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Cabomba as a model for studies of early angiosperm evolution

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INTRODUCTION

There are no widely accepted fossil angiosperms older than the Early Cretaceous, some 130 million years ago (Doyle, 2008). However, during the Cretaceous period, the angiosperms diversified to dominate almost all terrestrial environments. Molecular phylogenetic studies indicate that the orders Amborellales, Nymphaeales and Austrobaileyales, collectively termed the ANA grade, diverged as separate lineages from a remaining angiosperm clade at a very early stage in flowering plant evolution. By comparing these early diverging lineages, it is possible to infer the possible morphology and ecology of the last common ancestor of the extant angiosperms, and this analysis can now be extended to try to deduce the developmental mechanisms that were present in early flowering plants. However, not all species in the ANA grade form convenient molecular-genetic models.

The present study reviews the genus Cabomba (Nymphaeales), which shows a range of features that make it potentially useful as a genetic model. We focus on characters that have probably been conserved since the last common ancestor of the extant flowering plants. To facilitate the use of Cabomba as a molecular model, we describe methods for its cultivation to flowering in the laboratory, a novel hybridization protocol and a measurement of the nuclear genome size of C. caroliniana. We discuss the features required for species to become tractable models, and discuss the relative merits of Cabomba and other ANA-grade angiosperms in molecular-genetic studies aimed at understanding the origin of the flowering plants.

Key words: Cabomba, Cabombaceae, Trithuria, Hydatellaceae, Nymphaeales, ANA grade, angiosperm, flowering plant evolution, expressed sequence tags, EST, c-value, in situ hybridization, evo-devo.

Background

The angiosperms, or flowering plants, diversified in the Cretaceous to dominate almost all terrestrial environments. Molecular phylogenetic studies indicate that the orders Amborellales, Nymphaeales and Austrobaileyales, collectively termed the ANA grade, diverged as separate lineages from a remaining angiosperm clade at a very early stage in flowering plant evolution. By comparing these early diverging lineages, it is possible to infer the possible morphology and ecology of the last common ancestor of the extant angiosperms, and this analysis can now be extended to try to deduce the developmental mechanisms that were present in early flowering plants. However, not all species in the ANA grade form convenient molecular-genetic models.

Scope

The present study reviews the genus Cabomba (Nymphaeales), which shows a range of features that make it potentially useful as a genetic model. We focus on characters that have probably been conserved since the last common ancestor of the extant flowering plants. To facilitate the use of Cabomba as a molecular model, we describe methods for its cultivation to flowering in the laboratory, a novel hybridization protocol and a measurement of the nuclear genome size of C. caroliniana. We discuss the features required for species to become tractable models, and discuss the relative merits of Cabomba and other ANA-grade angiosperms in molecular-genetic studies aimed at understanding the origin of the flowering plants.

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Hydatellaceae, which together constitute approx. 90 species in eight or nine genera (depending on whether Ondinea is grouped within Nymphaea: Löhne et al., 2009). Finally, Austrobaileyales contains three families of woody plants: Austrobaileyaceae, Trimeniaceae and Schisandraceae, which include approx. 70 species in five genera. Most recent molecular phylogenetic analyses support the sequence of divergence of the three ANA-grade lineages as first Amborellales, then Nymphaeales and finally Austrobaileyales, although some studies have suggested an alternative topology in which Amborellales and Nymphaeales together form a first-diverging clade (Qiu et al., 2001, 2006; Zanis et al., 2002).

