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Micropylar Pollen Tube Guidance by Egg Apparatus 1 of Maize

Mihaela L. Márton,[1] Simone Cordts,[1] Jean Broadhvest,[2] Thomas Dresselhaus[1]*

Pollen tube guidance precedes the double fertilization of flowering plants. Here, we report the identification of a small maize protein of 94 amino acids involved in short-range signaling required for pollen tube attraction by the female gametophyte. *ZmEA1* is exclusively expressed in the egg apparatus, consisting of the egg cell and two synergids. Chimeric ZmEA1 fused to green fluorescent protein (ZmEA1:GFP) was first visible within the filiform apparatus and later was localized to nucellar cell walls below the micropylar opening of the ovule. Transgenic down-regulation of the *ZmEA1* gene led to ovule sterility caused by loss of close-range pollen tube guidance to the micropyle.

In contrast to most animal and many lower plant species, sperm cells of flowering plants are nonmotile and are transported from the stigma to the female gametophyte (embryo sac) via pollen tube growth to allow double fertilization (*1*). Genetic and physiological studies have shown the involvement of both female sporophytic and gametophytic tissues in pollen tube guidance of different plant species (*2*). Molecules involved in sporophytic guidance have been identified as γ-aminobutyric acid (GABA), arabinogalactans, and small secreted proteins (*3–6*), but little is known about the molecules produced by the female gametophyte required for pollen tube guidance. The synergids have been identified as the source of producing a short-range pollen tube attractant or attractants in *Torenia fournieri*, but the molecular nature of the attractant(s) is still unknown (*7*).

We report the identification of *ZmEA1* (*Zea mays EGG APPARATUS1*) from an unfertilized maize egg cell cDNA library (*8*) and its role for short-range pollen tube attraction by the female gametophyte.

ZmEA1 is produced by the cells of the egg apparatus and represents a highly hydrophobic small protein of 94 amino acids with a predicted transmembrane domain (Fig. 1A). Tissue and single-cell reverse transcription polymerase chain reaction (RT-PCR) analyses showed that *ZmEA1* is exclusively expressed in the maize egg apparatus (Fig. 1, B and C) before fertilization. Lower RNA levels were detected in zygotes after in vitro fertilization and at an even lower level in two-celled proembryos. Expression of *ZmEA1* was no longer detectable at later embryo stages, which suggests a rapid down-regulation of the gene after fertilization. In situ hybridization of the maize ovary (Fig. 2B) and of female gametophyte isolated cells confirmed the restricted expression in the egg apparatus, which lacked detectable signals in the central cell, antipodals, and nucellar and integumental cells. Out of 988 ESTs generated from the originating cDNA library, 32 *ZmEA1* cDNA clones were identified, which suggests a high level of expression in mature egg cells. Further studies showed that *ZmEA1* is an intronless single gene in maize but may represent a member of a relatively heterologous gene family based on weak genomic Southern hybridization signals. Homologous sequences are also present as single genes in barley, pearl millet, and *Tripsacum dactyloides*. Interestingly, two

closely physically linked (4-kb) homologs appear to be present in rice [*Oryza sativa EA-like* 1 (*OsEAL1*), BAC83883.1, and *OsEAL2*, BAC83885.1 (Fig. 1A)], as shown by genomic Southern and database analyses. No obvious homologs were identified in *Arabidopsis thaliana* or other dicotyledonous plant species, which suggests a possible Gramineae specific conservation of *EA1*-like genes.

