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Double Fertilization in *Ephedra*, a Nonflowering Seed Plant: Its Bearing on the Origin of Angiosperms

WILLIAM E. FRIEDMAN

Double fertilization and the associated formation of endosperm have long been considered unique and defining characters (autapomorphies) of the angiosperms. During normal fertilization in *Ephedra nevadensis*, a nonflowering seed plant, fusion of a second sperm nucleus with the ventral canal nucleus occurs regularly within the egg cytoplasm. The occurrence of double fertilization in *Ephedra* assumes added significance in light of its critical phylogenetic position as a basal member of the most closely related extant group of seed plants (Gnetales) to angiosperms. Thus, double fertilization in angiosperms and *Ephedra* may represent an evolutionary homology.

THE PROCESS OF DOUBLE FERTILIZATION in angiosperms, whereby one sperm fertilizes an egg while a second sperm fuses with the polar nuclei of the female gametophyte (embryo sac), was first reported by Navashin in 1898 (*1*). Subsequently, double fertilization and the associated formation of polyploid endosperm have been considered unique and defining characters (autapomorphies) of the angiosperms (*2–6*). Indeed, many plant biologists have suggested that double fertilization and endosperm represent significant reproductive features that are intimately associated with the ecological and evolutionary success of flowering plants (*7, 8*). Although considerable attention has been paid to the biological or "adaptive" significance of double fertilization and endosperm (*9*), relatively little work has been directed toward understanding the evolutionary origins of these important and apparently unique features of sexual reproduction in flowering plants.

Since the initial discovery of double fertilization in angiosperms, there have been occasional, poorly substantiated reports of anomalous double fertilization–like events in nonflowering seed plants, particularly the genus *Ephedra* (*10–15*). The possible occurrence of double fertilization in *Ephedra* is significant in view of recent phylogenetic studies of seed plants (*6, 16*). These cladistic analyses indicate that the Gnetales (*Ephedra, Gnetum*, and *Welwitschia*) are monophyletic, form part of a larger clade that includes angiosperms, and are more closely related to flowering plants than is any other extant group of seed plants. Finally, *Ephedra* is basal within the Gnetales and has retained many primitive characteristics of the group. Thus, *Ephedra* occupies a critical position in the phylogeny of seed plants. Character traits (such as double fertilization) that are shared by angiosperms and Gnetales are potentially evolutionarily homologous (synapomorphous), having been inherited from a common ancestor.

Extensive collections of *Ephedra nevadensis* took place in 1987 and 1988 from populations growing north of Tucson, Arizona. Ovules were dissected, fixed in glutaraldehyde, dehydrated, and embedded in glycol methacrylate. Each ovule was serially sectioned at 3 to 5 μm into several hundred sections. Complete sets of serial sections ensured that all nuclei from a specific male gametophyte and archegonium could be accounted for and correctly interpreted.

Within hours of pollination, the central cell nucleus, located at the micropylar end of the central cell, divides to produce a ventral canal nucleus and egg nucleus. There is no evidence of wall formation between the two daughter nuclei. The ventral canal nucleus remains in situ at the extreme micropylar and vacuolate end of the egg cytoplasm. The incipient egg nucleus migrates in a chalazal direction where it enters and becomes immersed in an unusual columnar zone of cytoplasm, which has been shown to be rich in mitochondria and plastids in *E. distachya* (*17*).

In *E. nevadensis*, the ventral canal nucleus

Department of Botany, University of Georgia, Athens, GA 30602.

