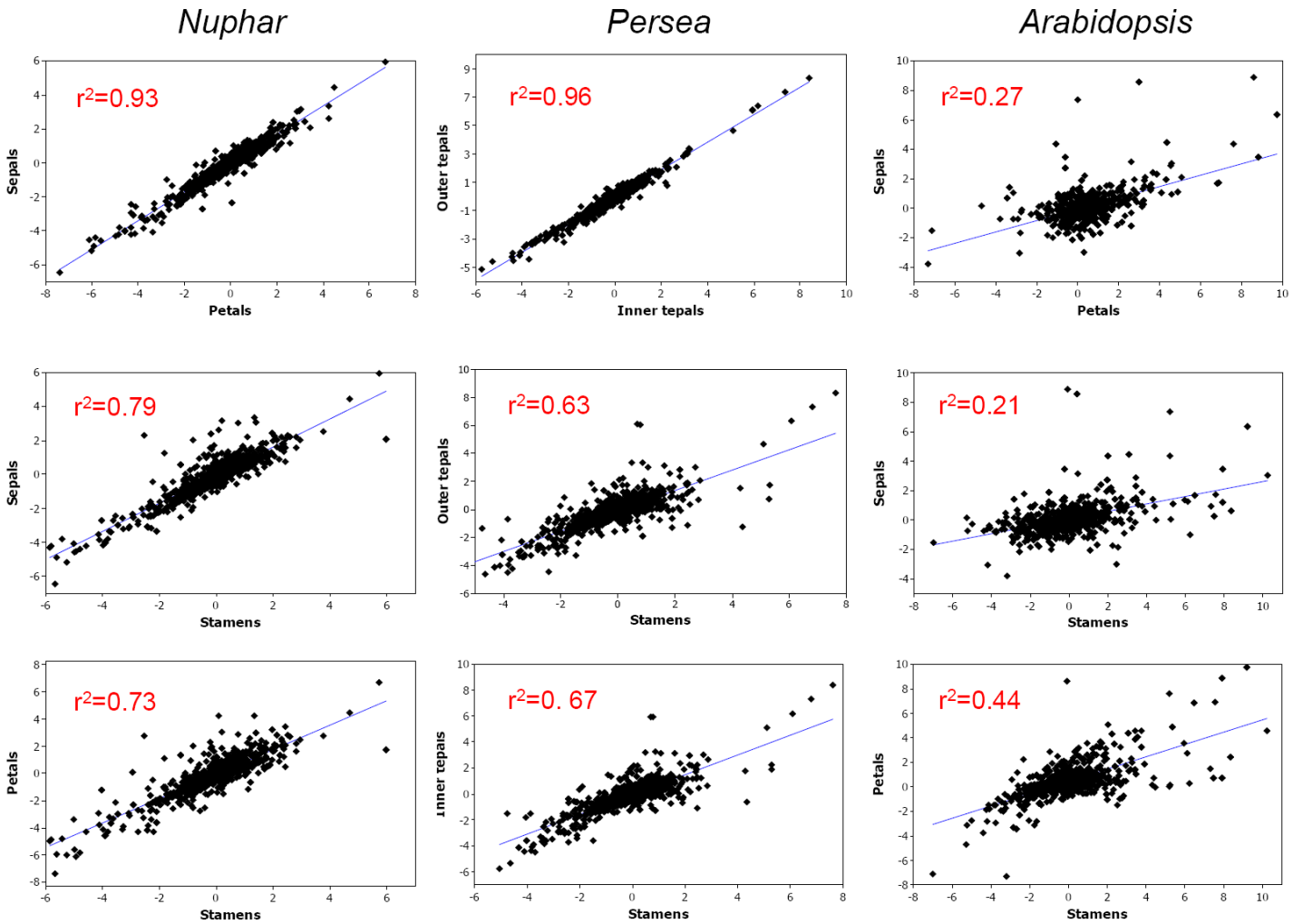


Figure 5-8. Scatter plots of spatial gene expression patterns in floral organs of *Nuphar*, *Persea*, and *Arabidopsis*. For this graphs we used the whole data set from *Nuphar*, *Persea* (Chanderbali et al. submitted), and *Arabidopsis* (Schmid et al. 2005). The $\log_2$ transformed expression data were used, so the positive value indicates up-regulation and the negative value indicates down-regulation in each floral organ relative to leaf. The r^2-value of the regression line (blue) is presented.

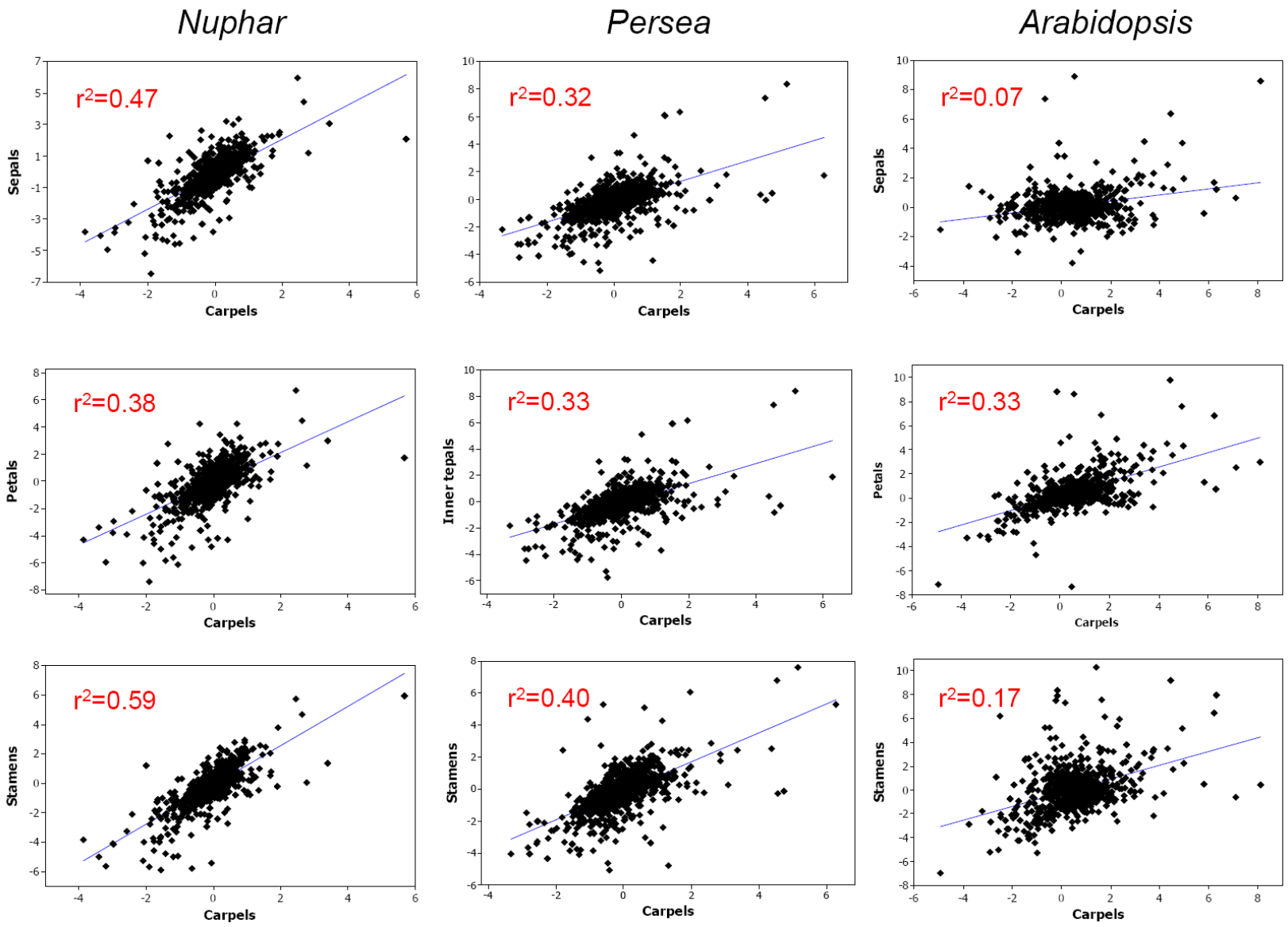


Figure 5-8. Continued.

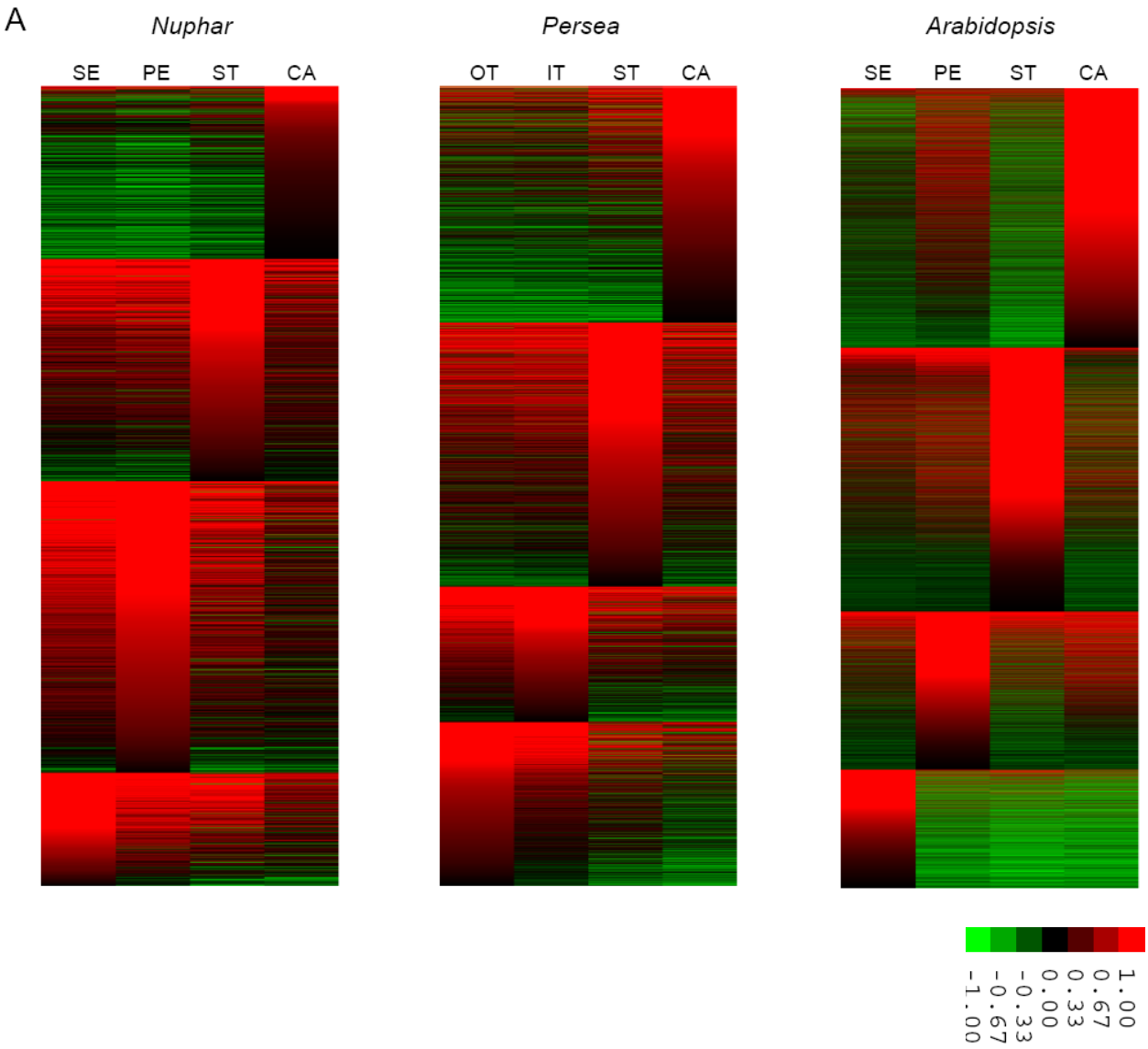


Figure 5-9. Expanse of gene expression domain across floral organs of *Nuphar*, *Persea*, and *Arabidopsis*. Genes overexpressed in each floral organ compared to other are sorted by their positive value according to primary expression domain. *A*, from the whole data set of *Nuphar, Persea* (Chanderbali et al. submitted), and *Arabidopsis* (Schimid et al. 2005). *B*, from *Nuphar*, *Persea*, and *Arabidopsis* homologues. SE: sepals, PE: petals, ST: stamens, CA: carpels, OT: outer tepals, IT: inner tepals. The color scale is presented at the bottom; red indicates up-regulation, and green shows down-regulation in each floral organ relative to leaf.