The *ZmEA1* promoter (*ZmEA1*p) was isolated as 1570 base pairs of genomic sequence 5′ of the AUG initiation codon and used for transgenic analyses of promoter and protein functions in the deeply embedded female gametophyte of maize (Fig. 2A). The promoter was fused to the β-glucuronidase (*Gus*) reporter gene. GUS activity in three of four independent functional *ZmEA1*p::*GUS* transgenic lines was exclusively detected in the egg apparatus of unpollinated mature ovules, confirming the egg apparatus specificity of the *ZmEA1*p transcriptional regulation (Fig. 2D). Unfertilized ovules from a transgenic maize line expressing GUS under control of a rice actin promoter (*OsACT*p::*GUS*) were used to show that staining in all cells of the female gametophyte could be detected (Fig. 2E). A ZmEA1:GFP C-terminal fusion protein regulated by the *ZmEA1*p was secreted from the egg apparatus to the micropylar region of the nucellus of four independent transgenic lines in a manner dependent on floral developmental stage. After silk emergence, the ZmEA1:GFP first accumulated in the filiform apparatus (egg apparatus cell walls) (Fig. 3, A and B). After silk elongation (>10 cm), GFP signal was extended to a restricted area of the nucellus of unfertilized ovules (Fig. 3, C to F). The increase of GFP signal was well correlated with maturation of the egg apparatus during the female receptive period (*9*). Confocal laser scanning microscopy (CLSM) observations confirmed the presence of cell wall–localized GFP signals spreading from the egg apparatus toward the surface of the nucellus at the micropylar opening of the ovule (Fig. 3, E and F). This suggests possible proteolysis of the conserved C-terminal region from the predicted ZmEA1 transmembrane domain to allow secretion and transport of

[1]Biocenter Klein Flottbek, Developmental Biology and Biotechnology, University of Hamburg, Ohnhorststrasse 18, D-22609 Hamburg, Germany. [2]Bayer BioScience N.V., Technologiepark 38, B-9052 Ghent, Belgium.

*To whom correspondence should be addressed. E-mail: dresselh@botanik.uni-hamburg.de

a mature protein to its target tissues. GFP fluorescence was not detectable at the surface of the inner integument (Fig. 3F) and was drastically reduced 24 hours after in vitro pollination. This rapid loss of GFP signal suggests proteolysis as a regulatory pathway to degrade ZmEA1 protein after fertilization.

We performed functional analyses of *ZmEA1* loss of activity using the highly expressing maize ubiquitin promoter via maize transgenic RNA interference (RNAi) and antisense (AS) lines A188 (R and AS lines, respectively) or hybrid A188 × H99 (Rh and ASh lines, respectively). We gen-