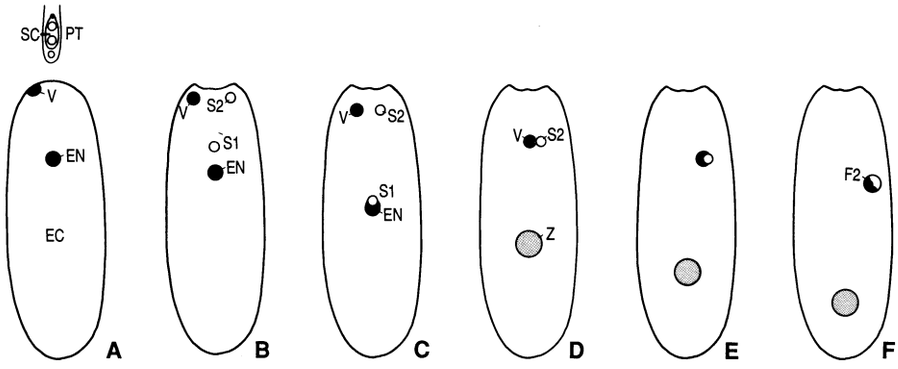

Fig. 1. Schematic of double fertilization in *E. nevadensis*. (**A**) Pollen tube (PT) with binucleate sperm cell (SC) growing to egg cell (EC); (**B** to **D**) fertilization of egg nucleus (EN) by first sperm nucleus (S1) to produce a zygote nucleus (Z); (**D** to **F**) migration of zygote nucleus to base of former egg cell; and (**C** to **F**) displacement of the ventral canal nucleus (V) from the apex of the egg cell, leading to a fusion (F2) with the second sperm nucleus (S2).

is almost always persistent through the time of fertilization. In ovules in which some, but not all, of the eggs were fertilized (*18*), each unfertilized egg contained a prominent ventral canal nucleus at the apex of the egg cytoplasm. Although no data are available on the time between pollination and fertilization in *E. nevadensis*, estimates for this time interval in *E. trifurca* and *E. distachya* range from 10 to 36 hours (*10, 19*). Thus, the extremely brief (for a nonflowering seed plant) time interval between pollination and fertilization in *Ephedra* may result in the persistence of the ventral canal nucleus through the time of fertilization.

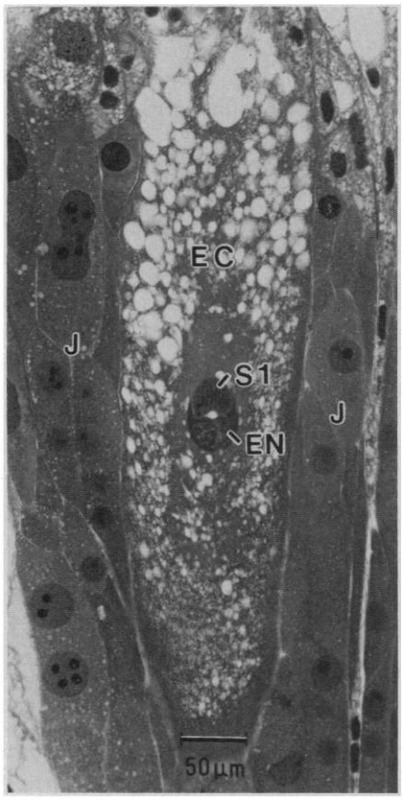

Fig. 2. Fusion of first sperm nucleus (S1) and egg nucleus (EN) showing characteristic invagination of egg nucleus in *E. nevadensis*. Jacket cells (J) surround the egg cell (EC). Bar, 50 µm.

Four nuclei are present in pollen tubes growing toward an egg: the tube nucleus advances first, followed by the two nuclei of a single binucleate sperm cell, and, tightly appressed to the posterior of the sperm cell, an intensely staining sterile cell nucleus. Numerous small circular inclusions are distributed throughout each sperm nucleus. As a consequence, sperm nuclei can be easily distinguished from other nuclei, and their fate closely followed.

With the arrival of the male gametophyte at the apex of the egg, a vacuolate region, the "fertilization chamber," forms directly above the egg cell (at the base of the archegonial neck). The tube nucleus, sterile cell nucleus, and small amounts of pollen tube cytoplasm are always seen within the fertilization chamber after the entry of sperm into the egg. When more than one male gametophyte grows toward a single egg, supernumerary pollen tubes are blocked at the apex of the egg, and their contents (including the binucleate sperm cell) are impounded in the fertilization chamber.