The relatively low number of extant ANA-grade species indicates a large number of extinctions in these ancient lineages, and/or the possibility that these groups have undergone fewer speciation events than the remaining angiosperm clade (Fig. 1). A relatively low speciation rate could have been a factor in the retention in the ANA grade of a high proportion of ancestral characters (Endress, 2004). Accordingly, comparisons of ANA-grade species have been used to speculate on the likely characteristics of the last common ancestor of the extant angiosperms, including both morphological/ anatomical (Endress and Igersheim, 2000; Endress, 2001a, b; Rudall et al., 2007, 2009a, b; Endress and Doyle, 2009; Friedman and Ryerson, 2009; Rudall and Bateman, 2010) and ecological (Feild et al., 2003, 2004) studies. A major challenge is to extend these views of early angiosperms to include information on the mechanisms that controlled their development. Furthermore, by including gymnosperms in these comparisons as an outgroup, it may be possible to infer something of the molecular events that contributed to the evolution of the first flowers (Scutt et al., 2006; Ferrandiz et al., 2010; Rudall and Bateman, 2010; Rudall et al., 2011). Thus, the aims of this article are to provide novel information and well-adapted protocols to facilitate the use of Cabomba as a potentially useful model within the ANA grade, and review the literature on Cabomba that is relevant to evo-devo studies.

CRITERIA FOR SELECTING MODEL ANA-GRADE ANGIOSPERMS

To facilitate molecular studies aimed at understanding the early evolution of angiosperms, it is useful to focus on some ANA-grade species, preferably covering all three orders.

Fig. 1. Phylogenetic position of Cabomba. Schematic phylogeny based on Bremer et al. (2009) and related internet resources. Approximate species numbers are given in parentheses.
Species used for in-depth investigation should be amenable for study using a wide variety of molecular biological and genetic methods (Bolker, 1995). Full genome sequencing of such models should be envisaged (see Soltis et al., 2008), and manageable genome sizes are therefore an advantage. Ideally, model species should also contain a high number of likely pleiomorphic features. However, the plesiomorphic states are notoriously difficult to determine in angiosperms, because of the high morphological diversity among ANA-grade angiosperms and the absence of a closely related extant outgroup that would allow polarization of characters and reconstruction of the hypothetical ancestral states (Bateman et al., 2006).

Recent molecular phylogenetic analyses (e.g. Löhr et al., 2007; Borsch et al., 2008) have resulted in the recognition of three families in Nymphaeales: Cabombaceae (two genera), Hydatellaceae (one) and Nymphaeaceae (six). In common with all other ANA-grade families, Nymphaeaceae show some derived traits, and the relatively large overall size and large flowers of most Nymphaeaceae could make these species problematic as model angiosperms. Despite these potential disadvantages, expressed sequence tag (EST) resources are available for Nuphar (Albert et al., 2005), and this genus, together with Nymphaea, has already been incorporated into several evolutionary-developmental (‘evo-devo’) studies (Yamada et al., 2003; Kim et al., 2004, 2005; Yoo et al., 2010).

Hydatellaceae, previously classified with the monocots, was recently shown to represent the earliest diverging extant lineage of Nymphaeales (Saarela et al., 2007). This family contains a single genus, Trithuria, with ten species in Australia, one in New Zealand and one in southern India (Sokoloff et al., 2008). Several species of Trithuria form bisexual reproductive axes, but these contain stamens at the centre, surrounded by carpels, and develop centripetally. These reproductive units, termed ‘non-flowers’ by Rudall et al. (2009a), could represent inside-out flowers, or cymose inflorescences in which each floral organ corresponds to a reduced flower. Set against their unusual reproductive arrangement, Trithuria plants are very small, and at least some species have a rapid generation time and can easily be grown to maturity in the laboratory (Rudall et al., 2009b). Although relatively few molecular biological procedures have so far been tested on Trithuria, the important practical considerations of growth habit, size and life cycle make these plants potentially well adapted as molecular-genetic models.

The remaining family of Nymphaeales, Cabombaceae, consists of two genera: Cabomba (five species: Orgaard, 1991) and Brasenia (one: B. schreberi). All Cabombaceae possess trimerous flowers with a whorled perianth arrangement and non-fused ascidiate carpels. Although Cabomba and Brasenia share many morphological features, Cabomba seems better suited as a model given its smaller overall size. In addition, all submerged parts of Brasenia are covered in a thick, viscous slime that could cause serious problems for molecular biological procedures such as in situ hybridization.