erated 11 independent R/Rh and 5 ASh transgenic lines, and all contained multiple and complete transgene insertions (≥2 to >10) in multiple loci (most lines), with the exception of 4 R/Rh lines that had incomplete transgene integrations. Transgenic lines were selfed or crossed with wild-type pollen and used as pollen donor to fertilize wild-type (A188) plants (Fig. 4A). Half of the transgenic lines with complete transgene insertions (6 out of 12) showed a reduced seed set (0 to 75%) upon selfing. The four nonfunctional transgenic lines displayed a seed set comparable to wild type (95 to 100%). Clonal transgenic lines (lines with identical transgene integration pattern) displayed very similar seed set. All wild-type plants crossed with transgenic pollen displayed full seed set (Fig. 4A), which suggests that the male gametophyte was not affected by the lower ZmEA1 activities. Although there was little variation between plants with the same integration pattern, the strong variation among independent lines is probably due to the segregation of the multiple transgenes in multiple chromosomal loci. We also assume that some integrations do not completely remove ZmEA1 activity. Because transgenic female reproductive structures were not different from wild type, we used a transgenic pollen tube expressing GUS line (*10*) to visualize the fertilization process to identify the cause of transgenic female sterility. In wild-type lines, many pollen tubes reached the surface of the inner integument and continued growth toward the micropylar region (Fig. 4B), and only one pollen tube turned abruptly at the micropyle to fertilize the embryo sac (Fig. 4C). GUS staining was visible in 82% of wild-type female gametophytes 24 to 30 hours after in vitro pollination (Fig. 4F). In the two selfed transgenic lines analyzed (Rh6.1 and Rh15), pollen tubes were visible on the inner integument surface and grew close to the
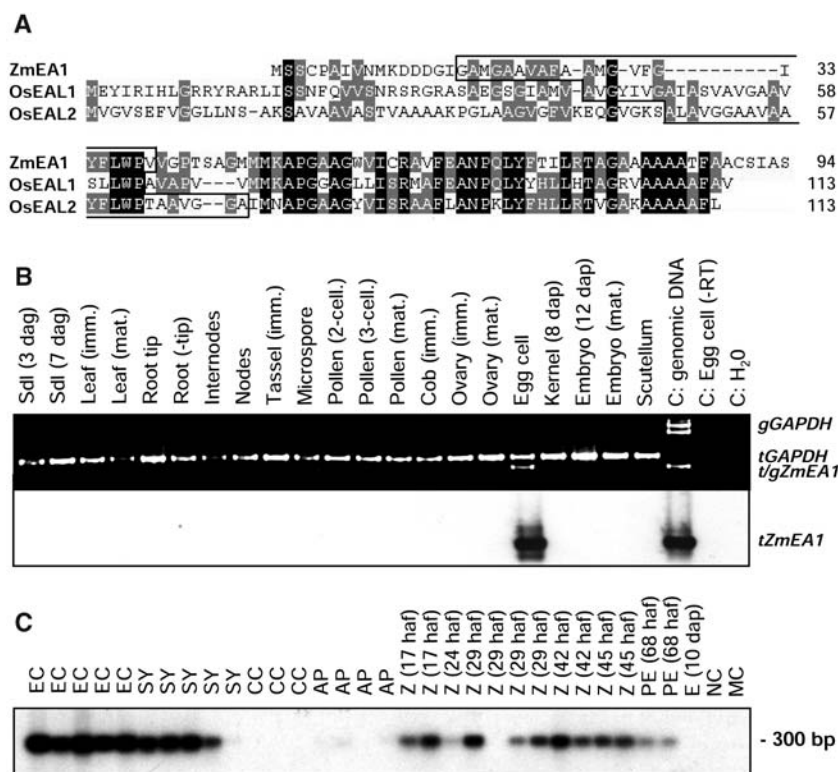

Fig. 1. *ZmEA1* gene expression and protein structure. (**A**) The *ZmEA1* predicted 94–amino acid protein shares C-terminal identity (shadowed) and transmembrane domains (boxed) with two predicted rice proteins (*OsEAL1/2*). (**B**) Multiplex RT-PCR with different tissues showed *ZmEA1* expression exclusively in the egg cell. *GAPDH* mRNA is present in all tissues. (**C**) RT-PCR analyses of *ZmEA1* expression in maize cells and tissues. *ZmEA1* is expressed in egg cells (EC) and synergids (SY) but not in other cells of the embryo sac (CC, central cell; AP, antipodals). *ZmEA1* expression is down-regulated in in vitro zygotes (Z), and transcript amounts are even less in the two-celled proembryo (PE). *ZmEA1* mRNA was not detected in nucellus cells (NC), at later embryo stages (E), or in leaf mesophyll cells (MC). Dag, days after germination; dap, days after pollination; haf, hours after in vitro fertilization; imm., immature; mat., mature; sdl, seedling.
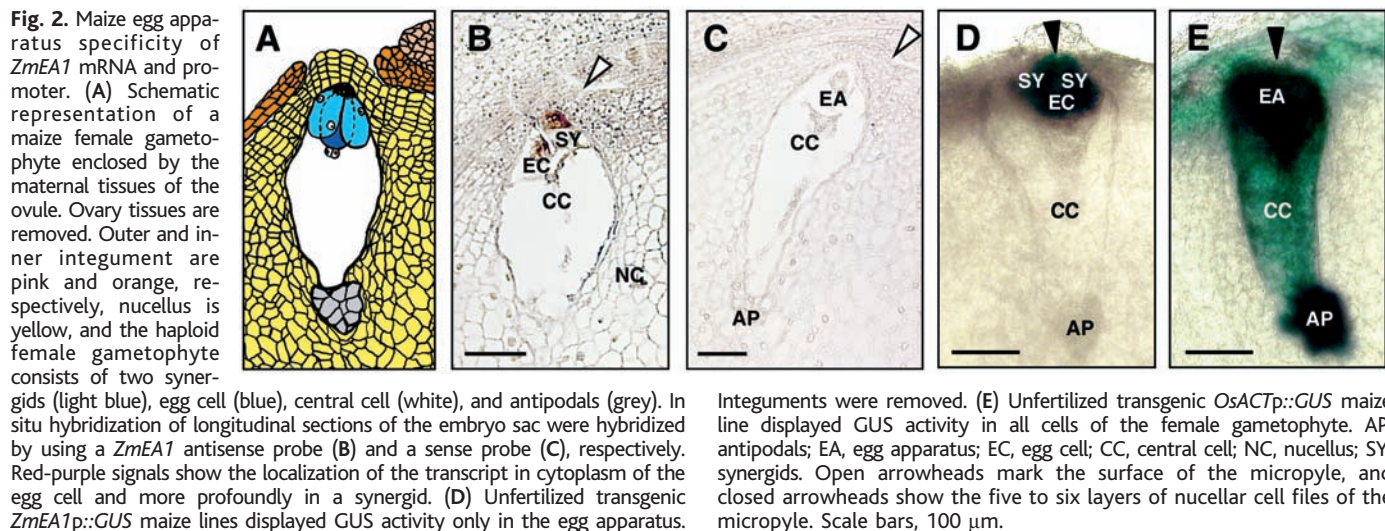