The ability to count impounded male nuclei in the fertilization chamber permits a direct estimate of the number of pollen tubes that reach the apex of each egg, and by deduction, an account of the fate of the sperm nuclei from the successful pollen tube. Of 22 serially sectioned archegonia at the fertilization stage, the fertilization chambers of 19 archegonia contained two fewer sperm nuclei than would be expected from the number of pollen tubes that had arrived at the apex of the egg. Thus, in almost every case, two sperm nuclei can be presumed to have entered the egg.

After entry of two sperm nuclei into the egg cytoplasm, one sperm nucleus migrates

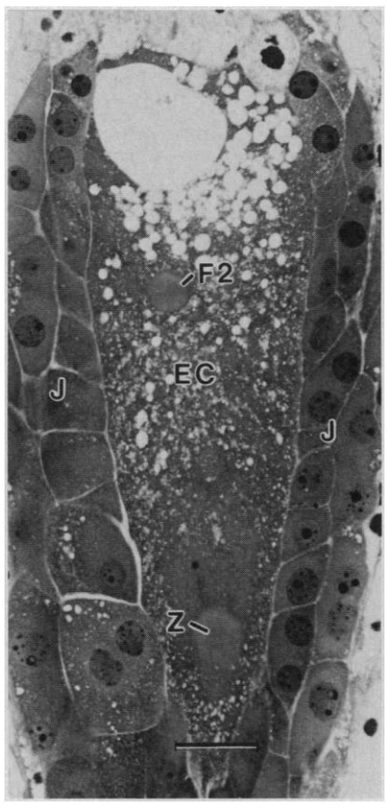

Fig. 3. Double fertilization in an egg cell (EC). The zygote nucleus (Z) has migrated to the base of the archegonium and the second fertilization product (F2) of the displaced ventral canal nucleus and second sperm nucleus is also apparent (this nucleus is shown enlarged in Fig. 4C). J, jacket cells. Bar, 50 µm.

directly toward the egg nucleus. Initial contact between these two nuclei results in a characteristic invagination of the receptive egg nucleus (Figs. 1C and 2). Measurements of nine pairs of sperm and egg nuclei undergoing fusion indicate that fusion is initiated and completed within a relatively limited horizontal band of egg cytoplasm. Vertical displacements (from the apex of the egg cytoplasm) of fusing sperm and egg nuclei were between 120 and 375 µm with a mean ± SD of 226.8 ± 81.0 µm (*20*). As fertilization proceeds, the fusing sperm and egg nuclei migrate toward the chalazal end of the egg cell. Measurements of fully formed zygote nuclei in eight archegonia show a range of vertical displacements between 400 and 570 µm (475.0 ± 63.6 µm), confirming the continued migration of the zygote nucleus toward the base of the former egg cell.

Shortly after the entry of sperm nuclei into the egg cell, the ventral canal nucleus dislodges from its apical position and begins to migrate to a location deeper within the egg cytoplasm (Figs. 1 and 3). Of nine archegonia with sperm and egg nuclei in the process of fusing, average displacement of