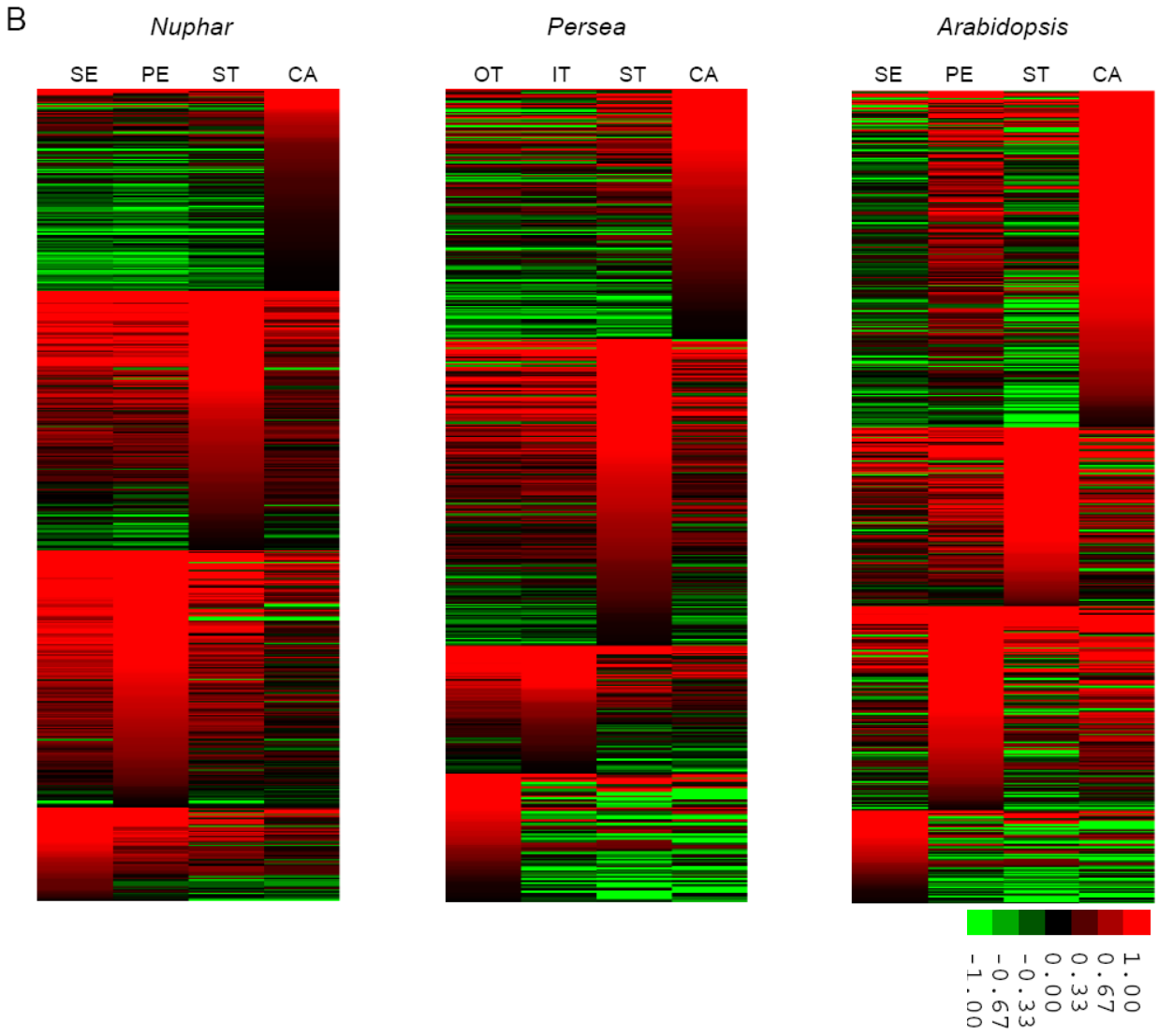


Figure 5-9. Continued.

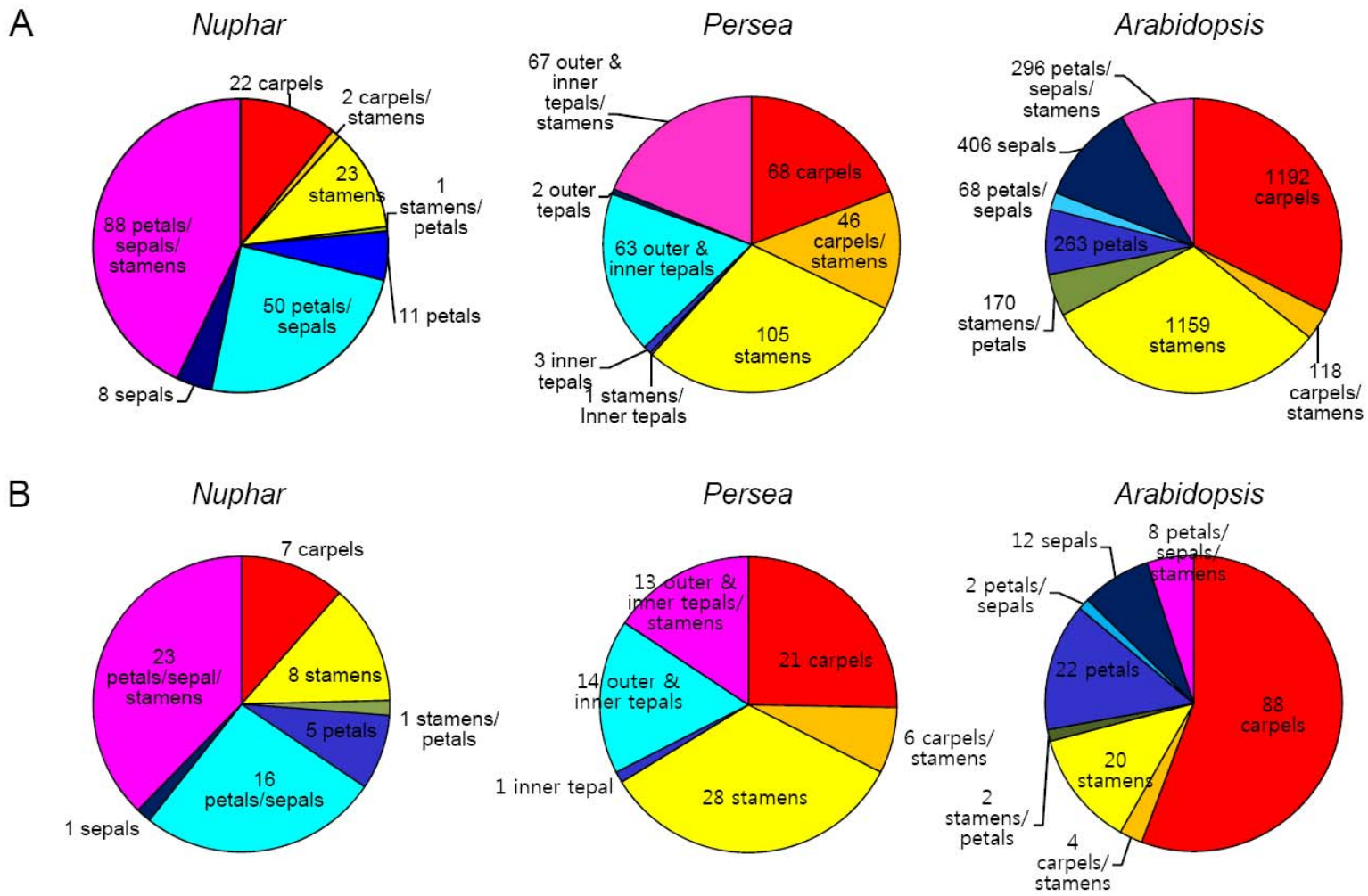


Figure 5-10. Number of genes with at least two-fold up-regulated in one organ and less than 1.5-fold in all other floral organs of *Nuphar*, *Persea*, and *Arabidopsis*. *A*, genes up-regulated in organ-specific and organ-combination from the whole data set of *Nuphar*, *Persea* (Chanderbali et al. submitted), and *Arabidopsis* (Schimid et al. 2005). *B*, genes up-regulated in organ-specific and organ-combination from from *Nuphar*, *Persea*, and *Arabidopsis* homologues.

CHAPTER 6
CONCLUSIONS

The goals of this study were to understand the phylogeny and timing of diversification of the waterlily clade (Nymphaeales) as well as the floral development and developmental genetics in this clade. Three species from Cabombaceae and Nymphaeaceae were used to construct floral developmental series for these families and to examine floral gene expression patterns. One species, *Nuphar advena* (Nymphaeaceae), was chosen for detailed floral transcriptome analysis using microarrays.

First, to elucidate the ages of Nymphaeales lineages, we estimated their divergence times. Using published nuclear 18S rDNA and plastid *rbcL* and *matK* DNA sequences and a published topology for Nymphaeales, we estimated the divergence times of genera in this clade. We applied four different methods, a strict molecular clock, nonparametric rate smoothing (NPRS), penalized likelihood (PL), and a Bayesian method, to estimate divergence times. We calibrated the trees by using the minimum age of the angiosperm crown group constrained to 131.8 mya. Our results indicate that extant Nymphaeales diversified into two major clades corresponding to Cabombaceae and Nymphaeaceae during the Eocene (44.6±7.9 mya); extant genera of Nymphaeaceae date to 41.1±7.7 mya, and extant Cabombaceae diversified during the Miocene (19.9±5.6 mya). Whereas the stem lineage of Nymphaeales is old based on fossil evidence (125-115 mya), our results indicate that extant Nymphaeales diversified relatively recently. In another set of analyses we used PL to estimate the age of the angiosperms using two prominent Nymphaeales fossils as calibration points. These analyses suggest that these "Nymphaeales" fossils may be attached at deeper nodes than proposed in earlier studies, that is, these fossils may not represent Nymphaeales. Using dispersal-vicariance analysis, we infer that the ancestor of Nymphaeales occupied the American and Eurasian continents during the Eocene and that the

present distributional patterns require several subsequent dispersal and extinction events. This biogeographic inference is supported by the fossil record.