Fig. 2. Maize egg apparatus specificity of *ZmEA1* mRNA and promoter. (**A**) Schematic representation of a maize female gametophyte enclosed by the maternal tissues of the ovule. Ovary tissues are removed. Outer and inner integument are pink and orange, respectively, nucellus is yellow, and the haploid female gametophyte consists of two synergids (light blue), egg cell (blue), central cell (white), and antipodals (grey). In situ hybridization of longitudinal sections of the embryo sac were hybridized by using a *ZmEA1* antisense probe (**B**) and a sense probe (**C**), respectively. Red-purple signals show the localization of the transcript in cytoplasm of the egg cell and more profoundly in a synergid. (**D**) Unfertilized transgenic *ZmEA1*p::*GUS* maize lines displayed GUS activity only in the egg apparatus. Integuments were removed. (**E**) Unfertilized transgenic *OsACT*p::*GUS* maize line displayed GUS activity in all cells of the female gametophyte. AP, antipodals; EA, egg apparatus; EC, egg cell; CC, central cell; NC, nucellus; SY, synergids. Open arrowheads mark the surface of the micropyle, and closed arrowheads show the five to six layers of nucellar cell files of the micropyle. Scale bars, 100 μm.
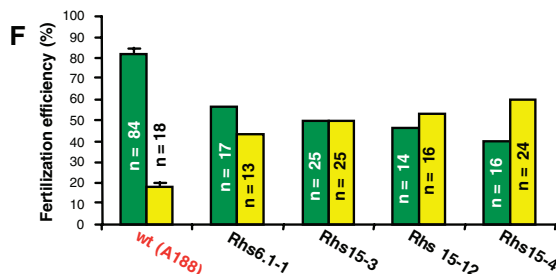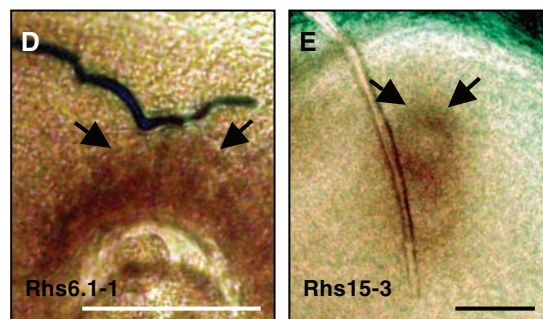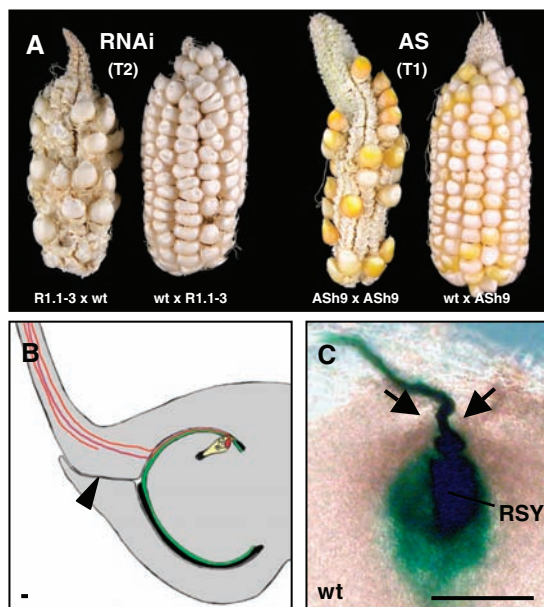
Fig. 3. Ovular localization of ZmEA1:GFP fusion protein by using epifluorescence microscopy (A and C), CLSM (E and F), and light microscopy (B and D). (**A** and **B**) GFP fluorescence is visible in the synergid cell walls (arrowheads) and filiform apparatus with faint signals within the overlaying nucellus cells below the micropyle of a young ovule (silks <5 cm). (**C** and **D**). A stronger fluorescence was observed at the micropylar region of older ovules between synergid cell walls (closed arrowheads) and the surface of the nucellus cells at the micropylar opening (open arrowheads) of a mature ovule (silks >10 cm). (**E** and **F**) Fluorescence is visible within the cell walls and the surface of the six nucellus cell layers at the micropyle but not at the surface of the inner integument. Arrowheads mark the surface of the nucellus cells underlying the inner integument. AP, antipodals; EA, egg apparatus; EC, egg cell; CC, central cell; II, inner integument; OI, outer integument; SY, synergids. Scale bars, 100 μm.
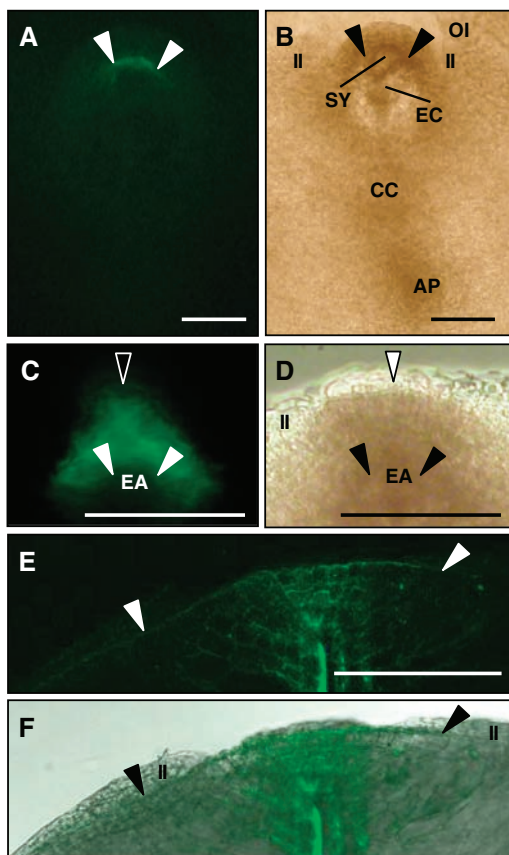
micropylar opening of the ovule (Fig. 4D), but instead of penetrating the nucellus, continued growth at random directions at the surface of the inner integument (Fig. 4E). Only 40 to 55% of the analyzed ovules of these RNAi lines showed blue staining within the female gametophyte. Such loss of pollen tube guidance was never observed in wild-type ovules and thus links transgenic expression with loss of pollen tube guidance.