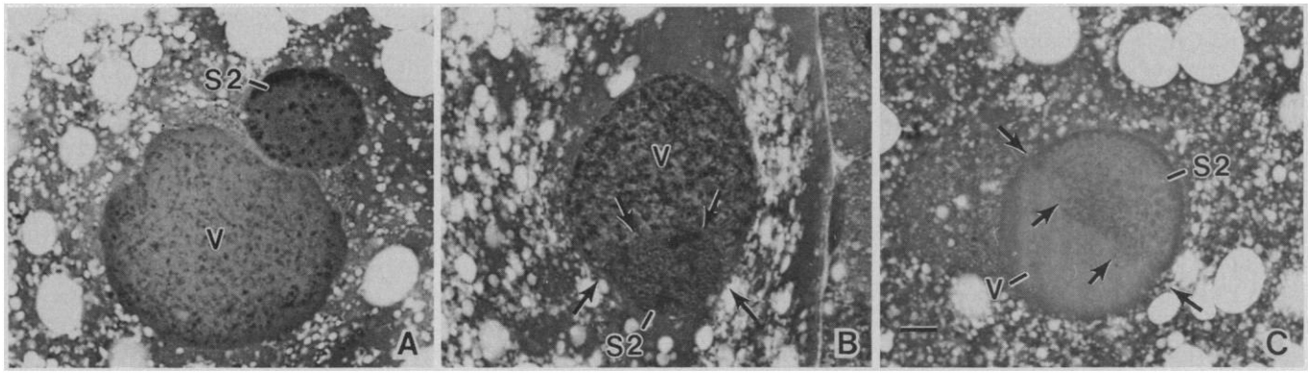

Fig. 4. Fusion of the ventral canal nucleus (V) with the second sperm nucleus (S2). The ventral canal nucleus displays the same invagination behavior (**A** and **B**) as the egg nucleus in Fig. 2. A fully fused second fertilization product appears (**C**) with half of the nucleus containing nuclear inclusions that are characteristic of the sperm nucleus. Arrows indicate interface between sperm nucleus and ventral canal nucleus. Bar, 10 μm.

the ventral canal nucleus from the apex of the egg cell was 32.0 ± 26.9 μm. In nine additional archegonia, each containing a fully formed zygote nucleus, vertical displacement of the ventral canal nucleus increased to an average of 169.0 ± 83.6 μm. In four of these archegonia, the second sperm nucleus was observed in various stages of fusion with the ventral canal nucleus (Figs. 3 and 4). In each of these situations, the second fertilization event occurred within the same horizontal band of cytoplasm (average displacement, 212.5 ± 20.2 μm) in which the first sperm nucleus and egg had likely fused earlier. The consistent displacement of the ventral canal nucleus (in 15 of 18 archegonia), entry of two sperm nuclei into the egg (in 19 of 22 archegonia), and observed fusion events suggest that double fertilization is a regular feature of sexual reproduction in *Ephedra nevadensis*.

With the completion of fertilization, the zygote nucleus embarks on a series of approximately three relatively synchronous free nuclear divisions within the cytoplasm of the former egg cell. Mitotic divisions of the zygote nucleus (and its derivatives) may be amplified (resulting in more than eight free nuclei) or deleted (resulting in less than eight free nuclei). Each of the resulting nuclei develops into a separate embryo. Therefore, it is uncertain whether the second fusion nucleus produces an additional embryo (genetically identical to the other zygotic embryos) or eventually degenerates. It is clear that no tissue similar to endosperm ever develops from the second fertilization event in *E. nevadensis*.

The series of fusion events involving the ventral canal nucleus and second sperm nucleus (Figs. 1, 3, and 4) clearly shows that the ventral canal nucleus, when displaced from its normal apical position within the egg cytoplasm to a position deeper within the egg (where the original egg and sperm nuclei first made contact and began to fuse), behaves similarly to its sister nucleus, the egg. It is capable of attracting a sperm nucleus to its immediate vicinity, it undergoes the characteristic invagination of a receptive female nucleus, and can completely fuse with a sperm nucleus.