In chapter 3, we examined the floral developmental series of three species (*Cabomba caroliniana*, *Nuphar advena*, and *Nymphaea odorata*) and aligned them. Although there are previous studies of flower development of Nymphaeales, comparative analyses have not been conducted. We found that the three study species follow the same 10 developmental stages in the very same order. *Nuphar*, which is sister to other members of Nymphaeaceae, exhibits *Nymphaea*-like or *Cabomba*-like developmental features, and its phylogenetic position suggests that sharing features between *Nuphar* and *Cabomba* might be ancestral characteristics to core Nymphaeales. The problematic origin of the third whorl of floral organs in *Nuphar*, is investigated in detail developmentally and morphologically. Comparison of these organs with stamens of *Nuphar* indicates that third-whorled organs originated from stamens. However, when compared with perianth organs from *Cabomba* and *Nymphaea*, it is found that those organs may be not homologous with perianth organs. For elucidating its organ identity, more comprehensive genetic research is required for other members of Nymphaeales.

In chapter 4, to provide insights into the floral developmental genetics of Nymphaeales, I investigated the expression patterns of floral organ identity genes representing major lineages of MADS-box genes using relative quantitative RT-PCR in *Cabomba*, *Nuphar*, and *Nymphaea.* Because of the similarity in floral structure between Nymphaeaceae and the basal eudicot *Nelumbo*, we conducted the same experiments in the latter as well. I focused on (1) perianth differentiation in all species examined and (2) the transition of petaloid staminodes to stamens in *Nymphaea*. For B-class gene homologues, in *Cabomba*, expression patterns fit the sliding boundaries model, whereas *Nuphar* and *Nymphaea* have generally broad patterns of gene

expression. Other gene homologues from these species follow the classic ABCDE eudicot model except *AP1* homologues. In contrast, the expression of floral gene homologues in the basal eudicot *Nelumbo* follows the classic ABCDE eudicot model except *AP1* homologue. Considering morphology together with developmental and genetic data, "sepaloid or petaloid tepals" are more appropriate terms than "sepals or petals" for perianth organs of Nymphaeales. Also, the gene expression patterns in the transitional sequence of stamens of *Nymphaea* suggest that petals of *Nymphaea* originated from petaloid staminodes or stamens. Despite the superficial floral similarity of *Nelumbo* to *Nymphaea*, expression of floral genes in *Nelumbo* differs from that of *Nuphar*, *Nymphaea*, and other basal angiosperms. In addition, based on expression data obtained from this study, I infer that the 'out-of-male' hypothesis, derivation of floral parts from male structures, might be true for ancestors of Nymphaeaceae.

In chapter 5, I investigated the floral transcriptomes of *Nuphar advena* in detail. Most of our understanding of the floral transcriptome comes from analyses of model eudicots and monocots. I present the first floral transcriptome profiling of one of the basalmost angiosperms, *Nuphar advena* (Nymphaeaceae). I used custom microarrays from Agilent Technologies containing 60-mer oligonucleotide probes, and representing approximately 6,200 unique *Nuphar* floral transcripts obtained from the Floral Genome Project (http://fgp.bio.psu.edu/fgp/index.html). We investigated gene expression in two floral buds from different floral developmental stages, sepals, petals, stamens, carpels, fruits, and leaves using a double loop design. I identified 3,333 floral transcripts that were significantly differentially expressed among eight tissues (with a significance of $P<0.05$; FDR=0.0381), and 1,624 genes showed a minimum of two-fold differential expression in at least one of the reproductive tissue relative to leaves. In particular, homologues of *AGAMOUS-LIKE 6* (*AGL6*), *AGAMOUS* (*AG*), *APETALA3* (*AP3*), *PISTILLATA*

(*PI*) and *AGAMOUS-LIKE 2* (*AGL2*) were up-regulated in floral tissues compared to leaves. These results are consistent with the expression patterns previously reported for these tissues in *Nuphar* using RQ-RT-PCR. Hierarchical clustering was performed to group tissues and genes based on similarities in gene expression patterns, and perianth members clustered together with stamens in hierarchical clustering, indicating a strong similarity in their gene expression profiles. The analysis identified four regulatory modules, and each module exhibited a distinctive expression pattern. I compared the floral transcriptome of *Nuphar* with those of the core eudicot *Arabidopsis* and the basal anigiosperm *Persea* (Lauraceae) with regard to spatial gene expression patterns and correlation of genes expressed in each floral tissue. I found that *Nuphar* has a less well defined floral transcriptional program compared to *Arabidopsis*. Floral transcriptional programs of sepals, petals, and stamens are not well-defined in *Nuphar*. There is overlap in gene expression among these organs. Similar results have been reported for *Persea*, other basal angiosperm. In *Arabidopsis*, in contrast, each floral organ has a well defined transcriptional program. These findings are in agreement with the floral morphologies and phylogenetic positions of these three species. Furthermore, most floral organ identity genes exhibited similar expression patterns among the three species, but genes involved downstream of floral organ formation and other pathways show distinct expression patterns. Therefore, modification and refinement in spatial expression patterns of those genes may, in part, be responsible for floral diversification across angiosperms.

During the period of this researh, Hydatellaceae was added to Nymphaeales based on molecular phylogenetics. Therefore, future developmental genetics work should include this new member of this clade. First, estimation of divergence times in Nymphaeales should be done including this new member. Hydatellaceae is sister to Cabombaceae + Nymphaeaceae, and they

are found in India, Australila, and New Zealand. Thus, inclusion of this family may alter divergence time estimates of the major lineages and also impact inferences on historical biogeography in Nymphaeales. Also, Hydatellaceae exhibit unique floral developmental features: the presence of involucral bracts, no perianth, and separate staminate and carpellate flowers. Therefore, application of the same approaches performed in this study will shed additional light on floral development genetics in Nymphaeales and, further, in early angiosperms.

LIST OF REFERENCES

Adam, H, S Jouannic, Y Orieux, F Morcillo, F Richaud, Y Duval, JW Tregear 2007 Functional characterization of MADS box genes involved in the determination of oil palm flower structure. J Exp Bot 58: 1245-1259.

Albert, VA, MHG Gustafsson, LD Laurenzio 1998 Ontogenetic systematic, molecular developmental genetics, and the angiosperm petal. *in*: DE Soltis, PS Soltis, JJ Doyle eds. Molecular systematic of plants II DNA sequencing. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Albert, VA, DE Soltis, JE Carlson, WG Farmerie, PK Wall, DC Ilut, TM Solow, LA Mueller, LL Landherr, Y Hu, M Buzgo, S Kim, MJ Yoo, MW Frohlich, R Perl-Treves, SE Schlarbaum, BJ Bliss, X Zhang, SD Tanksley, DG Oppenheimer, PS Soltis, H Ma, CW DePamphilis, JH Leebens-Mack 2005 Floral gene resources from basal angiosperms for comparative genomics research. BMC Plant Biol 5: 5.

Alves-Ferreira, M, F Wellmer, A Banhara, V Kumar, JL Riechmann, EM Meyerowitz 2007 Global expression profiling applied to the analysis of *Arabidopsis* stamen development. Plant Physiol 145: 747-762.

Angenent, GC, J Franken, M Busscher, L Colombo, AJ van Tunen 1993 Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1*. Plant J 4: 101-112.

Anzotegui, M 2004 Megaflora of the chiquimil formation (Late Miocene) in the Santa Maria and Villavil valleys, Catamarca and Tucuman provinces, Argentina. Ameghiniana 41: 303-314.

APG 1998 An ordial classification for the families of flowering plants. Ann Mo Bot Gard 85: 531-553.

APG II 2003 An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants. Bot J Linn Soc 141: 339-436.

Arora, R, P Agarwal, S Ray, AK Singh, VP Singh, AK Tyagi, S Kapoor 2007 MADS-box gene family in rice: genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics 8: 242.

Baldwin, BG, MJ Sanderson 1996 Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc Natl Acad Sci U S A 95: 9402-9406.

Baum, DA, LC Hileman 2006 A developmental genetic model for the origin of the flower. *in*: C Ainsworth ed. Flowering and its manipulation. Blackwell Publishing, Sheffield, UK.

Becker, A, K Kaufmann, A Freialdenhoven, C Vincent, MA Li, H Saedler, G Theissen 2002 A novel MADS-box gene subfamily with a sister-group relationship to class B floral homeotic genes. Mol Genet Genomics 266: 942-950.

Becker, A, H Saedler, G Theissen 2003 Distinct MADS-box gene expression patterns in the reproductive cones of the gymnosperm *Gnetum gnemon*. Dev Genes Evol 213: 567-572.

Becker, A, G Theissen 2003 The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol 29: 464-489.

Becker, A, KU Winter, B Meyer, H Saedler, G Theissen 2000 MADS-Box gene diversity in seed plants 300 million years ago. Mol Biol Evol 17: 1425-1434.

Becker, S, S Gleissbergy, DR Smyth 2005 Floral and vegetative morphogenesis in California poppy (*Eschscholzia californica* Cham.). Int J Plant Sci 166: 537-555.

Bell, CD, DE Soltis, PS Soltis 2005 The age of the angiosperms: a molecular timescale without a clock. Evolution 59: 1245-1258.

Benlloch, R, I d'Erfurth, C Ferrandiz, V Cosson, JP Beltran, LA Canas, A Kondorosi, F Madueno, P Ratet 2006 Isolation of *mtpim* proves *Tnt1* a useful reverse genetics tool in *Medicago truncatula* and uncovers new aspects of *AP1*-like functions in legumes. Plant Physiol 142: 972-983.

Birnbaum, K, DE Shasha, JY Wang, JW Jung, GM Lambert, DW Galbraith, PN Benfey 2003 A gene expression map of the *Arabidopsis* root. Science 302: 1956-1960.

Borsch, T 2000 Phylogeny and evolution of the genus *Nymphaea* (Nymphaeaceae). Dissertation. Universität Bonn, Bonn, Germany.

Borsch, T, KW Hilu, D Quandt, V Wilde, C Neinhuis, W Barthlott 2003 Noncoding plastid *trnT-trnF* sequences reveal a well resolved phylogeny of basal angiosperms. J Evol Biol 16: 558-576.

Borsch, T, KW Hilu, JH Wiersema, C Löhne, W Barthlott, V Wilde 2007 Phylogeny of *Nymphaea* (Nymphaeaceae): Evidence from substitutions and microstructural changes in the chloroplast trnT-trnF region. Int J Plant Sci 168: 639-671.