All above data support a major role for ZmEA1 as a signaling molecule in maize short-range pollen tube guidance. These include transcription and protein production specific to the egg apparatus, secretion and transport to the micropylar opening of the ovule, rapid transcriptional down-regulation, and protein degradation after fertilization, as well as female sterility observed when the activity of the protein is reduced. We also found that ZmEA1:GFP was restricted only to the micropylar region, which suggests also some degradation or negative regulation of the transport in the surrounding integuments and the adjacent nucellar cells. However, even if ZmEA1 has many of the characteristics for a candidate signal, it is still unclear whether ZmEA1 represents a molecule that is directly sensed by receptors of the pollen tube tip or whether ZmEA1 stimulates secretion of pollen tube guidance signals by

Fig. 4. Loss of close-range pollen tube guidance in maize transgenic lines with reduced *ZmEA1* activity. (**A**) Left, seed set of the transgenic line R1.1-3 (T_1) that was propagated via pollen of the R1.1 line (T_0) to a wild-type (wt) cob is shown after self-pollination. Pollen of this line was also used as donor to fertilize the wild-type cob shown at the right. Seed set of a self-pollinated ASh9 (T_0) and out-crossed wild-type cob with pollen of this line is shown at the right. Segregation of the transgene affects female but not male fertility. (**B**) Schematic representation of pollen tube growth in maize. Each ovary encloses a single bitegmic ovule. In maize, the outer integument (black) is reduced compared with the inner integument (green), which itself encloses most of the nucellus except in the vicinity of the female gametophyte (yellow). This region corresponds to the micropyle. Multiple pollen tubes (red) grow through the transmitting tissues of the silk to the inner integument, but only one pollen tube penetrates the micropyle and releases sperm cells in the receptive synergid. Arrow head points toward the stylar channel. (**C** to **E**) Transgenic *OsACTp::GUS* maize pollen was used to monitor pollen tube growth and sperm discharge 24 to 30 hours after in vitro pollination in ovules of RNAi plants. (**C**) Direction of pollen tube growth is abruptly changed at the micropyle of a wild-type ovule where the tube penetrates the cell walls of nucellus cells and releases its contents into the receptive synergid. (**D** and **E**) Pollen tubes within

around 50% ovaries of transgenic lines (examples show lines Rhs6.1-1 and Rhs15-3) grew at the surface of the inner integument without entering the micropyle, instead continuing growth at random directions. This phenotype was never observed in wild-type ovules. (**F**) Summary of female gametophyte fertilization in wild-type and transgenic ovules. In wild-type, 82% of the embryo sacs were fertilized 24 to 30 hours after in vitro pollination. GUS staining and thus fertilization of embryo sacs from R lines investigated was reduced to 40 to 55%. *n* gives the number of excised ovules, showing the indicated phenotype; green color, fertilized female gametophyte; and yellow color, unfertilized female gametophyte. RSY, receptive synergid. Arrows mark the micropylar opening of the ovule. Scale bars, 100 μm.

the nucellus cells. We are currently trying to identify the mature ZmEA1 protein, which will then be used to study in vitro pollen tube guidance in maize. Nevertheless, our results bring potentially new insights into the fertilization process. It had been suggested in 1918 (*11*) that the filiform apparatus is required for pollen tube attraction, and in 1964, the existence of chemotropic substances, which are produced by the synergids, was postulated (*12*). Also, numerous secretory vesicles have been observed at the micropylar region of maize synergids but not the egg cell (*13*). Loss of pollen tube guidance via synergid ablation was recently substantiated in *Torenia fournieri* (*7*). Our data suggest that, at least in maize, not only the synergids but the whole egg apparatus could be involved in micropylar pollen tube guidance. Whether the egg cell is also involved in synthesizing a ZmEA1 precursor that is transported toward the synergids via the endoplasmic reticulum or whether the egg cell is capable of ZmEA1 secretion itself is a matter of further experimentation. Rapid down-regulation of the pollen tube guidance signal(s) is a major prerequisite to preventing polyspermy, and fertilization is first sensed by the synergids and egg cell, features of *ZmEA1* gene activity and protein.