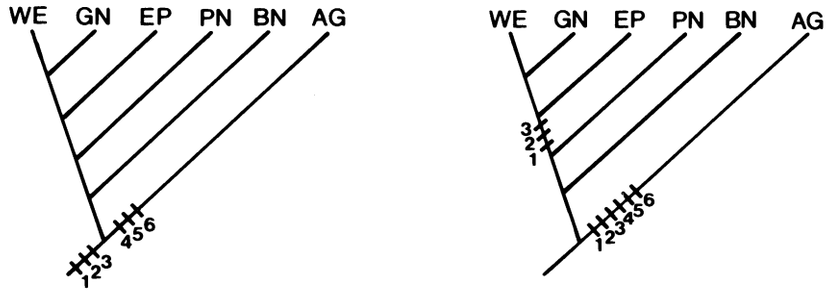

Fig. 5. Cladograms indicating two alternatives for the evolution of double fertilization and endosperm in the angiosperms (AG) and their sister group [phylogenetic relationships based on Doyle and Donoghue (*6*)]. Left: characters 1 to 3 are homologous in *Ephedra* and angiosperms. Right: characters 1 to 3 evolve separately in angiosperms and Gnetales [*Ephedra* (EP), *Gnetum* (GN), and *Welwitschia* (WE)] [see (*23*)]. Character states of fossil groups Pentoxylon (PN) and Bennettitales (BN) are unknown. Character states: 1, acceleration of time interval between pollination and fertilization: 2, persistence of sister nucleus of egg; 3, acquisition of egglike properties by sister nucleus of egg and origin of double fertilization; 4, reduction of female gametophyte (to embryo sac); 5, addition of second female nucleus to second fertilization event; 6, de novo development of second fertilization product into endosperm.

These findings show that double fertilization can no longer be assumed to be a unique and defining feature of angiosperms. Double fertilization events in *Ephedra* and angiosperms both involve fusion of the sister nucleus of the egg with a second sperm nucleus from a male gametophyte. In *Ephedra*, the sister nucleus of the egg is the ventral canal nucleus. Among angiosperms with a primitive monosporic pattern of embryo sac development (*Polygonum* type) (*7, 9*), one of the two polar nuclei with which the second sperm fuses is typically the sister nucleus of the egg (*21, 22*). Thus, it is possible that the establishment of double fertilization preceded the origin and evolutionary divergence of angiosperms and their sister group (which includes the Gnetales) (*23, 24*), as well as the developmental reduction of the female gametophyte (embryo sac) in the angiosperm line (Fig. 5).

Although it may ultimately be impossible to determine whether double fertilization in *Ephedra* and angiosperms represents an evolutionary convergence or homology, consideration must now be given to two alternatives for the evolutionary history of reproductive characters associated with double fertilization and endosperm. The occurrence of double fertilization in *Ephedra* establishes the possibility that persistence of the sister nucleus of the egg and its acquisition of egglike features (the ability to attract and fuse with sperm) are synapomorphous characters in *Ephedra* and angiosperms (Fig. 5). The addition of a second female nucleus to the second fertilization event (resulting in a typically triploid fusion product), as well as the development of a specialized nutritive tissue (endosperm) from the second fertilization event, may well be the only innovations of angiosperms with respect to their fertilization biology.

It is likely that the developmental reduction of the female gametophyte, from a significant nourishing tissue for the embryo in nonflowering seed plants to the structurally reduced embryo sac of angiosperms, was compensated for by the origin of endosperm as a nutritive tissue for the embryo (character advances 4 to 6, Fig. 5). If double fertilization in *Ephedra* and angiosperms was inherited from a common ancestor, the evolution of triploid endosperm, and not double fertilization per se, would appear to have been the significant reproductive innovation, in part, responsible for the evolutionary and ecological radiation of angiosperms.