Borsch, T, C Löhne, K Müller, KW Hilu, S Wanke, A Worberg, W Barthlott, C Neinhuis, D Quandt 2005 Towards understanding basal angiosperm diversification: recent insights using rapidly evolving genomic regions. Nova Acta Leopold 92: 85-110.

Borsch, T, C Löhne, JH Wiersema 2008 Phylogeny and evolutionary patterns in Nymphaeales: integrating genes, genomes and morphology. Taxon 57: 1052-1081.

Bowman, JL, J Alvarez, D Weigel, EM Meyerowitz, DR Smyth 1993 Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development 119: 721-743.

Bowman, JL 1997 Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. J Biosci 22: 515-527.

Bowman, JL 2000 The YABBY gene family and abaxial cell fate. Curr Opin Plant Biol 3: 17-22.

Buzgo, M, AS Chanderbali, S Kim, Z Zheng, D Oppenheimer, PS Soltis, DE Soltis 2007 Floral developmental morphology of *Persea americana* (avocado, Lauraceae): the oddities of male organ identity. Int J Plant Sci 168: 261-284.

Buzgo, M, PS Soltis, DE Soltis 2004 Floral developmental morphology of *Amborella trichopoda* (Amborellaceae). Int J Plant Sci 165: 925-947.

Buzgo, M, PS Soltis, S Kim, DE Soltis 2005 The making of the flower. Biologist 52: 149-154.

Caspary, R 1888 Nymphaeaceae. *in*: A Engler, K Prantl eds. Die Natürlichen Pflanzenfamilien. Vol. 3. Wilhelm Engelmann, Leipzig, Germany.

Cevallos-Ferriz, SRS, RA Stockey 1989 Permineralized fruits and seeds from the Princeton Chert (Middle Eocene) of British Columbia: Nymphacaeceae. Bot Gaz 150: 207-217.

Chanderbali, AS, S Kim, M Buzgo, Z Zheng, DG Oppenheimer, DE Soltis, PS Soltis 2006 Genetic footprints of stamen ancestors guide perianth evolution in *Persea* (Lauraceae). Int J Plant Sci 167: 1075-1089.

Chase, MW, DE Soltis, RG Olmstead, D Morgan, DH Les, BD Mishler, MR Dunvall, RA Price, HG Hills, Y-L Qiu, KA Kron, JH Rettig, E Conti, JD Palmer, JH Manhart, KJ Sytsma, HJ Michaels, WJ Kress, KG Karol, WD Clark, M Hedren, BS Gaut, RK Jansen, KJ Kim, CF Wimpee, JF Smith, GR Furnier, SH Strauss, QY Xiang, GM Plunkett, PS Soltis, SM Swensen, SE Williams, PA Gadek, CJ Quinn, LE Eguiarte, E Golenberg, GH Learn, SW Graham, SCH Barrett, S Dayanadan, VA Albert 1993 Phylogenies of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. Ann Mo Bot Gard 80: 528-580.

Chaw, SM, H Long, BS Wang, A Zharkikh, WH Li 1993 The phylogenetic position of Taxaceae based on 18S rRNA sequences. J Mol Evol 37: 624-630.

Chaw, SM, CL Parkinson, Y Cheng, TM Vincent, JD Palmer 2000 Seed plant phylogeny inferred from all three plant genomes: monophyly of extant gymnosperms and origin of Gnetales from conifers. Proc Natl Acad Sci U S A 97: 4086-4091.

Chaw, SM, A Zharkikh, HM Sung, TC Lau, WH Li 1997 Molecular phylogeny of extant gymnosperms and seed plant evolution: analysis of nuclear 18S rRNA sequences. Mol Biol Evol 14: 56-68.

Chen, I, SR Manchester, Z Chen 2004 Anatomically preserved seeds of *Nuphar* (Nymphaeaceae) from the early Eocene of Wutu, Shandong Province, China. Am J Bot 91: 1265-1272.

Chen, MK, IC Lin, CH Yang 2008 Functional analysis of three lily (*Lilium longiflorum*) *APETALA1*-like MADS box genes in regulating floral transition and formation. Plant Cell Physiol 49: 704-717.

Chen, Q, A Atkinson, D Otsuga, T Christensen, L Reynolds, GN Drews 1999 The *Arabidopsis FILAMENTOUS FLOWER* gene is required for flower formation. Development 126: 2715-2726.

Cheng, Y, RG Nicolson, K Tripp, SM Chaw 2000 Phylogeny of Taxaceae and Cephalotaxaceae genera inferred from chloroplast *matK* gene and nuclear rDNA ITS region. Mol Phylogenet Evol 14: 353-365.

Cho, Y, J Fernandes, SH Kim, V Walbot 2002 Gene-expression profile comparisons distinguish seven organs of maize. Genome Biol 3: research0045.

Coen, ES, EM Meyerowitz 1991 The war of the whorls: genetic interactions controlling flower development. Nature 353: 31-37.

Collinson, ME 1980 Recent and Tertiary seeds of the Nymphaeaceae sensu lato with a revision of *Brasenia ovula* (Brong.) Reid and Chandler. Ann Bot 46: 603-632.

Colombo, L, J Franken, E Koetje, J van Went, HJ Dons, GC Angenent, AJ van Tunen 1995 The *Petunia* MADS box gene *FBP11* determines ovule identity. Plant Cell 7: 1859-1868.

Crepet, WL 1998 The abominable mystery. Science 282: 1653-1654.

Crepet, WL 2000 Progress in understanding angiosperm history, success, and relationships: Darwin's abominably 'perplexing phenomenon'. Proc Natl Acad Sci U S A 97: 12939-12941.

Cronquist, A 1981 An integrated system of classification of flowering plants. Columbia University Press, New York, NY.

Cronquist, A 1988 The evolution and classification of flowering plants. New York Botanical Garden, Bronx, New York.

Crow, GE, CB Hellquist 2000 Cabombaceae and Nymphaeaceae Aquatic and wetland plants of northeastern North America: a revised and enlarged edition of Norman C. Fassett's A manual of aquatic plants. University of Wisconsin Press, Madison, WI, USA.

Cutter, EG 1957a Studies of morphogenesis in the Nymphaeaceae. I. Introduction: some aspects of the morphology of *Nuphar lutea* (L.) Sm. and *Nymphaea alba* L. Phytomorphology 7: 45-56.

Cutter, EG 1957b Studies of morphogenesis in the Nymphaeaceae. II. Floral development in *Nuphar* and *Nymphaea*: bracts and calyx. Phytomorphology 7: 57-73.

Cutter, EG 1959 Studies of morphogenesis in the Nymphaeaceae. IV. Early floral development in species of *Nuphar*. Phytomorphology 9: 263-275.

Cutter, EG 1961 The inception and distribution of flowers in the Nymphaeaceae. Proc Linn Soc Bot 172: 93-100.

Davies, B, M Cartolano, Z Schwarz-Sommer 2006 Flower development: The *Antirrhinum* perspective. Adv Bot Res 44: 280-321.

Davies, B, P Motte, E Keck, H Saedler, H Sommer, Z Schwarz-Sommer 1999 *PLENA* and *FARINELLI*: redundancy and regulatory interactions between two *Antirrhinum* MADS-box factors controlling flower development. EMBO J 18: 4023-4034.

Davis, CC, CD Bell, PW Fritsch, S Mathews 2002a Phylogeny of *Acridocarpus-Brachylophon* (Malpighiaceae): implications for tertiary tropical floras and Afroasian biogeography. Evolution 56: 2395-2405.

Davis, CC, CD Bell, S Mathews, MJ Donoghue 2002b Laurasian migration explains Gondwanan disjunctions: evidence from Malpighiaceae. Proc Natl Acad Sci U S A 99: 6833-6837.

de Folter, S, AV Shchennikova, J Franken, M Busscher, R Baskar, U Grossniklaus, GC Angenent, RG Immink 2006 A Bsister MADS-box gene involved in ovule and seed development in *Petunia* and *Arabidopsis*. Plant J 47: 934-946.

de Hoon, MJ, S Imoto, S Miyano 2002 Statistical analysis of a small set of time-ordered gene expression data using linear splines. Bioinformatics 18: 1477-1485.

Ditta, G, A Pinyopich, P Robles, S Pelaz, MF Yanofsky 2004 The *SEP4* gene of *Arabidopsis thaliana* functions in floral organ and meristem identity. Curr Biol 14: 1935-1940.

Dorofeev, PI 1973 Systematics of ancestral forms of *Brasenia*. Paleontological Journal 2: 212-227.

Dorofeev, PI 1974 Nymphaeales. *in*: A Takhtajan ed. Magnoliophyta Fossilia URSS. Vol. 1. Nauka, Leningrad, Russia.

Doyle, JA, H Eklund, PS Herendeen 2003 Floral evolution in Chloranthaceae: Implication of a morphological phylogenetic analysis. Int J Plant Sci 164 Suppl: S365-S382.

Doyle, JA, PK Endress 2000 Morphological phylogenetic analysis of basal angiosperms: Comparison and combination with molecular data. Int J Plant Sci 161: 5121-5153.

Doyle, JJ, JL Doyle 1987 Rapid DNA isolation from small amount of fresh leaf tissue. Phytochem Bull 19: 11-15.

Drinnan, AN, PR Crane, SB Hoot 1994 Patterns of floral evolution in the early diversification of non-magnoliid dicotyledons (eudicots). Pl Syst Evol Suppl 8: 93-122.

Druka, A, G Muehlbauer, I Druka, R Caldo, U Baumann, N Rostoks, A Schreiber, R Wise, T Close, A Kleinhofs, A Graner, A Schulman, P Langridge, K Sato, P Hayes, J McNicol, D Marshall, R Waugh 2006 An atlas of gene expression from seed to seed through barley development. Funct Integr Genomics 6: 202-211.

Duarte, JM, L Cui, PK Wall, Q Zhang, X Zhang, J Leebens-Mack, H Ma, N Altman, CW dePamphilis 2006 Expression pattern shifts following duplication indicative of subfunctionalization and neofunctionalization in regulatory genes of *Arabidopsis*. Mol Biol Evol 23: 469-478.

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

Eklund, H, JA Doyle, PS Herendeen 2004 Morphological phylogenetic analysis of living and fossil Chloranthaceae. Int J Plant Sci 165: 107-151.

Endress, PK 2001 The flowers in extant basal angiosperms and inferences on ancestral flowers. Int J Plant Sci 162: 1111-1140.

Endress, PK 2006 Angiosperm floral evolution: Morphological developmental framework. Adv Bot Res 44: 1-61.

Endress, PK 2008 Perianth biology in the basal grade of extant angiosperms. Int J Plant Sci 169: 844-862.

Fan, J, W Li, X Dong, W Guo, H Shu 2007 Ectopic expression of a *Hyacinth AGL6* homolog caused earlier flowering and homeotic conversion in *Arabidopsis*. Sci China C Life Sci 50: 676-689.