The occurrence of *ZmEA1*-related genes in cereals, but not in dicotyledonous species, might be one explanation; wide crosses involving successful pollen tube guidance and fertilization are possible within genera of the Gramineae but not between species spanning wider taxonomic boundaries (*14*). In *Torenia fournieri*, pollen tubes of related species do not respond to the synergids attraction signal, which suggests species-specific short-range attractants (*2*). A similar observation was made conducting interspecific crosses using *Arabidopsis* and other Brassicaceaes where pollen tubes grew normally through the transmitting tissue but rarely arrived at the funiculus and did not enter the micropyle (*15*). Sequence identity between ZmEA1 from two maize inbred lines (A188 and H99) is 91.5% (*16*) and less than 45 and 43% between the maize and the two rice homologs (Fig. 1A). The low homology between EA1 proteins provides further support that specific short-range guidance signals may be involved in the species-barrier concept. The identification of further molecules involved in female gametophyte pollen tube guidance in maize and other plant species should not only help understanding of many outstanding issues in plant reproductive biology but may also be used for future plant breeding to overcome some of the current crossing barriers and to allow hybridization between plant genera that cannot be crossed today.

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A Brief History of Seed Size

Angela T. Moles,[1,2]* **David D. Ackerly,**[3]† **Campbell O. Webb,**[4]
John C. Tweddle,[5,6] **John B. Dickie,**[6] **Mark Westoby**[2]

Improved phylogenies and the accumulation of broad comparative data sets have opened the way for phylogenetic analyses to trace trait evolution in major groups of organisms. We arrayed seed mass data for 12,987 species on the seed plant phylogeny and show the history of seed size from the emergence of the angiosperms through to the present day. The largest single contributor to the present-day spread of seed mass was the divergence between angiosperms and gymnosperms, whereas the widest divergence was between Celastraceae and Parnassiaceae. Wide divergences in seed size were more often associated with divergences in growth form than with divergences in dispersal syndrome or latitude. Cross-species studies and evolutionary theory are consistent with this evidence that growth form and seed size evolve in a coordinated manner.

Seed mass affects many aspects of plant ecology. Small-seeded species are able to produce more seeds for a given amount of energy than are large-seeded species (*1*, *2*), whereas large-seeded species have seedlings that are better able to tolerate many of the stresses encountered during seedling establishment (*3*). Seed mass is also correlated with the environmental conditions under which species establish (*4–6*) and with traits such as plant size, dispersal syndrome, plant life-span, and the ability to form a persistent seed bank (*7–13*). Present-day species have seed masses ranging over 11.5 orders of magnitude, from the dust-like seeds of orchids (some of which weigh just 0.0001 mg) to the 20-kg seeds of the double coconut (Fig. 1A). Improving our knowledge of the changes in seed mass that occurred as the angiosperms radiated out of the tropics, colonized a wide range of habitats, developed a range of growth forms and dispersal strategies, and became the most abundant and diverse group of plants on earth (*14–18*) will greatly enhance our understanding of the ecological history of plants.

Using newly developed software (*19*, *20*), we integrated a large seed mass data set with current best opinion for the phylogeny of seed plants. Our seed mass data set included 12,669 angiosperms and 318 gymnosperms. This is ~5% of all extant angiosperm and 38% of all extant gymnosperm species. The angiosperm species are from 3158 genera (22% of the global total) and 260 families (57%) and include representatives from all extant orders. The gymnosperm species are from 52 genera (63%) and 10 families (63%) (*21*).

[1]National Center for Ecological Analysis and Synthesis, 735 State Street, Santa Barbara, CA 93101–5304, USA. [2]Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia. [3]Department of Biological Sciences, Stanford University, Stanford, CA 94305–5020, USA. [4]Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06520–8106, USA. [5]The Natural History Museum, Cromwell Road, London, SW7 5BD, UK. [6]Royal Botanic Gardens (RBG), Kew, Wakehurst Place, Ardingly, West Sussex, RH17 6TN, UK.

*To whom correspondence should be addressed. E-mail: amoles@bio.mq.edu.au
†Present address: Department of Integrative Biology, University of California, Berkeley, CA 94720, USA.