**CABOMBA: A BRIEF DESCRIPTION**

Cabomba is a New World genus whose distribution extends from the north-eastern United States to northern Argentina, although most species are restricted to tropical or subtropical regions (Orgaard, 1991). All Cabomba species are aquatic and can grow in both stagnant and flowing fresh water. Cabomba plants form sparsely branched, erect stems and horizontal rhizomes that give rise to adventitious roots at leaf nodes. A marked foliar dimorphism is present in Cabomba, which forms submerged leaves that are highly dissected to reduce water drag, and floating leaves that have entire margins (Fig. 2). Both leaf types carry out photosynthesis, and the floating leaves also function to anchor and stabilize the plant to the water’s surface and thus allow the flowers to be borne above the surface to enable pollination. As in many other aquatic plants, air canals ramify through the stems and petioles of Cabomba to facilitate gaseous exchange in the submerged parts of the plant. This and other aspects of the anatomy of Cabomba were described by Moseley et al. (1984), including a detailed treatment of the vascular arrangement.

A marked phase change to reproductive development is present in Cabomba, which can be manipulated by adjusting the light conditions (see below). Prior to phase change, the primordia arising from the shoot apical meristem develop into submerged leaves, whereas the primordia that arise after phase change develop into floating leaves that each subtend an axillary flower bud. Each shoot in its reproductive phase thus produces a succession of single flowers at the water’s surface. Cabomba flowers are trimerous and contain two whorls of three petaloid tepals that form in alternating positions (Figs 2 and 3). Interestingly, a whorled, rather than a spiral, floral phyllotaxy is sometimes regarded as a derived condition in the angiosperms, but is present throughout both Nymphaeaceae and Cabombaceae, and may represent the basal condition in Nymphaeales. In Cabomba, the inner tepals are developmentally retarded with respect to the outer tepals and other floral organs (Tucker and Douglas, 1996; Endress, 2001a; Rudall et al., 2009a), so that the reproductive organs are visible without dissection at early developmental stages (Fig. 3B): an unusual feature, also present in Trithuria (Rudall et al., 2009a), that is potentially useful in a model organism. Three or six stamens are typically present in Cabomba, and one to four (most commonly three) separate carpels, which each contain one to five pendulous, anatropous ovules (Yamada et al., 2001). The carpels of Cabomba are ascidiate and almost radially symmetric (Fig. 2D–F), resembling those of Trithuria, especially at early developmental stages (Endress, 2001; Schneider et al., 2003; Rudall et al., 2007). The carpels of Cabomba are not closed by cell fusion but by substances secreted into a narrow, open canal: a condition termed Type 1 angiosperm by Endress and Igersheim (2000), who concluded that this represents the likely ancestral condition in angiosperms because it occurs in many ANA-grade taxa (but not all; exceptions include Nymphaea and Illicium, in which carpels are postgenitally closed at anthesis; Endress and Igersheim, 2000).

The stigmatic surface in Cabomba is composed of multicellular protrusions, rather than unicellular papillae. Multicellular stigmatic hairs also occur in some other ANA-grade taxa, most notably Trithuria (Przychid et al., 2011), so this could represent a plesiomorphic angiosperm feature (Endress and Igersheim, 2000). Cabomba is insect pollinated, and nectaries are
present at the base of the inner tepals (Fig. 3F, G; Orgaard, 1991) to reward visiting pollinators, which include bees, wasps and flies (Schneider and Jeter, 1982). In *Cabomba*, as in many members of the ANA grade, protogyny acts as an out-breeding mechanism. Accordingly, each *Cabomba* flower opens on two successive days and closes during the intervening night. For fertilization to occur, the carpels of a first-day flower must be pollinated from the stamens of a second-day flower. Once pollination has occurred, the flower is rapidly pulled below the water by bending of the penduncle (Schneider and Jeter, 1982). The fruit then forms and, after approx. 1 month, releases its seeds directly into the water by decomposition of the fruit wall (Fig. 3). Vegetative propagation in the wild is also very common, and fragments of *Cabomba* stem can rapidly generate new plants under appropriate conditions: a capacity that has caused the clogging of ponds and waterways where *Cabomba* has been inadvertently introduced in Australia and elsewhere (Wilson et al., 2007; Schooler, 2008).