Fassett, N 1953 A monograph of *Cabomba*. Castanea 13: 116-128.

Felsenstein, J 1981 Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol 17: 368-376.

Felsenstein, J 1984 Distance methods for inferring phylogenies: a justification. Evolution 38: 16-24.

Felsenstein, J 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Felsenstein, J 1988 Phylogenies from molecular sequences: inference and reliability. Annu Rev Genet 22: 521-565.

Fornara, F, G Marziani, L Mizzi, MM Kater, L Colombo 2003 MADS-box genes controlling flower development in rice. Plant Biology 1: 16-22.

Fraser, CM, LW Rider, C Chapple 2005 An expression and bioinformatics analysis of the *Arabidopsis serine carboxypeptidase*-like gene family. Plant Physiol 138: 1136-1148.

Friedman, WE 2008 Hydatellaceae are water lilies with gymnospermous tendencies. Nature 453: 94-97.

Friis, EM 1997 Fossil history of magnoliid angiosperms. *in*: K Iwatsuki, PR Raven eds. Evolution and diversification of land plants. Springer, Tokyo, Japan.

Friis, EM, KR Pedersen, PR Crane 1994 Angiosperm floral structures from the Early Cretaceous of Portugal. Plant Syst Evol 8: 31-49.

Friis, EM, KR Pedersen, PR Crane 1999 Early angiosperm diversification: the diversity of pollen associated with angiosperm reproductive structures in Early Cretaceous floras from Portugal. Ann Mo Bot Gard 86: 259-296.

Friis, EM, KR Pedersen, PR Crane 2001 Fossil evidence of water lilies (Nymphaeales) in the Early Cretaceous. Nature 410: 357-360.

Frohlich, MW 1999 MADS about Gnetales. Proc Natl Acad Sci U S A 96: 8811-8813.

Frohlich, MW, DS Parker 2000 The mostly male theory of flower evolutionary origins: from genes to fossils. Syst Bot 25: 155-170.

Frohlich, MW 2003 An evolutionary scenario for the origin of flowers. Nat Rev Genet 4: 559-566.

Frohlich, MW 2006 Recent developments regarding the evolutionary origin of flowers. Adv Bot Res 44: 63-127.

Fukui, M, N Futamura, Y Mukai, Y Wang, A Nagao, K Shinohara 2001 Ancestral MADS box genes in Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), homologous to the B function genes in angiosperms. Plant Cell Physiol 42: 566-575.

Gandolfo, MA, KC Nixon, WL Crepet 2004 Cretaceous flowers of Nymphaeaceae and implications for complex insect entrapment pollination mechanisms in early angiosperms. Proc Natl Acad Sci U S A 101: 8056-8060.

Goldman, N 1993 Statistical tests of models of DNA substitution. J Mol Evol 36: 182-198.

Golz, JF, A Hudson 1999 Plant development: YABBYs claw to the fore. Curr Biol 9: R861-863.

Gomez-Mena, C, S de Folter, MM Costa, GC Angenent, R Sablowski 2005 Transcriptional program controlled by the floral homeotic gene *AGAMOUS* during early organogenesis. Development 132: 429-438.

Goremykin, VV, KI Hirsch-Ernst, S Wolfl, FH Hellwig 2003 Analysis of the *Amborella trichopoda* chloroplast genome sequence suggests that *Amborella* is not a basal angiosperm. Mol Biol Evol 20: 1499-1505.

Goto, K, EM Meyerowitz 1994 Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. Genes Dev 8: 1548-1560.

Graham, SW, RG Olmstead 2000 Utility of 17 chloroplast genes for inferring the phylogeny of the basal angiosperms. Am J Bot 87: 1712-1730.

Guo, Y, S Gan 2006 AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46: 601-612.

Hardenack, S, D Ye, H Saedler, S Grant 1994 Comparison of MADS box gene expression in developing male and female flowers of the dioecious plant white campion. Plant Cell 6: 1775-1787.

Hayes, V, EL Schneider, S Carlquist 2000 Floral development of *Nelumbo nucifera* (Nelumbonaceae). Int J Plant Sci 161: 183-191.

Heinsbroek, PG, WA Van Heel 1969 Note on the bearing of the pattern of vascular bundles on the morphology of the stamens of *Victoria amazonica* (Poepp.) Sowerby. Proc K ned A kad Wet, Ser C 72: 431-444.

Henkel, F, F Rehnelt, L Dittmann 1907 Das Buch der Nymphaeaceen oder Seerosengewächse. Verlag F. Henkel, Darmstadt, Germany.

Hennig, L, W Gruissem, U Grossniklaus, C Kohler 2004 Transcriptional programs of early reproductive stages in *Arabidopsis*. Plant Physiol 135: 1765-1775.

Heywood, VH 1993 Flowering plants of the world. Oxford University Press, New York.

Hiepko, P 1965 Vergleichend-morphologische und entwicklungsgeschichtliche Untersuchungen uber das Perianth bei den Polycarpicae. Bot Jahrb Syst 84: 359-508.

Hilu, KW, T Borsch, K Müller, DE Soltis, PS Soltis, V Savolainen, MW Chase, MP Powell, LA Alice, R Evans, H Sauquet, C Neinhuis, TAB Slotta, JG Rohwer, CS Campbell, LW Chatrou 2003 Angiosperm phylogeny based on *matK* sequence information. Am J Bot 90: 1758-1776.

Honma, T, K Goto 2001 Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409: 525-529.

Hoot, SB, S Magallon, PR Crane 1999 Phylogeny of basal eudicots based on three molecular data sets: *atp*B, *rbc*L, and 18S nuclear ribosomal DNA sequences. Ann Mo Bot Gard 86: 1-32.

Hord, CL, C Chen, BJ Deyoung, SE Clark, H Ma 2006 The *BAM1*/*BAM2* receptor-like kinases are important regulators of *Arabidopsis* early anther development. Plant Cell 18: 1667-1680.

Hsu, HF, CH Huang, LT Chou, CH Yang 2003 Ectopic expression of an orchid (*Oncidium Gower Ramsey*) *AGL6*-like gene promotes flowering by activating flowering time genes in *Arabidopsis thaliana*. Plant Cell Physiol 44: 783-794.

Huijser, P, J Klein, WE Lonnig, H Meijer, H Saedler, H Sommer 1992 Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene *squamosa* in *Antirrhinum majus*. EMBO J 11: 1239-1249.

Irish, VF, IM Sussex 1990 Function of the *apetala-1* gene during *Arabidopsis* floral development. Plant Cell 2: 741-753.

Ito, M 1983 Studies in the floral morphology and anatomy of Nymphaeales. I. The morphology of vascular bundles in the flower of *Nymphaea tetragona* George. Acta Phytotax Geobot 34: 18-26.

Ito, M 1984 Studies in the floral morphology and anatomy of the Nymphaeales. II. Floral anatomy of *Nymphaea tetragona* George. Acta Phytotax Geobot 35: 18-26.

Ito, M 1986 Studies in the floral morphology and anatomy of Nymphaeales. III. Floral anatomy of *Brasenia schreberi* Gmel. and *Cabomba caroliniana* A. Gray. Bot Mag (Tokyo) 99: 169-184.

Ito, M 1987 Phylogenetic systematics of the Nymphaeales. Bot Mag (Tokyo) 100: 17-36.

Jack, T 2004 Molecular and genetic mechanisms of floral control. Plant Cell 16 Suppl: S1-17.

Jack, T, LL Brockman, EM Meyerowitz 1992 The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 68: 683-697.

Jack, T, GL Fox, EM Meyerowitz 1994 *Arabidopsis* homeotic gene *APETALA3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell 76: 703-716.

Jager, M, A Hassanin, M Manuel, H Le Guyader, J Deutsch 2003 MADS-box genes in *Ginkgo biloba* and the evolution of the *AGAMOUS* family. Mol Biol Evol 20: 842-854.

Jakoby, M, B Weisshaar, W Droge-Laser, J Vicente-Carbajosa, J Tiedemann, T Kroj, F Parcy 2002 bZIP transcription factors in *Arabidopsis*. Trends Plant Sci 7: 106-111.

Jansen, RK, Z Cai, LA Raubeson, H Daniell, CW Depamphilis, J Leebens-Mack, KF Muller, M Guisinger-Bellian, RC Haberle, AK Hansen, TW Chumley, SB Lee, R Peery, JR McNeal, JV Kuehl, JL Boore 2007 Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. Proc Natl Acad Sci U S A 104: 19369-19374.

Jones-Rhoades, MW, JO Borevitz, D Preuss 2007 Genome-wide expression profiling of the *Arabidopsis* female gametophyte identifies families of small, secreted proteins. PLoS Genet 3: 1848-1861.

Judd, WS, CS Campbell, EA Kellogg, PF Stevens, MJ Donoghue 2002 Nymphaeales Plant systematics: a phylogenetic approach. Sinauer Associates, Sunderland, MA.

Kang, HG, YS Noh, YY Chung, MA Costa, K An, G An 1995 Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. Plant Mol Biol 29: 1-10.

Kater, MM, L Colombo, J Franken, M Busscher, S Masiero, MM Van Lookeren Campagne, GC Angenent 1998 Multiple *AGAMOUS* homologs from cucumber and *Petunia* differ in their ability to induce reproductive organ fate. Plant Cell 10: 171-182.

Kater, MM, L Dreni, L Colombo 2006 Functional conservation of MADS-box factors controlling floral organ identity in rice and *Arabidopsis*. J Exp Bot 57: 3433-3444.

Kaya, H, KI Shibahara, KI Taoka, M Iwabuchi, B Stillman, T Araki 2001 *FASCIATA* genes for chromatin assembly factor-1 in *Arabidopsis* maintain the cellular organization of apical meristems. Cell 104: 131-142.

Kempin, SA, MA Mandel, MF Yanofsky 1993 Conversion of perianth into reproductive organs by ectopic expression of the tobacco floral homeotic gene *NAG1*. Plant Physiol 103: 1041-1046.

Kenrick, P, PR Crane 1997 The origin and early diversification of land plants: a cladistic study. Smithsonian Institution Press, Washington, D.C.

Kerner von Marilaun, A 1891 Pflanzenleben, Bd. 2: Geschichte der Pflanzen. Verlag des Bibliographischen Instituts, Leipzig, Germany.

Kerstetter, RA, K Bollman, RA Taylor, K Bomblies, RS Poethig 2001 *KANADI* regulates organ polarity in *Arabidopsis*. Nature 411: 706-709.

Khanna, P 1964 Morphological and embryological studies in the Nymphaeaceae. I. *Euryale ferox* Salisb. Proc Indian Acad Sci 59: 237-243.

Khanna, P 1967 Morphological and embryological studies in the Nymphaeaceae. III. *Victoria cruziana* d'Orb. and *Nymphaea stellata* Willd. . Bot Mag (Tokyo) 80: 305-312.

Kim, GT, K Shoda, T Tsuge, KH Cho, H Uchimiya, R Yokoyama, K Nishitani, H Tsukaya 2002 The *ANGUSTIFOLIA* gene of *Arabidopsis*, a plant *CtBP* gene, regulates leaf-cell expansion, the arrangement of cortical microtubules in leaf cells and expression of a gene involved in cell-wall formation. EMBO J 21: 1267-1279.