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Novel Fluorogenic Substrates for Assaying Retroviral Proteases by Resonance Energy Transfer

EDMUND D. MATAYOSHI,* GARY T. WANG, GRANT A. KRAFFT, JOHN ERICKSON

The 11-kD protease (PR) encoded by the human immunodeficiency virus 1 (HIV-1) is essential for the correct processing of viral polyproteins and the maturation of infectious virus, and is therefore a target for the design of selective acquired immunodeficiency syndrome (AIDS) therapeutics. To facilitate the identification of novel inhibitors of HIV-1 PR, as well as to permit detailed studies on the enzymology and inhibition of this enzyme, a continuous assay for its activity was developed that was based on intramolecular fluorescence resonance energy transfer (RET). The assay used the quenched fluorogenic substrate 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)–Ser Gln Asn Tyr Pro Ile Val Gln–5-[(2-aminoethyl)amino]naphthalene-1 sulfonic acid (EDANS), whose peptide sequence is derived from a natural processing site for HIV-1 PR. Incubation of recombinant HIV-1 PR with the fluorogenic substrate resulted in specific cleavage at the Tyr-Pro bond and a time-dependent increase in fluorescence intensity that was linearly related to the extent of substrate hydrolysis. An internally quenched fluorogenic substrate was also designed that was selectively cleaved by the related PR from avian myeloblastosis virus (AMV). The fluorescence quantum yields of the HIV-1 PR and AMV PR substrates in the RET assay increased by 40.0- and 34.4-fold, respectively, per mole of substrate cleaved. Because of its simplicity, rapidity, and precision in the determination of reaction rates required for kinetic analysis, this method offers many advantages over the commonly used high-performance liquid chromatography– or electrophoresis-based assays for peptide substrate hydrolysis by retroviral PRs.

As with other classes of positive-strand RNA viruses, retroviruses encode proteins that are initially synthesized as large polyprotein precursors and are later processed by posttranslational cleavage (*1*). Translation of the polycistronic viral mRNA results in the synthesis of two precursor polyproteins: Pr^{gag}, which contains the structural capsid proteins, and $Pr^{gag\text{-}pol}$, which contains information for both the structural proteins and the replicative enzymes. The *env* gene products are translated as a precursor polyprotein from a separately spliced mRNA transcript. Retroviruses also encode a small, 10- to 12-kD protease (PR) that is generally expressed as part of the $Pr^{gag\text{-}pol}$ precursor, except in the case of the avian retroviruses, where it is synthesized as the COOH-terminal portion of Pr^{gag} (*2*). The retroviral PR is required for the processing of both the Pr^{gag} and $Pr^{gag\text{-}pol}$ precursor polyproteins at specific cleavage sites. These cleavages are believed to occur during or just after virion assembly and have been shown for HIV-1 and murine leukemia virus (MuLV) to be required for the maturation of infectious virus particles (*3*). Thus, inhibition of the viral PR has become an important target for the design of antiretroviral agents, including new therapeutic agents for AIDS.

Three-dimensional crystal structures of the PRs for Rous sarcoma virus (RSV) (*4*) and HIV-1 (*5*) have verified predictions (*6, 7*) that the retroviral PRs are structurally and functionally related to the eukaryotic aspartic proteinase family of enzymes. Strategies used to design inhibitors of human renin, a related aspartic proteinase that specifically cleaves angiotensinogen to angiotensin I and initiates a hypertensive response, are now being applied to the design of HIV-1 PR inhibitors. A variety of techniques have been previously used to measure retroviral proteolytic activity, including protein immunoblot analysis of the *gag* polyprotein and its cleavage products (*7, 8*), and high-performance liquid chromatography (HPLC) (*9, 10*) or thin-layer electrophoretic (*11*) analysis of synthetic peptide-cleavage fragments. All of these methods are relatively time-consuming and impractical for screening and characterizing large numbers of inhibitors. In addition, they are not well suited for enzymological studies because continuous measurement of reaction kinetics is not possible (*12*).

As an alternative approach, we have developed a fluorescence assay based on the quenched fluorogenic substrates **1** and **2** (Fig. 1A). These substrates consist of an

E. D. Matayoshi and J. Erickson, Pharmaceutical Discovery Division, Abbott Laboratories, Abbott Park, IL 60064.
G. T. Wang and G. A. Krafft, Abbott Diagnostics Division, Abbott Laboratories, Abbott Park, IL 60064.

*To whom correspondence should be addressed.