**HOW TO GROW CABOMBA IN THE LABORATORY**

For a plant to be considered as a potential molecular-genetic model for the study of its flowers, it must be at least reasonably easy to grow to flowering in a laboratory or greenhouse environment. We have accordingly developed procedures to grow *Cabomba* through to flowering and seed-set in an aquarium of 200 L. Using such an aquarium, we have grown both *C. aquatica* and *C. caroliniana* plants rooted in a substrate composed of 50% sand, 35% ericaceous compost and 15% kaolin (by volume). A 5-cm layer of this substrate was used, retained by a 2.5-cm layer of well-washed horticultural pumice. The aquarium was initially half-filled with deionized water, supplemented with ‘Flourish’ and ‘Flourish Iron’ nutrient solutions (Seachem, Madison, GA, USA), according to the manufacturer’s recommendations. The use of peat-containing compost in the aquarium substrate was found to maintain the aquarium water at approx. pH 5.5–6.0, which proved suitable for growing both *Cabomba* species. The aquarium water was constantly filtered using an external pump through layers of both aquarium cell foam and active peat granules (JBL GmbH, Neuhausen, Germany). A UV lamp (Aqua Cristal; JBL GmbH) was also incorporated into the pump circuit to reduce algal growth. Once the initial turbulence of the water had subsided, after approx. 2 d of filtration, groups of *Cabomba* stems approx. 12 cm in length were planted into the substrate. *C. caroliniana* and *C. aquatica* were grown at water temperatures of 22 and 25°C, respectively. The aquarium water was supplemented with carbon dioxide from a cylinder at approx. 120 bubbles per minute, these being retained for maximum dissolution in an inverted chamber attached to the return from the water pump. The aquarium water was also oxygenated by allowing part of the return...
from the water pump to fall from holes drilled into a pipe running along one side of the aquarium, approx. 5 cm above the water’s surface. The water level was gradually increased, to keep up with plant growth.

To produce rapid vegetative growth of *Cabomba*, we used a purely artificial light source consisting of three specialized aquarium lamps (Floraset; Osram, Mississauga, Canada) fitted with 80-W discharge light bulbs (HDL-R; Osram) suspended at approx. 20 cm above the maximum water level. However, to induce flowering, it was necessary to add three domestic 60-W tungsten light bulbs, which had the effect of enriching the overall illumination in far-red wavelengths (700–750 nm). Under these conditions, *Cabomba* could be grown from short pieces of stem to flowering in approx. 6 weeks.

We have successfully pollinated *C. caroliniana* plants to generate fruits by taking dehisced anthers from flowers open on their second day and using these to manually pollinate flowers open on their first day. We have not, however, investigated seed viability, longevity or germination requirements.

**AN EST RESOURCE FOR THE *CABOMBA* FLOWER**

Publicly available EST resources of Nymphaeales have up to now been limited to *Nuphar* (Albert et al., 2005). To facilitate molecular studies of *Cabomba*, we have generated a database of 3097 ESTs from a uni-directional *C. aquatica* flower cDNA bank constructed in the Bacteriophage λ Uni-Zap II vector (Stratagene, La Jolla, CA, USA). cDNAs for inclusion in this database were selected randomly from bacterial colonies harbouring recombinant pBluescriptII SK- plasmids which resulted from mass *in vivo* excisions of the λ-cloned bank, performed using ExAssist M13 helper phage (Stratagene) according to the manufacturer’s protocols. The
selected cDNAs were sequenced from their 5′-ends and the resulting EST database has been made publicly available and can be interrogated by Blast and other procedures at http://www.ncbi.nlm.nih.gov/ and http://urgi.versailles.inra.fr/index.php/urgi/Data/Sequences/Run-GnpSeq.