Kim, GT, H Tsukaya, H Uchimiya 1998 The *CURLY LEAF* gene controls both division and elongation of cells during the expansion of the leaf blade in *Arabidopsis thaliana*. Planta 206: 175-183.

Kim, S, J Koh, MJ Yoo, H Kong, Y Hu, H Ma, PS Soltis, DE Soltis 2005 Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. Plant J 43: 724-744.

Kim, S, LM Zahn, Z Zheng, AS Chanderbali, DG Oppenheimer, H Ma, PS Soltis, DE Soltis unpublished The evolution of the *AGL6*-like MADS-box genes.

Kishino, H, M Hasegawa 1989 Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in hominoidea. J Mol Evol 29: 170-179.

Kishino, H, JL Thorne, WJ Bruno 2001 Performance of a divergence time estimation method under a probabilistic model of rate evolution. Mol Biol Evol 18: 352-361.

Knobloch, E, DH Mai 1984 Neue Gattungen nach Früchten und Samen aus dem Cenoman bis Maastricht (Kreide) von Mitteleuropa. Feddes Repertorium 95: 3-41.

Kramer, EM, VS Di Stilio, PM Schluter 2003 Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the Ranunculaceae. Int J Plant Sci 164: 1-11.

Kramer, EM, RL Dorit, VF Irish 1998 Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. Genetics 149: 765-783.

Kyozuka, J, T Kobayashi, M Morita, K Shimamoto 2000 Spatially and temporally regulated expression of rice MADS box genes with similarity to *Arabidopsis* class A, B and C genes. Plant Cell Physiol 41: 710-718.

Langley, CH, WM Fitch 1974 An examination of the constancy of the rate of molecular evolution. J Mol Evol 3: 161-177.

Leebens-Mack, J, LA Raubeson, L Cui, JV Kuehl, MH Fourcade, TW Chumley, JL Boore, RK Jansen, CW depamphilis 2005 Identifying the basal angiosperm node in chloroplast genome phylogenies: sampling one's way out of the Felsenstein zone. Mol Biol Evol 22: 1948-1963.

Les, DH, DK Garvin, CF Wimpee 1991 Molecular evolutionary history of ancient aquatic angiosperms. Proc Natl Acad Sci U S A 88: 10119-10123.

Les, DH, EL Schneider, DJ Padgett, PS Soltis, DE Soltis, M Zanis 1999 Phylogeny, classification and floral evolution of water lilies (Nymphaeaceae; Nymphaeales): A synthesis of non-molecular, rbcL, matK, and 18S rDNA data. Syst Bot 24: 28-46.

Leys, RS, JB Cooper, MP Schwarz 2002 Molecular phylogeny and historical biogeography of the large carpenter bees, genus *Xylocopa* (Hymenoptera: Apidae). Biol J Linn Soc 77: 249-266.

Li, M, W Xu, W Yang, Z Kong, Y Xue 2007 Genome-wide gene expression profiling reveals conserved and novel molecular functions of the stigma in rice. Plant Physiol 144: 1797-1812.

Li, Y, K Sorefan, G Hemmann, MW Bevan 2004 *Arabidopsis NAP* and *PIR* regulate actin-based cell morphogenesis and multiple developmental processes. Plant Physiol 136: 3616-3627.

Litt, A 2007 An evaluation of A-function: evidence from the *APETALA1* and *APETALA2* gene lineages. Int J Plant Sci 168: 73-91

Litt, A, VF Irish 2003 Duplication and Diversification in the *APETALA1/FRUITFULL* Floral Homeotic Gene Lineage: Implications for the Evolution of Floral Development. Genetics 165: 821-833.

Llamas, KA 2003 Tropical flowering plants: A guide to identification and cultivation. Timber Press, Portland, OR.

Löhne, C, T Borsch 2005 Molecular evolution and phylogenetic utility of the *petD* group II intron: a case study in basal angiosperms. Mol Biol Evol 22: 317-332.

Löhne, C, T Borsch, JH Wiersema 2007 Phylogenetic analysis of Nymphaeales using fast-evolving and noncoding chloroplast markers. Bot J Linn Soc 154: 141-163.

Lü, S, X Du, W Lu, K Chong, Z Meng 2007 Two AGAMOUS-like MADS-box genes from *Taihangia rupestris* (Rosaceae) reveal independent trajectories in the evolution of class C and class D floral homeotic functions. Evol Dev 9: 92-104.

Lynch, M, JS Conery 2000 The evolutionary fate and consequences of duplicate genes. Science 290: 1151-1155.

Ma, H, C dePamphilis 2000 The ABCs of floral evolution. Cell 101: 5-8.

Ma, H, MF Yanofsky, EM Meyerowitz 1991 *AGL1-AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev 5: 484-495.

Magallón, S 2004 Dating lineages: Molecular and paleontological approaches to the temporal framework of clades. Int J Plant Sci 165 Suppl: S7-S21.

Mai, DH 1988 New nymphaealean fossils from the Tertiary of central Europe. Tertiary Research 9: 87-96.

Mapes, G, GW Rothwell 1984 Permineralized ovulate cones of Lebachia from the late Palaeozoic limestones of Kansas. Paleontology 27: 69-94.

Mapes, G, GW Rothwell 1991 Structure and relationships of primitive conifers. Neues Jahrbuch für Geologie und Paläontologie Abhandlungen 183.

Martinez-Castilla, LP, ER Alvarez-Buylla 2003 Adaptive evolution in the *Arabidopsis* MADS-box gene family inferred from its complete resolved phylogeny. Proc Natl Acad Sci U S A 100: 13407-13412.

Mathews, S, MJ Donoghue 1999 The root of angiosperm phylogeny inferred from duplicate phytochrome genes. Science 286: 947-950.

Mena, M, MA Mandel, DR Lerner, MF Yanofsky, RJ Schmidt 1995 A characterization of the MADS-box gene family in maize. Plant J 8: 845-854.

Miki, S 1960 Nymphaeaceae remains in Japan, with new fossil genus *Eoeuryale*. Journal of the Institute of Polytechnics, Osaka City University, Japan D-11: 63-78.

Moore, MJ, CD Bell, PS Soltis, DE Soltis 2007 Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc Natl Acad Sci U S A 104: 19363-19368.

Moore, RC, SR Grant, MD Purugganan 2005 Molecular population genetics of redundant floral-regulatory genes in Arabidopsis thaliana. Mol Biol Evol 22: 91-103.

Moore, RC, MD Purugganan 2005 The evolutionary dynamics of plant duplicate genes. Curr Opin Plant Biol 8: 122-128.

Morris, AB, CD Bell, JW Clayton, WS Judd, DE Soltis, PS Soltis 2007 Phylogeny and divergence time estimation in Illicium with implications for New World biogeography. Syst Bot 32: 236-249.

Moseley, MF 1958 Morphological studies of the Nymphaeaceae. I. The nature of the stamens. Phytomorphology 8: 1-29.

Moseley, MF 1961 Morphological studies of the Nymphaeaceae. II. THe flower of *Nymphaea*. Bot Gaz 122: 233-259.

Moseley, MF 1965 Morphological studies of the Nymphaeaceae. III. The floral anatomy of *Nuphar*. Phytomorphology 15: 54-84.

Moseley, MF 1972 Morphological studies of Nymphaeaceae. VI. Development of flower of *Nuphar*. Phytomorphology 21: 253-283.

Moseley, MF, IJ Mehta, PS Williamson, H Kosakai 1984 Morphological studies of the Nymphaeaceae (sensu lato). XIII. Contributions to the vegetative and floral structure of *Cabomba*. Am J Bot 71: 902-924.

Moseley, MF, EL Schneider, PS Williamson 1993 Phylogenetic interpretation from selected floral vasculature characters in the Nymphaeaceae sensu lato. Aquat Bot 44: 325-342.

Mouradov, A, B Hamdorf, RD Teasdale, JT Kim, KU Winter, G Theissen 1999 A *DEF/GLO*-like MADS-box gene from a gymnosperm: *Pinus radiata* contains an ortholog of angiosperm B class floral homeotic genes. Dev Genet 25: 245-252.

Nakai, T 1943 Ordines, familae, tribi, genera, sectiones, species, varietates, formae et combinationes novae a Prof. Nakai-Takenosin adhuc tu novis edita. Appendix:

Quaestiones characterium naturalium plantarum vel Extractus ex praelectionibus pro alumnis botanicus Universitatis Imperialis Tokyoensis per annos 1926–1941. Imperial University of Tokyo, Tokyo, Japan.

Nam, J, J Kim, S Lee, G An, H Ma, M Nei 2004 Type I MADS-box genes have experienced faster birth-and-death evolution than type II MADS-box genes in angiosperms. Proc Natl Acad Sci U S A 101: 1910-1915.

Nesi, N, I Debeaujon, C Jond, AJ Stewart, GI Jenkins, M Caboche, L Lepiniec 2002 The *TRANSPARENT TESTA16* locus encodes the *ARABIDOPSIS BSISTER* MADS domain protein and is required for proper development and pigmentation of the seed coat. Plant Cell 14: 2463-2479.

Ng, M, MF Yanofsky 2001 Function and evolution of the plant MADS-box gene family. Nat Rev Genet 2: 186-195.

Nijhawan, A, M Jain, AK Tyagi, JP Khurana 2008 Genomic survey and gene expression analysis of the basic leucine zipper transcription factor family in rice. Plant Physiol 146: 333-350.

Ørgaard, M 1991 The genus *Cabomba* (Cabombaceae)-a taxonomic study. Nord J Bot 11: 179-203.

Osborn, JM, EL Schneider 1988 Morphological studies of the Nymphaeaceae sensu lato. XVI. The floral biology Of *Brasenia schreberi*. Ann Mo Bot Gard 75: 778-794.

Padgett, DJ, DH Les, GE Crow 1999 Phylogenetic relationships in *Nuphar* (Nymphaeaceae): evidence from morphology, chloroplast DNA, and nuclear ribosomal DNA. Am J Bot 86: 1316-1324.

Padgett, DJ 2007 A monograph of *Nuphar* (Nymphaeaceae). Rhodora 109: 1-95.

Parkinson, CL, KL Adams, JD Palmer 1999 Multigene analyses identify the three earliest lineages of extant flowering plants. Curr Biol 9: 1485-1488.

Pelaz, S, GS Ditta, E Baumann, E Wisman, MF Yanofsky 2000 B and C floral organ identity functions require *SEPALLATA* MADS-box genes. Nature 405: 200-203.

Perez-Perez, JM, MR Ponce, JL Micol 2002 The *UCU1 Arabidopsis* gene encodes a SHAGGY/GSK3-like kinase required for cell expansion along the proximodistal axis. Dev Biol 242: 161-173.

Poupin, MJ, F Federici, C Medina, JT Matus, T Timmermann, P Arce-Johnson 2007 Isolation of the three grape sub-lineages of B-class MADS-box *TM6*, *PISTILLATA* and *APETALA3* genes which are differentially expressed during flower and fruit development. Gene 404: 10-24.