We have characterized this database by performing a functional annotation using version 2.4-5 of the Blast2GO procedure described by Conesa et al. (2005). The NCBI non-redundant (nr) nucleotide database was searched by Blastx with Cabomba ESTs using the following parameters: number of hits, 100; expected value, 1.0 × 10^{-3}; mode, QBlast-NCBI; HSP length cut-off, 33; filtering for low-complexity sequences, on. Following mapping onto GO databases, annotation was performed using the following parameters: E-value hit filter, 1.0 × 10^{-5}; annotation cut-off, 55; GO weight, 5; Hsp hit coverage cut-off, 0. Sequences were categorized for biological processes, molecular functions and cellular components.

The results of these functional annotations are shown in Fig. 4, and demonstrate that the Cabomba EST database described contains numerous categories of sequences likely to be involved in developmental, regulatory and reproductive processes (Fig. 4A), of potential interest in the context of flower evo-devo research. High proportions of ESTs in the database encode domains of known binding or catalytic activity, while some 145 ESTs show homology to known transcription factors (Fig. 4B). Preliminary phylogenetic analyses of the ESTs encoding transcription factors indicate the presence of potential orthologues of several regulators of flower development, including members of the MADS-box, NAC and AUXIN RESPONSE FACTOR families. The majority of encoded proteins appear to be localized in the cytoplasm or nucleus, while the second largest group is addressed to organelles. Probable membrane-associated and extracellular proteins are also represented in significant numbers (Fig. 4C).

AN IN SITU HYBRIDIZATION PROTOCOL ADAPTED TO CABOMBA

In situ hybridization is an extremely valuable technique for the study of plant development. Conservation of gene expression patterns between species can be used to argue for the probable conservation of gene function. This type of evidence can be particularly useful for the molecular analysis of species that occupy key phylogenetic positions, but which are not amenable to the direct analysis of gene function, as is currently the case for the entire ANA grade of basal angiosperms.

We have tested several methods for non-radioactive mRNA in situ hybridization on Cabomba and found the method used by Nikovics et al. (2006) to give the best results, combining high signal strength and excellent tissue preservation. Using this technique, in situ hybridizations to the shoot apical meristem on a HISTONE4 probe clearly show very high signal strength in actively dividing cells, with very little background elsewhere (Fig. 5). At high magnification, both individual cell contours and cell nuclei are clearly visible (Fig. 5C). As this method is described succinctly in its original publication, we reproduce it in the present work in the form of a user-friendly protocol (Supplementary Data).

COMPARISON OF NUCLEAR GENOME SIZES IN ANA-GRADE ANGIOSPERMS

Genome size is an important consideration in plant molecular biology, which must be taken into account when constructing and screening genomic libraries, performing Southern blots, or planning the sequencing of an entire genome. We measured the nuclear genome size of C. caroliniana material obtained from both Anthias SA (Les Chêres, Rhône, France) and Bleu Aquarium (Caveriac, France). Leaves of each sample, and of the internal standard Pisum sativum ‘Long Express’ (2C = 8.37 pg; Marie and Brown, 1993), were chopped using a razor blade in 600 μL of cold Galbraith buffer (Galbraith et al., 1983) supplemented with 1 % polyvinylpyrrolidone 10 000 and 50 μg mL^{-1} RNase A (Sigma Chemical Co., St. Louis, MO, USA). The suspension of nuclei thus produced was filtered through nylon mesh (pore size 48 μm), stained with 50 μg mL^{-1} propidium iodide (Sigma) and analysed on a flow cytometer (Elite, Beckman-Coulter, Villepinte, France). No significant difference was observed between the means of the two populations sampled, and the combined mean of the 23 measurements made was calculated as 2C = 6.73 ± 0.06 pg, giving a haploid genome size for C. caroliniana of 1C = 3290 ± 30 Mbp (taking 1 pg as equivalent to 978 Mbp, from Dolezel et al., 2003).

The genome of C. caroliniana appears to be considerably larger than average for Nymphaeae, but slightly smaller than average for the ANA grade as a whole (Table 1). This genome, at approx. 30 % greater than that of the monocot model Zea mays, remains sufficiently small to permit both genomic library construction and Southern blotting. In addition, with the current enormous increase in the speed of DNA sequencing technology, including recently published methods of up to 10^8 times faster than automated Sanger sequencing (Eid et al., 2009), the genomes of C. caroliniana and many other ANA-grade angiosperms should rapidly fall within the reach of complete sequence analysis. Perhaps a greater challenge for evo-devo studies aimed at understanding the origin of the flower will be the sequencing and assembly of the much larger genomes typically present in gymnosperms. The 1C values of gymnosperms range from 9.62 Mbp in Juniperus communis subsp. alpina (Sijak-Yakovlev et al., 2010) to 32.5 Mbp in Pinus gerardiana (Grotkopp et al., 2004, recalculated with the reference value of Bogunic et al., 2007): three to ten times bigger than that of Cabomba. Sequencing of gymnosperm genomes should provide an external reference to the angiosperms, which will surely be necessary if we are to identify the molecular events that generated the first flowers.

POTENTIAL OF CABOMBA AS A MODEL ANA-GRADE ANGIOSPERM

The present work examines the potential of Cabomba as a molecular-genetic model to represent Nymphaeae within the ANA grade. As explained above, all three families within Nymphaeae contain species of potential use as molecular models. Within Cabombaceae, it seems clear that Cabomba fulfills the requirements of a convenient model better than its sister genus Brasenia. However, within Nymphaeae as a whole, serious competition for super-model...
Fig. 4. Functional annotation of the *Cabomba* flower EST database. Sequences are annotated using Blast2GO to categorize ‘Gene Ontology level 2 terms’ matching with at least 100 sequences for: biological processes (A), molecular functions (B) and cellular components (C). Numbers of ESTs in each category are shown in parentheses.
models within Nymphaeales. Indeed, for evo-devo studies of floral patterning and perianth development, which are highly derived features in Trithuria, it would seem essential to include additional models of Nymphaeales. Cabomba, as discussed above, combines simplicity of floral structure, numerous pleiomorphic angiosperm characters, and practical features that make it amenable to study using a broad range of molecular biological techniques. We therefore argue that Cabomba can provide a useful contribution to evo-devo studies of angiosperm origin, alongside Trithuria in Nymphaeales, Amborella as the unique representative of Austrobaileyales and suitable further models chosen from Austrobaileyales. Indeed, Cabomba has already made some contributions to evo-devo studies, with respect to both protein-coding genes (Fourquin et al., 2005; Finet et al., 2010; Yoo et al., 2010; Vialette-Guiraud et al., 2011) and small regulatory RNAs (Jasinski et al., 2010).

Although Cabomba and several other ANA-grade taxa are clearly amenable to molecular biological analyses, a far greater challenge lies in the study of gene function in these plants. Conservation of gene expression between model and non-model species has provided a number of insights into probable gene functions in the ANA grade, although such data fall short of direct functional evidence and are relevant only to genes that show highly characteristic expression patterns, which is not the case for many important developmental regulators. In addition, we should not be content to study only what has been conserved during evolution: differences in gene function should also be investigated if we are to understand the genetic basis for morphological biodiversity. For evo-devo studies to engage fully with the question of the origin and early evolution of the angiosperms, we should develop methods of functional analysis for use in the ANA grade. As yet, almost no work has been performed in this direction, but it is worth considering at this stage the techniques and ANA-grade taxa that might be best adapted to the needs of evo-devo research.