Prance, GT, JR Arias 1975 A study of the floral biology of *Victoria amazonica* (Poepp.) Sowerby (Nymphaeaceae). Acta Amazonica 5: 109-139.

Qiu, YL, MW Chase, DH Les, CR Parks 1993 Molecular phylogenetics of the Magnoliidae: cladistic analysis of nucleotide sequences of the plastid gene *rbcL*. Ann Mo Bot Gard 80: 587-606.

Qiu, YL, O Dombrovska, J Lee, LB Li, BA Whitlock, F Bernasconi-Quadroni, JS Rest, CC Davis, T Borsch, KW Hilu, SS Renner, DE Soltis, PS Soltis, MJ Zanis, JJ Cannone, RR Gutell, M Powell, V Savolainen, LW Chatrou, MW Chase 2005 Phylogenetic analyses of basal angiosperms based on nine plastid, mitochondrial, and nuclear genes. Int J Plant Sci 166: 815-842.

Qiu, YL, J Lee, F Bernasconi-Quadroni, DE Soltis, PS Soltis, M Zanis, EA Zimmer, Z Chen, V Savolainen, MW Chase 1999 The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. Nature 402: 404-407.

Quinn, CJ, RA Price, PA Gadek 2002 Familial concepts and relationships in the conifers based on *rbcL* and *matK* sequence comparisons. Kew Bull 57: 513-531.

Richard, A 1828 Nouveaux éléments de botanique et de physiologie végétale. Bechet, Paris, France.

Riechmann, JL, BA Krizek, EM Meyerowitz 1996 Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. Proc Natl Acad Sci U S A 93: 4793-4798.

Rijpkema, A, T Gerats, M Vandenbussche 2006 Genetics of floral development in *Petunia*. Adv Bot Res 44: 237-278.

Ronquist, F 1996 DIVA: dispersal-vicariance analysis, vers. 1.1. Uppsala University, Uppsala, Sweden.

Ronquist, F 1997 Dispersal-vicariance analysis: a new approach to the quantification of historical biogeography. Syst Biol 46: 195-203.

Ronse De Craene, LP 2008 Homology and evolution of petals in the core eudicots. Syst Bot 33: 301-325.

Ronse De Craene, LP, EF Smets 2001 Staminodes: Their morphological and evolutionary significance. Bot Rev 67: 351-402.

Rounsley, SD, GS Ditta, MF Yanofsky 1995 Diverse roles for MADS box genes in Arabidopsis development. Plant Cell 7: 1259-1269.

Rudall, PJ, DD Sokoloff, MV Remizowa, JG Conran, JI Davis, TD Macfarlane, DW Stevenson 2007 Morphology of Hydatellaceae, an anomalous aquatic family recently recognized as an early-divergent angiosperm lineage. Am J Bot 94: 1073-1092.

Rutledge, R, S Regan, O Nicolas, P Fobert, C Cote, W Bosnich, C Kauffeldt, G Sunohara, A Seguin, D Stewart 1998 Characterization of an *AGAMOUS* homologue from the conifer

black spruce (*Picea mariana*) that produces floral homeotic conversions when expressed in *Arabidopsis*. Plant J 15: 625-634.

Ruzin, SE 1999 Plant microtechnique and microscopy. Oxford University Press, Oxford, UK.

Saarela, JM, HS Rai, JA Doyle, PK Endress, S Mathews, AD Marchant, BG Briggs, SW Graham 2007 Hydatellaceae identified as a new branch near the base of the angiosperm phylogenetic tree. Nature 446: 312-315.

Sablowski, RW, EM Meyerowitz 1998 A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. Cell 92: 93-103.

Saldanha, AJ 2004 Java Treeview--extensible visualization of microarray data. Bioinformatics 20: 3246-3248.

Sanderson, MJ 1997 A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol Biol Evol 14: 1218-1231.

Sanderson, MJ 1998 Estimating rate and time in molecular phylogenies: beyond the molecular clock? *in*: DE Soltis, PS Soltis, JJ Doyle eds. Molecular systematics of plants II, DNA sequencing. Kluwer, Boston, MA.

Sanderson, MJ 2002 Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Mol Biol Evol 19: 101-109.

Sanderson, MJ 2003 r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19: 301-302.

Sanderson, MJ, JA Doyle 2001 Sources of error and confidence intervals in estimating the age of angiosperms from *rbcL* and 18S rDNA data. Am J Bot 88: 1499-1516.

Sanderson, MJ, JL Thorne, N Wikström, K Bremer 2004 Molecular evidence on plant divergence times. Am J Bot 91: 1656-1665.

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

Schmid, M, NH Uhlenhaut, F Godard, M Demar, R Bressan, D Weigel, JU Lohmann 2003 Dissection of floral induction pathways using global expression analysis. Development 130: 6001-6012.

Schmidt, RJ, B Veit, MA Mandel, M Mena, S Hake, MF Yanofsky 1993 Identification and molecular characterization of *ZAG1*, the maize homolog of the *Arabidopsis* floral homeotic gene *AGAMOUS*. Plant Cell 5: 729-737.

Schneider, EL 1976 The floral anatomy of *Victoria* Schomb. (Nymphaeaceae). Bot J Linn Soc 72: 115-148.

Schneider, EL 1983 Gross morphology and floral biology of *Ondinea purpurea* den Hartog. Aust J Bot 31: 371-382.

Schneider, EL, JM Jeter 1982 Morphological studies of the nymphaeaceae. Xii. The floral biology of *Cabomba caroliniana*. Am J Bot 69: 1410-1419.

Schneider, EL, LA Moore 1977 Morphological studies of the Nymphaeaceae. VII. The floral biology of *Nuphar lutea* subsp. *macrophylla*. Brittonia 29: 88-99.

Schneider, EL, SC Tucker, PS Williamson 2003 Floral development in the Nymphaeales. Int J Plant Sci 164 Suppl 5: S279-S292.

Schneider, EL, PS Williamson 1993 Nymphaeaceae. *in*: K Kubitzki, JG Rohwer, V Bittrich eds. The families and genera of vascular plants. Vol. 2. Springer, Berlin, Germany.

Schwarz-Sommer, Z, I Hue, P Huijser, PJ Flor, R Hansen, F Tetens, WE Lonnig, H Saedler, H Sommer 1992 Characterization of the *Antirrhinum* floral homeotic MADS-box gene *DEFICIENS*: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J 11: 251-263.

Schwarz-Sommer, Z, P Huijser, W Nacken, H Saedler, H Sommer 1990 Genetic Control of Flower Development by Homeotic Genes in *Antirrhinum majus*. Science 250: 931-936.

Shan, H, K Su, W Lu, H Kong, Z Chen, Z Meng 2006 Conservation and divergence of candidate class B genes in *Akebia trifoliata* (Lardizabalaceae). Dev Genes Evol 216: 785-795.

Shan, H, N Zhang, C Liu, G Xu, J Zhang, Z Chen, H Kong 2007 Patterns of gene duplication and functional diversification during the evolution of the *AP1/SQUA* subfamily of plant MADS-box genes. Mol Phylogenet Evol 44: 26-41.

Smith, AG, DG Smith, BM Funnel 1994 Atlas of Mesozoic and Cenozoic Coastlines. Cambridge University Press, Cambridge, UK.

Smyth, DR, JL Bowman, EM Meyerowitz 1990 Early flower development in *Arabidopsis*. Plant Cell 2: 755-767.

Smyth, GK 2004 Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 3: Article3.

Smyth, GK, J Michaud, HS Scott 2005 Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21: 2067-2075.

Smyth, GK, M Ritchie, N Thorne, J Wettenhall 2006 LIMMA: Linear Models for Microarray Data User's Guide.

Sokoloff, DD, MV Retnizowa, TD Macfarlane, PJ Rudall 2008 Classification of the early-divergent angiosperm family Hydatellaceae: one genus instead of two, four new species and sexual dimorphism in dioecious taxa. Taxon 57: 179-200.

Soltis, DE, H Ma, MW Frohlich, PS Soltis, VA Albert, DG Oppenheimer, NS Altman, C dePamphilis, J Leebens-Mack 2007 The floral genome: an evolutionary history of gene duplication and shifting patterns of gene expression. Trends Plant Sci 12: 358-367.

Soltis, DE, PS Soltis, DL Nickrent, LA Johnson, WJ Hahn, SB Hoot, JA Sakamoto, RK Kuzoff, KA Kron, MW Chase, SM Swensen, EA Zimmer, SM Chaw, LJ Gillespie, KJ Systma 1997 Angiosperm phylogeny inferred from 18S ribosomal DNA sequences. Ann Mo Bot Gard 84: 1-49.

Soltis, DE, PS Soltis, MW Chase, ME Mort, DC Albach, M Zanis, V Savolainen, WH Hahn, SB Hoot, MF Fay, M Axtell, SM Swensen, LM Prince, WJ Kress, KC Nixon, JS Farris 2000 Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. Bot J Linn Soc 133: 381-461.

Soltis, DE, PS Soltis, PK Endress, MW Chase 2005 Phylogeny and evolution of angiosperms. Sinauer Associates, Sunderland, MA.

Soltis, PS, DE Soltis, MW Chase 1999 Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. Nature 402: 402-404.

Soltis, PS, DE Soltis, V Savolainen, PR Crane, TG Barraclough 2002 Rate heterogeneity among lineages of tracheophytes: integration of molecular and fossil data and evidence for molecular living fossils. Proc Natl Acad Sci U S A 99: 4430-4435.

Stellari, GM, MA Jaramillo, EM Kramer 2004 Evolution of the *APETALA3* and *PISTILLATA* lineages of MADS-box-containing genes in the basal angiosperms. Mol Biol Evol 21: 506-519.

Storey, BC 1995 The role of mantle plumes in continental breakup: case histories from Gondwanaland. Nature 377: 301-308.

Sundström, J, A Carlsbecker, ME Svensson, M Svenson, U Johanson, G Theissen, P Engström 1999 MADS-box genes active in developing pollen cones of Norway spruce (*Picea abies*) are homologous to the B-class floral homeotic genes in angiosperms. Dev Genet 25: 253-266.

Swofford, DL 2002 PAUP* 4.0b10: phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA, USA.

Takahashi, M 1992 Development of spinous exine in *Nuphar japonicum* De Candolle (Nymphaeaceae). Review of Palaeobotany and Palynology 75: 317-322.

Takhtajan, A 1980 Outline of the classification of flowering plants (Magnoliophyta). Bot Rev 46: 225-359.

Takhtajan, A 1991 Evolutionary trends in flowering plants. Columbia University Press, New York, NY, USA.

Takhtajan, A 1997 Diversity and classification of flowering plants. Columbia University Press, New York, NY.

Tandre, K, M Svenson, ME Svensson, P Engstrom 1998 Conservation of gene structure and activity in the regulation of reproductive organ development of conifers and angiosperms. Plant J 15: 615-623.