Traditionally, plant functional genetics has been performed through the production of mutants, which can be generated by pseudo-random T-DNA insertion, transposon mutagenesis, or exposure to mutagenic chemicals or radiation. The resulting mutant collections can be screened phenotypically by ‘forward genetic’ approaches, or for mutations in genes of interest through ‘reverse genetic’ approaches, including the TILLING technique, which does not require

status comes from Trithuria, which possesses the advantages of very small size, ease of cultivation and rapid generation time. Preliminary studies (R. Kynast and P. J. Rudall, unpubl. res.) also indicate the advantage of a relatively small genome size in Trithuria.

Despite the evident practical advantages of Trithuria, the need for a comparative approach is a compelling argument for the development of more than one model within each of the non-monotypic ANA-grade orders, Nymphaeales and Austrobaileyales. Furthermore, the unusual reproductive arrangement of Trithuria argues for the use of additional

### Table 1. Genome sizes of ANA-grade angiosperms

<table>
<thead>
<tr>
<th>ANA-grade species</th>
<th>Mass of 1C (pg)</th>
<th>Physical length (Mb)</th>
<th>Reference</th>
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<td>Mean for Amborellales</td>
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<tr>
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<td>Mean for ANA grade</td>
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</table>
genetic transformation (Wang et al., 2010). Both forward and reverse genetic screens require the growth of large numbers of plants and the long-term storage of mutant collections, usually in the form of seed. Unfortunately, Cabomba is not sufficiently easy to grow in large numbers to permit forward or reverse genetic screening, and seed production and storage issues have yet to be fully addressed in this genus. Trithuria, by contrast, is small and flowers rapidly, and its seed can be stored conveniently (Tuckett et al., 2010). Trithuria is thus potentially amenable to forward and reverse genetic screening, and indeed appears to be the only ANA-grade angiosperm that might readily lend itself to the use of these techniques.

Happily for the future of reverse genetics in the ANA grade, further techniques are available that do not depend on the generation of large mutant collections. In these methods, much smaller groups of plants can either be transformed to integrate a chosen transgene or, in an approach termed virus-induced gene silencing (VIGS), infected with a transgenic virus to reduce the expression of one or more endogenous genes (Burch-Smith et al., 2004). Through the transformation approach, gene expression may be either down-regulated by RNA interference (RNAi; Hellwell and Waterhouse, 2003), or misdirected through the use of a non-native or modified gene promoter. Further, more precise techniques for the analysis of gene function are also being developed, including the use of zinc-finger nucleases (Osakabe et al., 2010). These recombinant enzymes are engineered to generate double-stranded DNA breaks at pre-defined loci, whose subsequent repair by endogenous mechanisms can be exploited to perform precise gene replacements. Needless to say, the success of these approaches depends on the availability of efficient transformation and regeneration methods, or on a VIGS vector that is capable of infecting the species of interest. Almost no work has been performed to date on the development of any of these techniques in the ANA grade, and such studies must now be a high priority. Although several members of the ANA grade have been shown to be amenable to methods for molecular analysis, it is the successful development of stable transformation and/or VIGS methods that will define the long-term value of these species as experimental models. As discussed above, Trithuria may prove to be the only ANA-grade angiosperm that readily lends itself to large-scale forward and reverse genetic screening. However, the requirements of evo-devo research mean that smaller-scale forward and reverse genetic screening, and indeed appears to be the only ANA-grade angiosperm that might readily lend itself to the use of these techniques.

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SUPPLEMENTARY DATA
Supplementary data are available online at www.aob.oxfordjournals.org and consist of a protocol for mRNA in situ hybridization for tissue sections suitable for use with Cabomba.

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