Taylor, SA, JM Hofer, IC Murfet, JD Sollinger, SR Singer, MR Knox, TH Ellis 2002 *PROLIFERATING INFLORESCENCE MERISTEM*, a MADS-box gene that regulates floral meristem identity in pea. Plant Physiol 129: 1150-1159.

Teeri, TH, M Kotilainen, A Uimari, S Ruokolainen, YP Ng, U Malm, E Pollanen, S Broholm, R Laitinen, P Elomaa, VA Albert 2006 Floral developmental genetics of *Gerbera* (Asteraceae). Adv Bot Res 44: 323-351.

Theissen, G 2001a Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol 4: 75-85.

Theissen, G 2001b Flower development, genetics of. *in*: S Brenner, JH Miller eds. Encyclopedia of Genetics. Academic Press.

Theissen, G, A Becker 2004 Gymnosperm orthologues of class B floral homeotic genes and their impact on understanding flower origin. CRC Crit Rev Plant Sci 23: 129-148.

Theissen, G, A Becker, A Di Rosa, A Kanno, JT Kim, T Munster, KU Winter, H Saedler 2000 A short history of MADS-box genes in plants. Plant Mol Biol 42: 115-149.

Theissen, G, A Becker, K-U Winter, T Münster, C Kirchner, H Saedler 2002 How the land plants learned their floral ABCs: the role of MADS-box genes in the evolutionary origin of flowers. *in*: Q Cronk, R Bateman, J Hawkins eds. Developmental Genetics and Plant Evolution. Taylor & Francis, London, UK.

Theissen, G, JT Kim, H Saedler 1996 Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in the morphological evolution of eukaryotes. J Mol Evol 43: 484-516.

Theissen, G, R Melzer 2007 Molecular mechanisms underlying origin and diversification of the angiosperm flower. Ann Bot 100: 603-619.

Thompson, JD, TJ Gibson, F Plewniak, F Jeanmougin, DG Higgins 1997 The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876-4882.

Thorne, JL, H Kishino 2002 Divergence time and evolutionary rate estimation with multilocus data. Syst Biol 51: 689-702.

Thorne, JL, H Kishino, IS Painter 1998 Estimating the rate of evolution of the rate of molecular evolution. Mol Biol Evol 15: 1647-1657.

Tröbner, W, L Ramirez, P Motte, I Hue, P Huijser, WE Lonnig, H Saedler, H Sommer, Z Schwarz-Sommer 1992 *GLOBOSA*: a homeotic gene which interacts with *DEFICIENS* in the control of Antirrhinum floral organogenesis. EMBO J 11: 4693-4704.

Tucker, SC, AW Douglas 1996 Floral structure, development, and relationships of paleoherbs: *Saruma*, *Cabomba*, *Lactoris*, and selected Piperales. *in*: DW Taylor, LJ Hickey eds. Flowering plant origin, evolution, and phylogeny. Chapman & Hall, New York, NY, USA.

Tzeng, TY, HY Chen, CH Yang 2002 Ectopic expression of carpel-specific MADS box genes from lily and lisianthus causes similar homeotic conversion of sepal and petal in *Arabidopsis*. Plant Physiol 130: 1827-1836.

Vandenbussche, M, J Zethof, S Royaert, K Weterings, T Gerats 2004 The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in *Petunia hybrida* flower development. Plant Cell 16: 741-754.

Vogel, S, F Hadacek 2004 Contributions to the functional anatomy and biology of *Nelumbo nucifera* (Nelumbonaceae) III. An ecological reappraisal of floral organs. Pl Syst Evol 249: 173-189.

Wang, XQ, DC Tank, T Sang 2000 Phylogeny and divergence times in Pinaceae: evidence from three genomes. Mol Biol Evol 17: 773-781.

Wang, XR, Y Tsumura, H Yoshimaru, K Nagasaka, AE Szmidt 1999 Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast *rbcL*, *matK*, RPL20-RPS18 spacer, and *trnV* intron sequences. Am J Bot 86: 1742-1753.

Wang, Z, Y Liang, C Li, Y Xu, L Lan, D Zhao, C Chen, Z Xu, Y Xue, K Chong 2005 Microarray analysis of gene expression involved in anther development in rice (*Oryza sativa* L.). Plant Mol Biol 58: 721-737.

Warner, KA, PJ Rudall, WF Michael 2008 Differentiation of perianth organs in Nymphaeales. Taxon 57: 1096-1109.

Weigel, D 1995 The genetics of flower development: from floral induction to ovule morphogenesis. Annu Rev Genet 29: 19-39.

Wellmer, F, M Alves-Ferreira, A Dubois, JL Riechmann, EM Meyerowitz 2006 Genome-wide analysis of gene expression during early *Arabidopsis* flower development. PLoS Genet 2: e117.

Wellmer, F, JL Riechmann 2005 Gene network analysis in plant development by genomic technologies. Int J Dev Biol 49: 745-759.

Wellmer, F, JL Riechmann, M Alves-Ferreira, EM Meyerowitz 2004 Genome-wide analysis of spatial gene expression in *Arabidopsis* flowers. Plant Cell 16: 1314-1326.

Whipple, CJ, P Ciceri, CM Padilla, BA Ambrose, SL Bandong, RJ Schmidt 2004 Conservation of B-class floral homeotic gene function between maize and *Arabidopsis*. Development 131: 6083-6091.

Wiersema, JH 1988 A monograph of *Nymphaea* subgenus *Hydrocallis* (Nymphaeaceae). American Society of Plant Taxonomists, Ann Arbor, MI, USA.

Wiersema, JH, CB Hellquist 1998 Nymphaeaceae. *in*: FoNAE Committee ed. Flora of North America. Vol. 3. Oxford University Press, New York, NY, USA.

Williamson, PS, MF Moseley 1989 Morphological Studies of the Nymphaeaceae sensu lato. XVII. Floral anatomy of *Ondinea purpurea* subspecies *purpurea* (Nymphaeaceae). Am J Bot 76: 1779-1794.

Williamson, PS, EL Schneider 1993 Cabombaceae. *in*: K Kubitzki, JG Rohwer, V Bittrich eds. The families and genera of vascular plants. Vol. 2. Springer, Berlin, Germany.

Williamson, PS, EL Schneider 1994 Floral aspects of *Barclaya* (Nymphaeaceae): pollination, ontogeny and structure. Pl Syst Evol 8 suppl: 159-173.

Winter, KU, A Becker, T Munster, JT Kim, H Saedler, G Theissen 1999 MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. Proc Natl Acad Sci U S A 96: 7342-7347.

Winter, KU, C Weiser, K Kaufmann, A Bohne, C Kirchner, A Kanno, H Saedler, G Theissen 2002 Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. Mol Biol Evol 19: 587-596.

Worberg, A, D Quandt, AM Barniske, C Lohne, KW Hilu, T Borsch 2007 Phylogeny of basal eudicots: Insights from non-coding and rapidly evolving DNA. Organisms diversity & Evolution 7: 55-77.

Yang, YH, NP Thorne 2003 Normalization for two-color cDNA microarray data. *in*: DR Goldstein ed. Science and Statistics: A Festschrift for Terry Speed, IMS Lecture Notes - Monograph Series. Vol. 40. Institute of Mathematical Statistics, Beachwood, OH.

Yang, Z 1994 Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. J Mol Evol 39: 306-314.

Yang, Z 2000 Phylogenetic analysis by maximum likelihood (PAML). University College London, London, UK.

Yanofsky, MF, H Ma, JL Bowman, GN Drews, KA Feldmann, EM Meyerowitz 1990 The protein encoded by the *Arabidopsis* homeotic gene agamous resembles transcription factors. Nature 346: 35-39.

Yu, D, M Kotilainen, E Pollanen, M Mehto, P Elomaa, Y Helariutta, VA Albert, TH Teeri 1999 Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). Plant J 17: 51-62.

Yu, H, T Ito, F Wellmer, EM Meyerowitz 2004 Repression of *AGAMOUS-LIKE 24* is a crucial step in promoting flower development. Nat Genet 36: 157-161.

Yu, HJ, P Hogan, V Sundaresan 2005 Analysis of the female gametophyte transcriptome of Arabidopsis by comparative expression profiling. Plant Physiol 139: 1853-1869.

Zachgo, S, A Silva Ede, P Motte, W Trobner, H Saedler, Z Schwarz-Sommer 1995 Functional analysis of the *Antirrhinum* floral homeotic *DEFICIENS* gene in vivo and *in vitro* by using a temperature-sensitive mutant. Development 121: 2861-2875.

Zahn, LM, H Kong, JH Leebens-Mack, S Kim, PS Soltis, LL Landherr, DE Soltis, CW Depamphilis, H Ma 2005 The evolution of the *SEPALLATA* subfamily of MADS-box genes: a pre-angiosperm origin with multiple duplications throughout angiosperm history. Genetics 169: 2209-2223.

Zanis, MJ, DE Soltis, PS Soltis, S Mathews, MJ Donoghue 2002 The root of the angiosperms revisited. Proc Natl Acad Sci U S A 99: 6848-6853.

Zanis, MJ, PS Soltis, YL Qiu, E Zimmer, DE Soltis 2003 Phylogenetic analyses and perianth evolution in basal angiosperms. Ann Mo Bot Gard 90: 129-150.

Zhang, L-B, S Renner 2003 The deepest split in Chloranthaceae as resolved by chloroplast sequences. Int J Plant Sci 164 Suppl: S383-S392.

Zhang, L, Y Xu, R Ma 2008 Molecular cloning, identification, and chromosomal localization of two MADS box genes in peach (*Prunus persica*). J Genet Genomics 35: 365-372.

Zhang, P, HT Tan, KH Pwee, PP Kumar 2004 Conservation of class C function of floral organ development during 300 million years of evolution from gymnosperms to angiosperms. Plant J 37: 566-577.

Zhang, X, B Feng, Q Zhang, D Zhang, N Altman, H Ma 2005 Genome-wide expression profiling and identification of gene activities during early flower development in Arabidopsis. Plant Mol Biol 58: 401-419.

Zhao, D, Q Yu, C Chen, H Ma 2001 Genetic control of reproductive meristems. *in*: MT McManus, B Veit eds. Meristematic tissues in plant growth and development. Sheffield Academic Press, Sheffield, England.

Zomlefer, WB 1994 Nymphaeaceae. *in*: WB Zomlefer ed. Guide to flowering plant families. University of North Carolina Press, Chapel Hill, NC.

BIOGRAPHICAL SKETCH

Mi-Jeong Yoo was born in Chungju, Korea, and spent her childhood in the country, where she developed her interest in nature. She received her Bachelor of Science in biology from Seoul national university, Seoul, Korea, in March 1998. She began her graduate career in the Department of Biological Sciences at Seoul National University, and received her Master of Science in March 2000. After graduation, she decided to study abroad. While preparing her application, she worked at the herbarium of Seoul National University (SNU) as a specimen manager. Mi-Jeong was admitted to the University of Florida, so she started her doctoral work with Drs. Douglas and Pamela Soltis in Fall 2002. She studied the floral developmental genetics and evolution in water lilies. Mi-Jeong graduated with a PhD in botany in December 2008.